Ida Caroline Schrøder

Establishing tools to study the involvement of Keap1 in inflammatory signaling upon infection with *M. avium*

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Principal supervisor: Jane Atesoh Awuh
Co-supervisor: Trude Helen Flo

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine
Centre of Molecular Inflammation Research

NTNU
Norwegian University of Science and Technology

CEMIR
Centre of Molecular Inflammation Research

NTNU

Norwegian University of Science and Technology

Master thesis

Faculty of Medicine

Department of Cancer Research and Molecular Medicine

Center of Molecular Inflammation Research

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Abstract

Mycobacterium avium is a non-tuberculous mycobacteria that causes opportunistic infections in immunocompromised individuals such as AIDS patients. The pathogen is receiving more attention due to increasing resistance towards several antibiotics, and a high degree of genetic variability within the strain. The treatment towards *M. avium* is lengthy, highlighting the importance of elucidating the molecular mechanisms of infection in the host cells in search for novel drug targets. *M. avium* infects macrophages, where they reside and avoid degradation by blocking maturation of the phagosomes and fusion with lysosomes like the more virulent *M. tuberculosis*, the causative agent of Tuberculosis. Reactive oxygen species released due to activation of Pattern recognition receptors are thought to be important for killing intracellular mycobacteria, but excessive amounts could be damaging to the cells. Keap1 is a sensor for ROS and a substrate adaptor for the Cullin3-based E3 ubiquitin ligase, shown to regulate IKKβ and NF-kB signaling among other important targets. Our group has shown that Keap1 is recruited to mycobacterial phagosomes and regulates inflammatory signaling in human primary macrophages. The study further raised the evidence for a direct or indirect regulation of TBK1, involved in the IRF-pathways and production of type 1 Interferons. In addition it suggests the role of Keap1's contribution to increased survival of *M. avium* intracellulary. The limitations with the use of primary macrophages due to donor variations and difficulties in modulating protein levels, further addressed the need for a macrophage-like model cell-line to further detail the mechanism of Keap1's regulation of IKKβ and TBK1 using transfection or transduction of tagged full-length Keap1 and deletion constructs of Keap1.

In this project we examined the U373-CD14 and THP1 cell-lines for response towards *M. avium*, both phagocytic clearance and inflammatory cytokine responses. As the U373-CD14 cell-line did not show a high up-regulation of the cytokines of interest, the cell-line was transiently transfected with the endogenously absent TLR2 and TLR8, and an increased response was observed towards infection, especially for TLR2 transfected cells. But the transient transfection displayed low efficiency in the cells, and it was decided to modify the cell-line with lentiviral transduction and gateway cloning with TLR2 and TLR8 in separate cell-lines. The U373-CD14 TLR2 cell-line responded to *M. avium* with a significant high up-regulation of TNF-α and IL-8, and a significant low up-regulation of IFNβ, which highlighted the importance of the receptor for *M. avium* induced inflammation. The TLR8- expressing cell-line did not display an efficient up-regulation of screened cytokines towards *M. avium*, and this could either be explained by the short infection time or the incompetence of the TLR8 receptor in infection because of absent components in the pathway.

This project has provided new tools to study the regulation of IKKβ and TBK1 by Keap1 upon *M. avium* infection. In addition it has raised new questions regarding the TLR8 receptor's role in mycobacteria infection, and highlighted the importance of TLR2 for an efficient immune response. It further remains to investigate whether the knockdown of Keap1 leads to an up-regulation of the cytokines of interest, and to reveal the regulation mechanisms of the kinases upon infection.

Abbreviations

Ab: Antibody

AIDS: Acquired immune deficiency syndrome

AIM2: Absent in melanoma 2

APC: Antigen presenting cell

ATPase: Adenosine triphosphatase

BCG: *Bacille Calmette Guerin*

CARD: Caspase- recruitment domain

cGAMP: Cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP)

cGAS: cGAMP synthase

CD14: Cluster of differentiation 14

CFP: Cyan fluorescent protein

CFU: Colony forming units

CLR: C- type lectin receptor

CMV: Promoters for cytomegalovirus

Cul3: Cullin-3

CXCL: Chemokine (C-X-C motif) ligand

C3b: Complement component 3b

DC-SIGN: Dendritic cell-specific intracellular adhesion molecule-3 grabbing nonintegrin

DIC: Differential interference contrast

ESX-1: ESAT-6 system 1

Fc: Fragment crystallizable

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

Hrt1: RING-H2 finger protein

IKKβ: Inhibitor of nuclear factor kappa-B kinase subunit beta

IL: Interleukin

IFNAR: Interferon alpha- receptor

IFNβ: Interferon beta 1

IRAK: IL-1R- associated kinases

IRF: Interferon regulatory factor

IVR domain: Intervening region domain

IκB: Inhibitor of κB

JAK-STAT: Janus kinase-signal transducer and activator of transcription

Keap1: Kelch-like ECH-associated protein 1

LAM: Lipoarabinomannan

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

LGP2: Laboratory of genetics and physiology 2

LM: Lipomannan

LPS: Lipopolysaccharide

LRR: Leucine-rich repeats

MAC: *Mycobacterium Avium* complex

Mal: MyD88 adaptor-like protein

MAPK: Mitogen-activated protein kinase

MAVS: Mitochondrial antiviral-signaling protein

MDA5: Melanoma differentiation-associated protein 5

MDM: Monocyte derived macrophages

MDP: Muramyl dipeptide

MOI: Multiplicity of infection

Mtb: *Mycobacterium tuberculosis*

mTORC1: Mammalian target of rapamycin complex 1/ Mechanistic target of rapamycin complex 1

MTP: Muramyl tripeptide

MyD88: Myeloid differentiation factor-88

NEMO: NF-κB essential modifier

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

NO: Nitric oxide

NOX2: NADPH oxidase

NLR: NOD (nucleotide-binding oligomerization domain)- like receptors

Nfr2: Nuclear factor-erythroid 2 related factor 2

PAMP: Pathogen-associated molecular pattern

PIM: Phosphatidylinositol mannosides

Poly I:C: Polyinosinic-polycytidylic acid

PRR: Pattern recognition receptors.

P62/ SQSTM1: Sequestosome 1

PYHIN: Pyrin and HIN domain-containing protein

RIP: Receptor-interacting serine/threonine-protein kinase

ROC1/Rbx1: RING finger protein 1

ROS: Reactive oxygen species

RLR: RIG-1-like receptor

RRE: REV-responsive elements

RQ: Relative quantity

SARM: Sterile α- and armadillo-motif-containing protein

SLR: Sequestosome 1/p62-like receptors

STING: Stimulator of Interferon genes

TAB: TAK1 binding proteins

TAK1: Transforming growth factor-β-activated kinase-1

Tb: Tuberculosis

TBK1: TANK-binding kinase 1

TIR: Toll/IL-1 receptor

TLR: Toll- like receptor

TNF-α: Tumor necrosis factor alpha

TRAF: TNF receptor associated factors

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain containing adaptor protein inducing IFN-β

VSV-G: Vesicular stomatitis virus G

YFP: Yellow fluorescent protein

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1. Introduction

1.1 The genus Mycobacterium

There are about 60 species of the genus Mycobacterium known today, and they are divided into several groups, among them; the *Mycobacterium tuberculosis* complex (*Mycobacterium bovis* and *Mycobacterium tuberculosis), Mycobacterium africanum, Mycobacterium microti* and the *Mycobacterium avium* complex (MAC). MAC are widely distributed in the environment, mainly in water sources, and consists of the closely related species; *M. avium*, *M. intracellulare, M. paratuberculosis* and *M. hominissuius* [8,9].

The mycobacteria are Gram-positive, aerobic and non-motile with a thick cell wall. Its outer layer consists of lipids and lipid-linked polysaccharides like lipoarabinomannan (LAM), lipomannan (LM), phthiocerol-containing lipids, sulfolipids, and phosphatidylinositol mannosides (PIM), recognized by the immune system of the host upon infection. The membrane inner layer consists of peptidoglycan, arabinogalactan and mycolic acids. The thick and impermeable nature of their cell wall contributes to their resistance to many drugs [10].

The disease tuberculosis caused by *M. tuberculosis* (Mtb) poses one of the biggest global health problems in the world today. In 2013, 9 million new cases of tuberculosis were reported, and 1,5 million people died from the disease [11]. The immune responses initiated after infection do not eliminate the mycobacteria, but leads to a latent infection where Mtb can survive inside host cells, and later get reactivated. Mtb can modulate trafficking and maturation of phagosomes they reside in to avoid degradation. The ESX1 type VII secretion system that the bacteria possess allows them to escape the host cells and infect other phagocytes to spread inside the host and expand its population. Currently existing and leading vaccine *Mycobacterium bovis bacillus Calmette- Guérin* (BCG) was developed in the early twentieth century but has limited function for preventing active tuberculosis in adults, and the need for novel vaccine candidates on the market is therefore crucial [12]. The impropriate use of antibiotics and weak Tb control programs leads to increasingly development of Multi-resistant tuberculosis (MDR-TB) and Extensively drug-resistant tuberculosis (XDR-TB) world wide, and poses a global health threat. These strains of mycobacteria are resistant to antibiotics used today to treat Tb, and can be further transmitted to other individuals [11].

The non-tuberculous mycobacteria like MAC consists of pathogens that cause opportunistic infections in individuals that are immunocompromised. This could be individuals with predisposing lung abnormalities like emphymesa or chronic bronchitis, or acquired immunodeficiency syndrome [13,14]. In addition, MAC-associated disease has been found in some healthy individuals with genetic deficiencies in the cytokines interferon (IFN)-γ and interleukin (IL)-12 [15], and also in some children [16]. The most common immunocompromised patient group affected is AIDS patients; typically with blood CD4+ cell counts below 50 per mm^3 [14]. *M. avium* infection is initiated through the intestinal tract by swallowing, or by inhalation of aerosols containing bacteria leading to pulmonary disease similar to Tb [17]. The

subspecies *M. paratuberculosis* are linked to Crohn's disease, a chronic inflammatory bowel disease.

There are no existing vaccines to MAC diseases, and currently existing treatments are lengthy and expensive with poor outcomes [18]. Understanding the mechanisms of how MAC evades the immune system of the host and modulates the immune response are crucial for understanding disease and pathophysiology, develop diagnostic tools, and to be able to suggest novel treatments and drug targets in the future. Today, there are still a lot of unanswered questions regarding the immune response towards infection with *M. avium*, and the genetic variability of the strains leads to diverse phenotypes in the host [14].

1.3 Innate immunity recognition of mycobacteria and specifically *M. avium*

Upon infection, the mycobacteria are thought to go through the gastro intestinal and respiratory tracts, crossing the mucosal barrier where they are recognized and phagocytosed by tissue macrophages and other phagocytic cells [13,5].

Mycobacterium tuberculosis is known to possess the ESX-1 type VII secretion system that is thought to induce escape from the phagosomes and spread to neighboring cells [19]. As *M. avium* do not possess the ESX-1 secretion system, they are therefore thought to reside inside the macrophages where they modulate the maturation of the phagosomes, preventing acidification and degradation [14,20.] This allows them to replicate and survive inside the host cells, and is a conserved survival strategy. The mycobacteria remain in a stadium between early endosomal and late endosomal compartment outside the phagolysosomal pathway [9], where it can fuse with other endosomal vesicles and possibly this way have access to nutrients, at the same time avoiding degradation [14]. Macrophages may succeed in eliminating some mycobacteria thorugh production of reactive oxygen and nitrogen species, fusion with lysosomes for degradation and autophagy [5].

The immune system has evolved specific receptors, pattern recognition receptors (PRRs) to be able to recognize foreign pathogens and damaged self-molecules. PRRs recognize conserved molecules at the pathogens called pathogen-associated molecular patterns (PAMPs). These receptors participate in MAC internalization and transmitting of intracellular signals in macrophages and other phagocytosing cells like dendritic cells and neutrophils [14].

Activation of PRRs induces the production of reactive oxygen species (ROS) through a NOX2-enzymatically active complex present on phagosomes, as a defense mechanism to eliminate the mycobacteria [21]. *M. avium* infected mouse models have shown to be resistant to nitric oxide (NO) unlike the more pathogenic Mtb, which could suggests the more important role of other NO-independent mechanisms in controlling the infection [14].

The PRRs thought to be most important in sensing mycobacteria are presented in figure 1. These are surface Toll-like receptors (TLRs) reviewed in Section 1.4, surface C-type lectin receptors (CLRs), endosomal TLRs, RIG-I-like receptors (RLRs), NOD- like receptors (NLRs), and other cytosolic nucleic acid receptors [22].

1. Introduction

An important PRR towards recognition of mycobacteria is the C-type lectin mannose receptor involved in recognition of mannose-capped LAM from the mycobacteria cell wall [22]. Dendritic cell-specific intracellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) is another subtype of the CLR's, and are involved in the interaction with mannose-capped LAM and LM on the mycobacterial surface for cell maturation, migration and further T-cell interactions. The Dectin-1 receptor that is present on macrophages, dendritic cells, neutrophils, and some T-cells does not have a well characterized ligand for mycobacteria, but it has been shown to activate adaptive Th1 and Th17 responses in mycobacteria infections [5]. The C-type lectin receptors together with other phagocytic receptors like Fcγ and complement receptor 3 (CR3) are responsible for MAC ingestion [14].

Stimulator of the NOD- like receptor 2 (NOD2) is muramyl dipeptide (MDP), which is a common motif of the mycobacteria. The stimulation leads to the release of proinflammatory cytokines, type 1 Interferons, and nitric oxide (NO). NOD2 interacts further with the RIP2 adapter through caspase- recruitment domain (CARD) interactions that further activates NF-κB and MAPK signaling pathways [23]. In Mtb infections, RIP2 is shown to mediate signaling further through a TBK1- IRF5 pathway for production of type 1 Interferon's [24].

Inflammasomes are activation platforms in the cytosol, and their sensing of foreign un-modified nucleic acids leads to activation of caspases. They are established to play an important role in antimycobacterial host defense. The inflammasomes can contain either NLR's like NLRP1 and NLRP3, or PYHIN domain-containing sensors like absent in melanoma 2 (AIM2). The activation of caspase-1 leads to cleavage and activation of IL-1 and IL-18 proinflammatory cytokines [23,22].

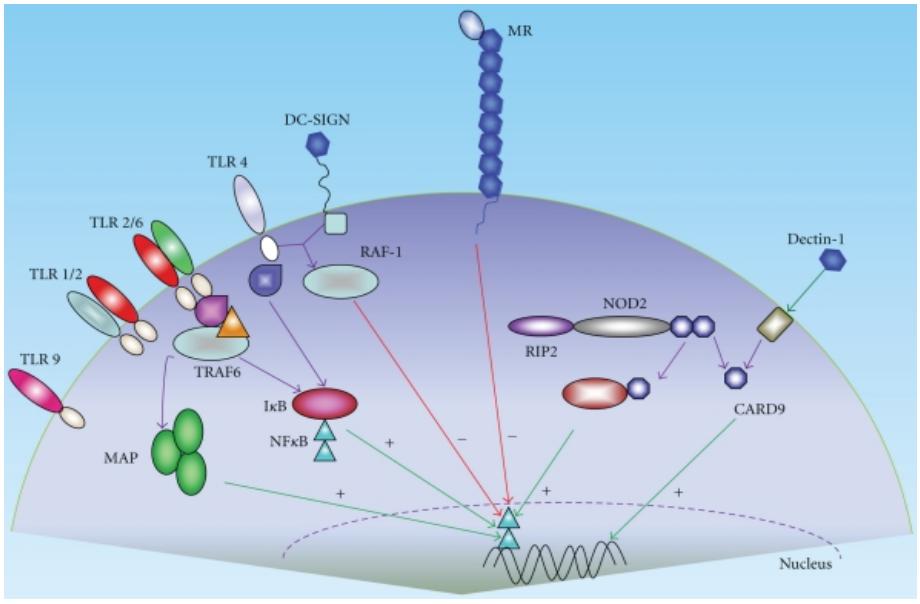

Figure 1: Important receptors involved in innate immunity towards The genus mycobacterium. Figure taken from Kleinnijenhuis et al, Innate Immune Recognition of Mycobacterium tuberculosis, 2011 [5].

As it is not fully elucidated where and how *M. avium* mediates immune intracellular signaling in host cells, the importance of the different nucleic acid receptors in the cytosol are unclear, especially if the mycobacteria are unable to escape the phagosomes and expose their DNA to the cytosol. It is, however, well known that PRRs like some of the TLRs are involved in the responses initiated upon infection with *M. avium,* both from the cell surface and probably intracellular compartments [14].

1.4 Toll-like receptors

Stimulation of Toll-like receptors by microbes increases the activity of the innate and adaptive immune responses by the release of pro-inflammatory cytokines, type 1 Interferon's, upregulation of cell surface receptors, increased effective phagocytosis and the ability to present antigens to T-cells [25]. Toll receptors was first acknowledged by their important role in dorsoventral development of the Drosophila embryo, and its absence leading to severely impaired immune defense towards fungi and Gram positive bacteria [26]. The receptors are present on various cells in the immune system like monocytes, macrophages, dendritic cells, natural killer cells, and B cells that are present or recruited to the site of infection. In addition they are present on other cells like endothelial and epithelial cells [25].

1.4.1 TLR structure

The receptors belong to the superfamily called the Toll/IL-1 receptor (TIR) family, because of their closely resemblance to receptor IL-1R, the first one in the group characterized. The type 1 single- pass transmembrane receptors are characterized by leucine-rich repeats (LRRs) in the extracellular space that interact with the ligands of the pathogens [26]. The receptors have membrane- spanning domains, and cytoplasmic TIR domains that can interact with TIR-containing adapters, creating a platform for mediating signal further downstream in the host cells [25]. The TIR adaptors known to mediate the signal further are myeloid differentiation factor-88 (MyD88), MyD88 adaptor-like protein (Mal), TIR domain- containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo- motif- containing protein (SARM) [27]. SARM is a negative regulator of TRIF-dependent signaling [28].

1.4.2 Types of TLRs, respective ligands, and localization in the cell

Each distinct TLR can sense different PAMPs from the pathogens and either alone or through cooperation, mediate the signal further downstream in the host cell [25]. Ligands from the outer layer of the complex cell wall of *M. avium* trigger the TLRs and further downstream activation [23].

Toll- like receptor 2 is present on the cell plasma membrane and senses bacterial lipopeptides. The receptor heterodimerizes with **TLR1** to recognice triacylated lipopeptides like the native mycobacterial 19-kDa lipoprotein [22], or **TLR6** to recognize diacylated lipopeptides [1]. The diacylated lipopeptide FSL-1 is synthesized based on an N-terminal structure of a 44 000 molecular weight lipoprotein from *Mycoplasma salvarium*, and is a

TLR2/6 ligand shown to enhance phagocytosis of bacteria by macrophages through signaling pathways [29]. PAM3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins. It is recognized thorugh dimerized TLR2/1 and leads to NF-κB activation [30]. TLR2 is the receptor responsible for recognizing most motives in the *M. avium* cell wall like different lipoproteins, phosphatidylinositol mannans and LM, and triggering of this receptor has shown to induce antimycobacterial activity. LM is arabinosylated to form lipoarabinomannan (LAM), which is the genuine TLR2 ligand [14,23]. TLR-2-deficient mice are slightly susceptible, and MyD88-deficient mice even more susceptible to *M. avium* infections [31,32].

Toll-like receptors 3, 7, 8, and 9 are located in the endosomes, and their ligands are microbial and host-derived nucleic acids [1]. TLR3 recognizes viral dsRNA and poly I:C [25]. TLR7 and TLR8 share similar structure and phylogenetics, and the same ssRNA ligands, and are known to have antiviral properties. In addition, synthetic imidazoquinoline-like molecules has been shown to activate NF- κB through TLR7 and 8. The thiazoloquinoline compound CL075 are shown to activate TLR8 specifically [33]. Previous research has shown that expression of TLR8 was up- regulated in macrophages after infection with BCG, and indicates a role of TLR8 in mycobacteria infections [5]. In addition some polymorphisms of the TLR8 gene is connected to susceptibility to Mtb [23]. TLR9 is identified to sense CpG-rich un-methylated DNA motifs frequently present in bacteria, among them mycobacteria [12]. TLR9 is also involved in the innate recognition of Mtb [34].

Toll- like receptor 4 can be present both on the cell plasma membrane where it signals through adaptor MyD88, and get endocytosed and signal from endosomes thorugh adaptor TRIF. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria [1], and the main recognized ligand for TLR4 in synergy with co-receptors MD2 and CD14 [35]. Infection with mycobacteria leads to recognition of PIM in the cell wall thorugh TLR4 [23].

Toll- like receptor 5 signals from the plasma membrane and senses bacterial flagellin that is a protein component of flagella. The receptor is connected to both innate and adaptive immunity particularly in the intestine [1], but is not essential for mycobacteria recognition as the bacteria are non- motile and do not have flagella.

Toll- like receptor 10's function is not yet elucidated, but it is known to have a similar structure to TLR1 and is therefore believed to heterodimerize with TLR2. The receptor ligand is not known, but it is recently linked to innate immune responses of viral infections [36].

Additional functional TLRs; 11, 12 and 13 have been recognized in mice, but not for humans [1]. Taken together, the only TLR receptor shown to be important for *M. avium* recognition so far is TLR2, while the role of the other TLRs are not elucidated or do not have a function. TLR4, 8, and 9 are previously connected to Mtb or other mycobacteria, and are interesting candidates for further studies.

1.4.3 NF-κB and IRF; transcription factors of two important TLR signaling pathways

The stimulation of TLRs activates signal transduction pathways thorugh adaptors such as MyD88 and TRIF, which facilitates activation of transcription factors like NF-κB and IRFs [5]. All TLR receptors except TLR3 use the MyD88 adaptor, while TLR3 uses the TRIF adaptor for signaling. TLR4 and possibly TLR2 [37,38] can use both MyD88 and TRIF to mediate its signals downstream from the cell surface (MyD88) or inside endosomes (TRIF). After adaptors have bound to the TIR-signaling platform of the cytosolic part of the receptors, they interact with IL-1R-associated kinases (IRAKs) and TNF receptor-associated factors (TRAFs). This in turn leads to activation of several pathways and transcription factors downstream, that initiates anti- microbial immune responses and expression of inflammatory cytokines, chemokines and type 1 Interferons that contribute to immune cell activation and migration [12, 39]. An overview of the different TLRs (except TLR10 that does not have an elucidated pathway or ligands yet), and their further downstream signaling in the cell, is presented in figure 2. All TLRs can initiate activation of the NF- κB pathway, while TLR2-4, and 7-9 can activate the IRF-pathways.

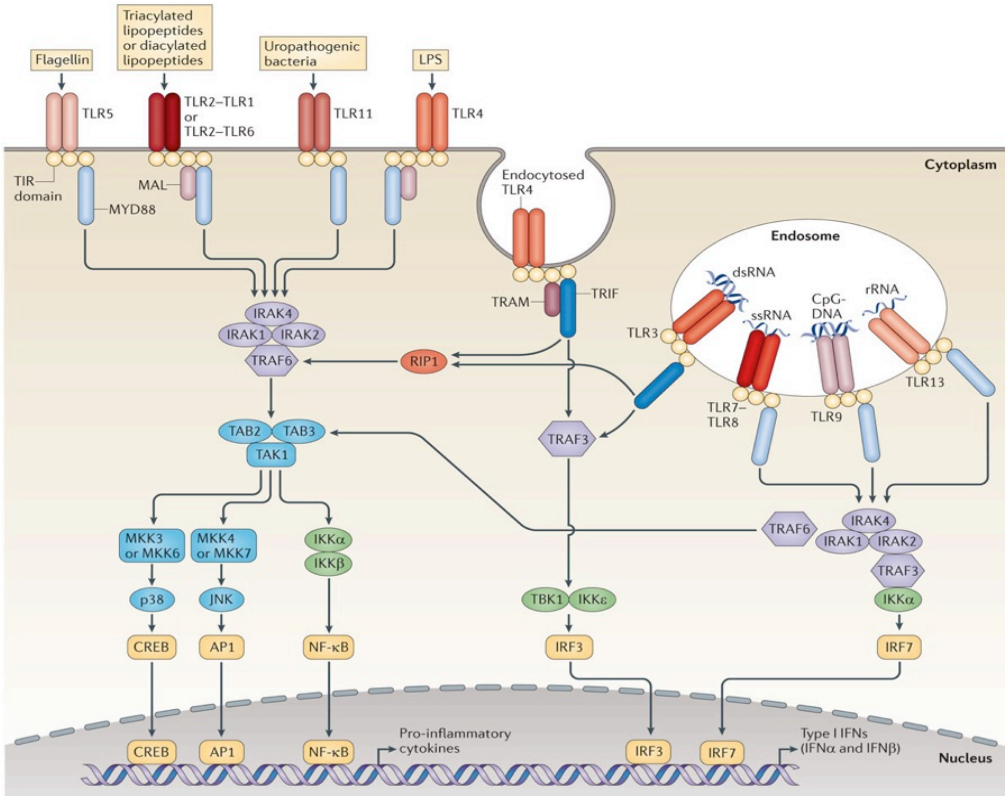

Figure 2: An schematic overview of the known Toll-like receptors 1-13 involved in innate immunity signaling, either from the cell membrane or inside endosomes. Only 10 functional are elucidated for humans, and 13 in mice. Picture taken from O'Neill et al, The history of Toll-like receptors- redefining innate immunity, 2013 [1].

1. Introduction

The canonical NF-κB pathway can be activated through both MyD88- dependent and TRIF-dependent adaptors and regulates expression of genes involved in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, stress responses and cancer progression.

MyD88 activates NF-κB through interaction with IRAKs; IRAK4 and IRAK1 get phosphorylated and bind to TRAF6 for further signal transduction. The IKK complex consists of subunits IKKα, IKKβ, and the regulatory component NF-κB essential modifier (NEMO/ IKKγ). TRAF6 function as an E3-ubiquitin ligase and promotes Lys63-linked poly-ubiquitination of NEMO, which in turn recruits a protein kinase complex with TAK1 (transforming growth factor-β- activated kinase-1) and TABs (TAK1 binding proteins). In the canonical NF-κB pathway the IKKβ subunit is responsible for the downstream cascade, and is assumed to be phosphorylated by TAK1, and get activated. The transcription factor NF-κB is present in the cell as homo- or heterodimers, bound to IκB (inhibitor of κB) proteins that masks the nuclear localization domain on the transcription factor in the cytosol. The IKK complex mediates phosphorylation of specific serine residues of IκB proteins, which leads to poly-ubiquitination and degradation in the 26S proteasomes. When IκB is degraded, NF-κB is free to translocate into the nucleus and mediate transcription of genes like tumor necrosis factor- α (TNF- α), Interleukin 6 (IL-6), IL-1 and IL-8 [40].

TRIF mediated NF-κB signaling is initiated thorugh recruitment and interaction with RIP1 (receptor- interacting- protein-1) and TRAF6. TRAF6 activates TAK1 in a similar fashion as described for the MyD88- dependent activation, and the rest of the cascade is similar to the MyD88- initiated one.

The IRF-pathway is induced either by MyD88- dependent or independent pathways. *The independent pathway* is initiated by stimulation of TLR3 or TLR4, when they signal through adaptors TRIF to activate transcription factor IRF3 (Interferon regulatory factor 3) [41]. IRF3 is usually expressed at high levels in most cells, and is responsible for both the early and late production of IFN-β. Two kinases related to the IKK kinases, inducible IKK (IKK-i/ε) and TRAF family member- associated NF- κB activator (TBK1), are believed to directly interact and phosphorylate IRF3 upon receptor stimulation [42,43]. As described previous TRIF activates TRAF-6 that leads to activation of the NF- κB pathway, but TRIF also interacts with TBK1 to facilitate phosphorylation and activation of IRF3. The phosphorylation of IRF3 causes a conformational change that exposes its IRF- association domain for dimerization, and DNA-binding domain for initiating transcription. The main target gene of the transcription factor is the IFNβ gene, and to a lesser degree the IFNα gene, that are transcribed with cooperation from NF-κB [42]. IFNβ expression activates a new transcription factor complex that induces transcription of IRF7 that in turn works as a later transcription factor for production of IFN-α/β. TBK1 and IKK-i can similarly phosphorylate and activate IRF7.

The MyD88 dependent pathway is initiated by stimulation of TLR7 and TLR9. Stimulation of the receptors leads to complex formation of the MyD88 adaptor with IRF7, IRAK4 and TRAF6 to activate the transcription factor IRF7, and induce transcription of IFNα/β [44, 45]. This pathway cannot activate IRF3.

Another receptor participating in the regulation of Interferon responses is STING (stimulator of interferon genes), located in the endoplasmic reticulum. When sensing pathogenic DNA in the cytoplasm through the cGAMP synthase cGAS [46], STING interacts with and activates TBK1 which further leads to phosphorylation of IRF3, and transcription of type 1 Interferon genes and induction of autophagy [47]. The role of Mtb in STING activation is suggested for Mtb [48], but not known for *M. avium*.

The IRF family has 9 members; IRF 1-9, where IRF 1, 3, 5, and 7 are involved in production of Type 1 Interferons [49]. IRF5 is reported to have a role in inducing type 1 Interferon cytokines as a response to mycobacteria infection [50], and thought to get activated thorugh a MyD88- and IRAK1- dependent pathway similar to IRF7 [42,51]. Activation of IRF5 thorugh IKKβ and TAK1 and production of IFNβ by stimulation of TLR8 by the Gram-positive bacteria *Staphylococcus aureus,* has recently been elucidated and has also surprisingly shown to be antagonized by co-activation of TLR2 [24]. IRF1 is activated through TLR9- MyD88 and is also known to be involved in Interferon gene production, and are suggested activated in Mtb infections [52].

1.4.4 Role of some early induced cytokines in mycobacteria infection

The induction of multiple pathways upon mycobacteria infections like NF-κB and IRF, leads to production of several important cytokines. Some of the important are listed below with their regulatory functions.

The cytokine **Tumor necrosis factor- α (TNF-α)** is induced upon infection with *M. avium* thorugh the NF-κB pathway, and is released by activated macrophages. The cytokine recruits immune cells to the site of infection, and stimulates formation and maintenance of granulomas. TNF-α also contributes to further activation of macrophages to increase the efficiency of engulfment and elimination of the bacteria [14]. It has been shown that blocking the activity of TNF can cause reactivation of latent Tb- infection [53]. TNF can bind to TNF-receptors, and activate canonical NF- κB signaling thorugh similar mechanisms as for the TLR receptors. TNFR's do not have TIR domains, but Death domains (DD), that signals through RIP and TRAFs [54].

Interleukin 8 (IL8, CXCL8) is a chemokine induced upon infection with *M. avium* from infected macrophages through the NF-κB pathway [55]. The cytokine is chemotactic for T-cells and neutrophils, and is induced by stimulation with lipopolysaccharides and cytokines like IL-1 and TNF- α in monocytes [56].

IFNβ: Production of IFN-α and β are up-regulated by macrophages as an immune response to bacterial nucleic acid exposure inside the cells. The cytokines have both pro- and anti-inflammatory functions. The cytokines are well known to induce antiviral function, while the role of the cytokines in mycobacteria infections are less characterized. IFN-α and β are shown to inhibit the production of IL-12, which leads to an increased susceptibility to intracellular bacteria [57], and more progressive *M. avium* infections [14]. Contradicting, IFN-α and β are shown to have antimicrobial activities for *M. avium* infection [58]. The type 1 Interferon's bind

to the IFNAR receptors, and mediates further signaling and immune functions through the JAK-STAT pathway.

Interferon inducible protein 10 (IP10, CXCL10) plays an important role in the innate and adaptive immune system in mycobacterial infections and is expressed in tissues primarily by monocytes and macrophages after stimulation of IFN-α/β/γ, lipopolysaccharide and other pro-inflammatory cytokines like TNF- α in synergy with IFN- γ [59]. In addition, expression can be directly induced by activation of IRF3 [49]. The cytokine's receptor is the CXC chemokine receptor (CXCR)3, and stimulation leads to trafficking of monocytes and T-helper cells to sites of inflammation [60].

1.5 Keap1- a sensor of ROS released upon infection with *M. avium*

As previously specified, the activation of PRRs induces the production of ROS as a defense mechanism to eliminate mycobacteria [21]. ROS could be damaging for the cells in large quantities, and regulation is therefore necessary. Kelch-like ECH-associated protein 1 (Keap1) function as a stress sensor that senses ROS released upon infection with *M. avium*. Keap1 has different binding domains for self-dimerization and regulation of components in autophagy and inflammation [61,62]. The protein's structure is presented in figure 3 with its different interaction domains and potential binding partners p62 and Nrf2.

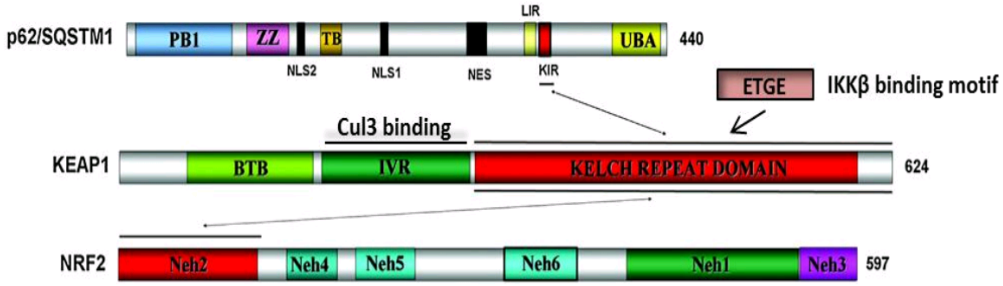

Figure 3: A schematic illustration of Keap1, p62 and Nrf2 proteins and their different binding domains and interactions with eachother. Figure is taken and modified from Ioannis P. Nezis et al, p62 at the interface of autophagy, oxidative stress signaling, and cancer, 2012 [4].

Keap1 has the role as an adaptor molecule for the Cullin (Cul)-based E3 ligase that consists of Cul 3, Roc1/Rbx1 and Hrt1. The Cul3 unit can interact directly with Keap1 thorugh the IVR domain, and cooperate in ubiquitinating target proteins for degradation [63]. The nuclear factor (NF)-erythroid 2 (E2)-related factor 2 (Nrf2) is a well-established mediator of cellular adaption to ROS, and is regulated through the Keap1- Cul3 ligase complex. In unstressed conditions Keap1 binds to Cul3 and to Nrf2 thorugh its Kelch domain, and targets Nrf2 for degradation by the 26 S proteasome. Upon ROS release one of the cysteine residues in the binding domain if Keap1 is modified, and the E3-complex fails to assemble and ubiquitinate Nrf2, which leads to nuclear translocation of Nrf2 and transcription of cytoprotective genes [64].

1. Introduction

Keap1 is seen associated with *M. avium*- containing phagosomes early after infection in a ROS- dependent manner [65].

Keap1 has also been linked to autophagy. The sequestosome 1/p62 receptor (p62) is an autophagy-adaptor protein that binds to ubiquitinated proteins to degrade them through autophagy, by co-interaction with LC3II at autophagosomes. p62 competes with Nrf2 for binding to the Kelch domain of Keap1, and targets Keap1 for autophagy-mediated degradation [61]. Phosphorylation of p62 through mTORC1 leads to increased affinity for Keap1, and Nrf2 binding is outcompeted leading to Nrf2 accumulation and induced expression of cytoprotective genes [66].

In addition to protect the cells from oxidative damage, Keap1 function has been linked to up-regulation of the NF-κB pathway and inflammation in cancer [62]. In normal conditions, Keap1 binds to IKKβ (previously described as a signal mediator of the NF-κB pathway), through the ETGE domain and Kelch domain, and thereby depress the pathway. This mechanism is dysregulated in some cancers with loss-of-function mutations in Keap1, and leads to failure of degradation of IKKβ and up-regulated inflammation [62]. Contradictory mechanisms of how Keap1 regulates IKKβ have been shown. Lee et al found that IKKβ was degraded in the proteasomes through ubiquitination by the E3-ligase complex [62], while Kim et al found that Keap1 regulates IKKβ in two ways; through ubiquitination and degradation by autophagy or inhibition of phosphorylation [67].

Our research group has, further investigated the role of Keap1 as a regulator of the kinases IKKβ and TBK1, for *M. avium* infections. Results displayed that knockdown of Keap1 induces increased cytokine production (TNF-α, IP10, IFNβ, IL-6 and IL-1β), increase in levels of IKK complex components, and TBK1 levels. This suggests a role of Keap1 in regulating these kinases upon *M. avium* infection [65]. TBK1 is in addition linked to phosphorylation of p62, enhancing LC3II affinity needed for efficient function in autophagy of the mycobacteria [68]. A schematic overview of Keap1's known and suggested interaction partners in inflammation and autophagy are presented in figure 4.

The findings highlights the potential role of Keap1 in down-regulation of inflammatory signaling (both through NF-κB and IRF-pathways) and autophagy, potentially increasing the survival of macrophages infected with *M. avium*.

1. Introduction

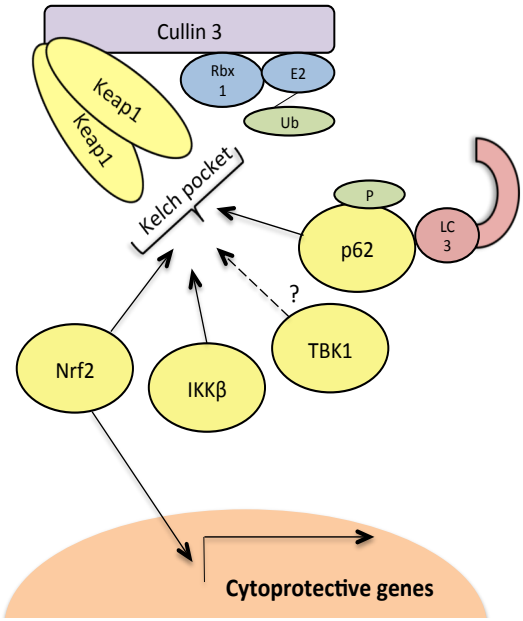

Figure 4: An overview of Keap1's known and suggested interaction partners in inflammation and autophagy upon M. avium infection. Arrows indicate interactions, and dashed arrow shows hypothesized interaction. (Image designed by author).

1. Introduction

2. Aims and objectives of the study

The overall aim of this project was to detail the mechanisms on how Keap1 interacts with IKKβ and TBK1 in macrophages to negatively regulate inflammatory cytokine production and type 1 IFN responses upon infection with *M. avium*. Lee et al showed that Keap1 directly bound to IKKβ thorugh an ETGE domain and Kelch domain, facilitating ubiquitination and degradation of IKKβ by the proteasome [62]. We hypothesize a similar mechanism for Keap1 regulation of IKKβ during *M. avium* infection, and in addition we aimed to determine if TBK1 might be regulated in a similar fashion, through direct interaction or through indirect regulations.

All the earlier findings from our research group were from studies in primary human macrophages [65]. These cells are hard to transfect and thus difficult to use for protein interaction studies needed to detail the regulatory mechanisms. Non- macrophage cell-lines do not necessarily respond to *M. avium* infection in a biological relevant manner, and experiments conducted in mice models cannot necessarily be extrapolated to humans. Thus to be able to study the pathways and the proteins involved, the main aim of the project was to:

- Establish a human macrophage cell-line to study how Keap1 might interact with and regulate IKKβ and TBK1 upon *M. avium* infection.

To be able to reach the overall aim, the specific objectives were:

- Find a human macrophage cell-line that could respond to *M. avium* in terms of phagocytosis and upregulation of inflammatory cytokines involved in the NF-κB and IRF- pathways.
- Make plasmids with full-length Keap1 and Keap1 deletion constructs, for studying domains involved in interactions
- Establish a stable knockdown of endogenous Keap1 in the chosen cell-line that responds to *M. avium,* and determine an inflammatory expression profile.
- Do pull-down experiments upon infection to determine potential binding partners of transient transfected Keap1. Use Keap1 deletion constructs to map presumed interaction domains.

3. Experimental methods

3.1 Reagents and kits

All reagents and kits used for experimental procedures are provided below, in table 1-8, and Appendix 1-3.

Reagents used for *M. avium* culturing and cell culturing: Dulbecco's Phosphate Buffered Saline (PBS), L-glutamine, Dimethylsulphoxide (DMSO) Hybri-Max, Phorbol 12-myristate 13- acetate (PMA, P1585), and Penicillin Streptomycin (Penstrep) were obtained from Sigma Aldrich Life Science. Dulbecco's modified Eagles medium and Ham's F12 medium (DMEM) and Trypsin-ethylenediaminetetraacetic acid (TE) were purchased from BioWhittaker, Lonza. Heat inactivated fetal bovine calf serum (FCS), Hepes Buffer Solution (1M), and Geneticin (G418) were obtained from Gibco Life Technologies. Gentamycin 2uM was ordered from Sanofi-Aventis. Roswell Park Memorial Institute (RPMI) medium were purchased from ATCC (1640 30-2001), and 2-Mercaptoethanol was obtained from Merck Millipore.

Table 1: Reagents and recipe for Middlebrook 7H9 liquid medium for mycobacteria culturing

Reagents	**Amount in 500 ml**	**Manufacturer**
Middlebrook 7H9 in 450 ml dH_2O	2,35 g	Difco/Beeton Dickinson
Glycerol (mix with medium, autoclave and cool)	1 ml	Merck Millipore
20 % Tween80 (sterile)	1,25 ml	Sigma- Aldrich
Albumin- dextrose catalase (ADC) enrichment	50 ml	Sigma- Aldrich

Table 2: Reagents and recipe for Middlebrook 7H10 plates for mycobacteria

Reagents	**Amount in 400 ml**	**Manufacturer**
Middlebrook 7H10 in 360 ml dH_2O	7,6 g	Difco
Glycerol (mix with medium, autoclave and cool)	2,0 ml	Merck Millipore
OADC	40 ml	Difco/ Beeton Dickinson

Reagents used for confocal microscopy: Paraformaldehyde (PFA) 16 % was purchased from Alfa Aesar and Draq5 4084L 5mM was obtained from Cell Signaling Technology.

3. Experimental methods

Reagents and kits used for RNA isolation and qPCR: RNase free DNase set and RNA isolation kit (RLT lysis buffer, RW1 wash buffer and RNase free water) were obtained from Qiagen. High Capacity RNA- to cDNA kit (2x RT buffer mix, 20 x RT Enzyme mix) was purchased from Applied Biosystems. Primers/probes GAPDH, IL-8, TNF-α, IFNβ, and CXCL10 were obtained from Applied Biosystems/ Life Technology. Perfecta qPCR FastMix was purchased from Quanta Bioscience Inc, MD, USA.

Reagents used for plasmid purification: PureYield Miniprep kit was obtained from Promega.

Table 3: Recipe for making Lysogeny broth (LB) medium and LB agar plates with antibiotics

Reagents	Amount	Manufacturer
Tryptone	10 g	LP0042, OXOID
Yeast extract	5 g	LP0021, OXOID
Sodium chloride Dissolved in 1 l destilled H2O, autoclaved	10 g	Merck, Millipore
Agar Added to make LB-plates before autoclaving Cool to <60°C before adding antibiotics	15 g	LP0011, OXOID

Reagents used for transient transfection: Gene Juice reagent was purchased from Novagen. Opti-MEM (1x) reduced serum medium was obtained from Gibco, Life Technologies.

Table 4: Plasmids used for transient transfection experiments

Plasmid backbone	Antibiotic resistance	Manufacturer/ provider
pDest GFP	Ampicillin, Sigma-Aldrich A9518	Ashish Jain, UiT
pDest GFP-Keap1	Ampicillin	Ashish Jain, UiT
pcDNA Cherry	Ampicillin	Nadra Nilsen, CEMIR
pcDNA TLR2 Cherry	Ampicillin	Nadra Nilsen, CEMIR
pcDNA TLR2 WT	Ampicillin	Nadra Nilsen, CEMIR
pUNO1 TLR8b	Blasticidin, Invivogen	Invivogen
pcDNA3.1	Ampicillin	Invitrogen

3. Experimental methods

Reagents and kits used for Western blotting and Immunoprecipitation: NuPage Novex 12 % Bis- Tris Gel mini, NuPage LDS gel loading buffer 4x, iBlot Gel Transfer Mini nitrocellulose membranes, SeeBlue Plus2 pre-stained marker and Magic mark XP, and MOPS 20x SDS running buffer (diluted to 1x working solution with deionized water) were purchased from Novex, Life Technologies. Dithiothreitol (DTT) 1 M was obtained from AppliChem. Dynabeads protein A was ordered from Life Technologies 10002D. Primary antibody GFP ab290 rabbit polyclonal was purchased from Abcam. Bis(sulfosuccinimidyl)suberate (BS^3) no weight format was ordered from Thermo Scientific.

Table 5: Primary and secondary antibodies towards proteins of interest, used for Western blotting and Immunoprecipitation.

Gene of interest	Primary antibody	Dilution	Antibody solution	Secondary antibody	Dilution	Antibody solution
GFP/GFP-Keap1	GFP ab290 rabbit, Abcam	1: 3500	5% non-fat milk in TBS-T	Goat anti-rabbit LI-COR IR dye 800 CW, Odyssey	1:5000	TBS-T
TLR2	TLR2 (D7G9Z) rabbit mAb#12276, CST	1:1000	5 % BSA in TBS-T	Goat anti-rabbit LI-COR IR dye 800 CW, Odyssey	1:5000	TBS-T
TLR8	TLR8 (D3Z6J) rabbit mAb#11886, CST	1:1000	5 % BSA in TBS-T	Goat anti-rabbit LI-COR IR dye 800 CW, Odyssey	1:5000	TBS-T
Endogenous control Actin	Actin AC-15 mouse ab6276, Abcam	1: 10000	5 % non-fat milk in TBS-T	Goat anti-mouse LI-COR IR dye 680 RD, Odyssey	1: 5000	TBS-T
GFP	GFP JL-8 mouse monoclonal, Living Colors	1:5000	5 % BSA in TBS-T	Goat anti-mouse LI-COR IR dye 680 RD, Odyssey	1:5000	TBS-T
Keap1	Keap1 rabbit polyclonal, 10503-2-AP, Proteintech	1:2000	5 % BSA in TBS-T	Goat anti-rabbit LI-COR IR dye 800 CW, Odyssey	1:5000	TBS-T

3. Experimental methods

Ligands used for stimulation experiments:

Table 6: Ligands used as control for the presence of different receptors in transfected cells, in stimulation experiments

Ligand	Derived from	Concentration experiments	Ligand for receptor	Manufacturer
LPS (Lipopolysaccharide)	*E.Coli* K12	200 ng/ml	TLR4	Invivogen
LM (Lipomannan)	*Mycobacterium Smegmatis*	200 ng/ml	TLR2	Invivogen
FSL-1	Synthetically made, represents parts of LP44 of *Mycoplasma salvarium*	200 ng/ml	TLR2/6	EMC microcollections GmbH
PAM3CSK4 (Pam3Cys-SKKKK)	Synthetically made, represents lipopeptide	200 ng/ml	TLR2/1	EMC microcollections GmbH
CL075	Thiazoloquinolone derivative	1,5 ug/ml	TLR7/8	Invivogen

Reagents used for PCR amplification of genes and purification: KOD Xtreme Hot start DNA polymerase kit was purchased from Novagen. Loading dye 6x was obtained from Thermo Scientific. Ladder 1 kB was ordered from manufacturer New England Biolabs Inc. QIAquick PCR purification kit and QIAquick Gel extraction kit were purchased from Qiagen.

Reagents and kits used for Gateway cloning: Gateway BP Clonase II Enzyme mix, LR Clonase II plus Enzyme mix, pDONR221, Proteinase K, pEXP7-tet fragment, and M13 forward primer and reverse primer were purchased from Invitrogen. Ampicillin was obtained from Sigma-Aldrich A9518. Restriction enzymes EcoRV and ApaLI, 10 x NEBuffer 2 pH 7,9 + 10 mg/ml (100x) BSA were purchased from New England Biolabs Inc. TE buffer endotoxin free, pH= 8,0 was ordered from manufacturer Qiagen. GoTaq Green mastermix x2 was obtained from Promega. Super optimal broth (SOC) medium, Kanamycin, and One Shot MAX Efficiency DH5-α-competent cells were purchased from Life Technologies.

3. Experimental methods

Table 7: An overview of plasmids used for gateway cloning, the genes of interest, the plasmids specific antibiotic resistance, att- sites and manufactures/ providers.

Vector backbone	Gene for recombination	Antibiotic resistance	Purchased from	att sites
pDest	GFP-Keap1	Ampicillin, 100 ug/ml	Kind gift from Ashish Jain, UiT	attB1, attB2
pDest	GFP	Ampicillin, 100 ug/ml	Kind gift from Ashish Jain, UiT	attB1, attB2
pcDNA YFP	TLR2	Ampicillin, 100 ug/ml	Addgene	-
pUNO1	TLR8b	Blasticidin, 50 ug/ml	Invivogen	-
pDONR221	ccdB	Kanamycin, 50 ug/ml	Invitrogen	attP1, attP2
pLenti CMV PURO Dest w118-1	ccdB	Ampicillin, 100 ug/ml	Addgene [69]	attR1, attR2
pDest-myc	ccdB	Ampicillin, 100 ug/ml	Kind gift from Marte Singsås Dragset	attR1, attR2

Reagents used for Immunofluorescence staining:

Table 8: Recipe for PBS/ Saponin solution

Reagents	Amount (ml)	Manufacturer
Saponin (1 g to 10 ml deonized water for 10 % , sterile filtrate)	0,25	Sigma Aldrich 7900
PBS	47,5	Sigma Aldrich

Quenching buffer: 2,5 ml NH_4Cl (1 M) from Sigma Aldrich was added to PBS/ Saponin solution.

Antibody buffer: 1 % heat inactivated A+ serum from the Department of Immunology and Transfusion Medicine at St-Olavs Hospital, Trondheim, was added to PBS/ Saponin solution.

Blocking buffer: 20 % heat inactivated A+ serum was added to PBS/ Saponin solution.

Primary antibody Keap1 rabbit polyclonal was obtained from Protein Tech 10503-2-AP. Secondary antibody Alexa Fluor dye 647 goat anti- rabbit was purchased from Life Technology.

Reagents and kits used for lentiviral transduction: pMDLg/pRRE, pRSV/REV, pMD2.G, and pLenti CMV Puro Dest w118-1 were purchased from Addgene. Puromycin dihydrochloride #540222 was obtained from Calbiochem. Polybrene was ordered from Sigma Aldrich.

3.2 *Mycobacterium Avium* maintenance

Transformants of the virulent *Mycobacterium Avium* strain clone 104 expressing either dsRed or CFP were used in all experiments [9].

Bacteria were grown in Middlebrook 7H9 broth medium supplemented with ADC enrichment, recipe provided above. Suspension was grown at 37°C under agitation in logarithmic phase, optical density (OD) between 0,3- 0,8.

Bacteria in logarithmic phase were frozen in glycerol stocks of 15 % autoclaved glycerol in 7H9 medium, – 80 °C. To recover the bacteria, a small inoculum was scraped with a pipette and streaked on a 7H10 agar plate, and incubated at 37 °C. Single colony of bacteria was selected and added into autoclaved 7H9 medium for making a liquid culture.

3.3 U373-CD14 cell-line

U373- CD14 is a human glioblastoma astrocytoma cell-line derived from a malignant tumor with stably transfected CD14. The cell-line was a kind gift from Dr. R. Thieringer (Merck research laboratories, New Jersey).

The adherent cells were grown in DMEM with 10 % heat inactivated FCS and 100 ug/ml L-glutamine. Two micromole Gentamycin was added to stocks to avoid contamination, and 1 % Geneticin (G418) was added to select for CD14 expressing cells with neomycin resistance. Cell stocks were incubated in T-75 flasks at 37 °C in 8 % CO2. Cells were passaged 1:8 two times a week to ensure proper confluent cells in culturing flask.

3.4 THP-1 cell-line

The THP1 cell-line was purchased from ATCC (TIB-202) and is a monocytic cell-line from peripheral blood tissue derived from a boy with acute monocytic leukemia [70].

The suspension cells were cultured in RPMI medium with high glucose levels and 100 µg/ml L-glutamine. One percent Hepes, 10 % FCS and 0,05 mM 2- Mercaptoethanol were added to obtain growth medium. To avoid contamination, 1 % penstrep was provided in culturing flasks. Cell stocks were incubated in T-75 flasks at 37 °C in 5 % CO2. Cells were passaged into 0,25 x 10^6 cells/ ml three times a week.

Cells used in experiments were differentiated into macrophage-type cells using 40 ng/ml PMA. Cells were left to rest for 48 hours, and medium was changed to complete growth medium. After 72 hours, cells were used for experiments.

3.5 HEK 293T cell-line

HEK 293T (293tsA1609neo) cells are embryonic kidney cells derived from the HEK 293 cell-line, but with stably transfected SV40 large T antigen. The cell-line was purchased from Thermo Scientific Open Biosystems, and is optimal for production of high titers of lentiviruses.

The adherent cells were grown in DMEM with 10 % FCS and 100 ug/ml L-glutamine. One percent Penstrep was added to cell culture flasks to prevent contamination. Cell stocks were incubated in T-75 flasks at 37 °C in 5 % CO2. Cells were passaged at 80 % confluence, 1:10 dilution about two times a week.

3.6 Cell-line maintenance

Adherent cells were washed gently with PBS before adding 3 ml Trypsin- EDTA to the culture flask, followed by 3 minutes incubation in 37 °C. Complete medium in a 1:1 ratio was added to neutralize the trypsin. Both adherent cells and suspension cells were spun down at 1500 rpm (1000 rpm for THP-1 cells) for 5 minutes, tube was flicked to loosen the pellet, and pellet was re-suspended carefully in complete medium and appropriate amount of cells passaged to a new T-75 flask with complete growth medium. Cell culture flasks were kept at passage numbers under 20.

Cells were cryopreserved down in 2 ml sterile cryovials, 1 million cells/ml diluted in 10 % sterile DMSO. Tubes were frozen immediately in -80 °C in an isopropanol chamber, and transferred to liquid nitrogen following day.

When cells were recovered from liquid nitrogen, the cryovials were thawed in a 37 °C water bath rapidly, vial was wiped with 70 % ethanol and cells were added to pre-warmed complete medium. Cells were spun down for 5 min at 1000 rpm, and re-suspended gently in complete medium in appropriate culture flask size.

3.7 Infection experiments with *M. avium*

Cell-lines U373- CD14 and THP1 were used for infection experiments with *M. avium* stably expressing CFP and dsRed, and imaged by confocal microscopy. In addition infection experiments were performed to assess cytokine levels detected by q-PCR as a response to the infection with U373-CD14 cells, TLR-transfected U373-CD14 cells, and TLR-transduced U373-CD14 cells.

Cells in suspension were counted using Cell Countess from Invitrogen. For U373-CD14 cells, 400 000 cells were seeded to each 35 mm glass bottom γ-irradiated tissue cell dish (from MatTek Corporation, Ashland, MA) in 2 ml complete medium without gentamycin, the day before the experiment. For THP1 cells, 200 000 cells were seeded in each dish, and differentiated as described previously.

M. avium was harvested by spinning down culture at maximum speed for 3 min, and cells were washed one time by re-suspension in PBS. The suspension was vortexed and sonicated for 30 seconds, 3 times. A syringe of 0,5x 16 mm was used to further separate clumps and make a single-celled suspension of bacteria. The OD was measured with Ultrospec 10 Cell density meter (Amersham Bio Sciences) before wash to determine the growth phase, and after wash to determine amount to add for right multiplicity of infection (MOI). An OD of 1 corresponding to 4,5 x 10^8 bacteria/ml was used to calculate number of bacteria corresponding to colony forming units (CFU) [9]. Different MOI's and time points of infection were investigated for each cell-line as presented in table 9.

Table 9: Cell-lines U373-CD14 and THP1 were infected with different MOI's of M. avium for different time-points

U373-CD14		THP-1	
Multiplicity of infection (MOI)	**Time course (hours)**	**Multiplicity of infection (MOI)**	**Time course (hours)**
10	4	10	4
	6		
50	4	20	4
	8		
100	4	50	4

3.8 Confocal imaging

Confocal imaging is an optical imaging technique, and was chosen based on its ability to obtain high- resolution images, reduce background from the focal plane and collect serial optical sections through the cells for a 3D image. Optical sections could also help to determine if bacteria are present inside the cells. A beam splitter can separate the light from the laser and allow only one laser light of a particular wavelength to reach the cells. The emitted fluorescent light from the cells goes thorugh a pinhole with selected size before reaching the photomultiplier detector, to remove out of focus light [71]. Imaging with confocal microscopy was performed for infection experiments with dsRed or CFP *M. avium* for U373-CD14 and THP1 cells, and transfection experiments.

35 mm glass bottom γ-irradiated tissue cell dishes with cells were washed in PBS, and fixed with 3 % PFA. Cells were kept on ice during fixation for 10 minutes, washed with PBS two times and left in 1 ml PBS in 4 °C. Some of the dishes were stained with 5 µM Draq5, a nuclear stain, after fixation.

Cells were studied with differential interference contrast (DIC) on confocal microscopy Zeiss LSM 510 Meta FCS with objective 63 x 1,4 Oil DIC (Zeiss). Different lasers and filters were used to study different flurophores, as presented in table 10. Pinhole was chosen to 1 airy unit (AU) and Z stacks were created thorugh the cells. When dsRed and Draq5, or dsRed and Alexa 647 were present in same cells, two tracks were used to avoid overlap of emitted light. Images were assembled and observed using the Fiji Image J software.

Table 10: Different lasers and filters at confocal microscopy used for the different flurophores in the observed cells

Fluorophore in cells	Laser (nm)	Filter
CFP	405	Bandpass 470-500 nm
GFP	Ar 488	Bandpass 505-530/ 570IR nm
dsRed	HeNe 543	Longpass filter 650 nm/ bandpass 560-615 nm
Draq5	HeNe 633	Longpass filter 650 nm
Alexa 647	HeNe 633	Longpass filter 650 nm

3.9 EVOS FL Auto Cell Imaging System

EVOS FL Auto (Life Technologies) is an auto imaging system that can be used for monitoring of cell cultures and time-lapse cell imaging. The microscope was used to study efficiency of transfection for U373-CD14 cells with different fluorescent plasmids, concentrations and time points. Light cubes used were GFP (excitation 470/22, emission 510/42) and Texas Red (585/29, 624/40), and objectives Plan Fluor 10x LWD PH, Plan Fluor 20x LWD and Plan Fluor 40x LWD PH. Both fluorescence and transmitted light imaging systems were used.

3.10 RNA extraction and q-PCR assessment of mRNA levels

Quantitative PCR was performed for analysis of inflammatory cytokine levels as a response to stimulation experiments with *M. avium* or different ligands, for cell-line U373-CD14, TLR-transfected U373-CD14 or TLR-transduced U373-CD14.

To extract RNA from cells, 350 ul RLT lysis buffer with 0,1 % beta- mercaptoethanol were used to lyse the cells. Cell lysate were added to 2 ml collection tubes and RNA was purified using the QIAcube instrument and the Qiagen RNeasy mini kit. Program "RNA mini for animal cells and tissue with DNase" was used for the purifications. RNA concentrations and purity was measured with NanoDrop ND-1000 from Saveen Werner. Two different ratios of absorbance were used to assess the purity, and a ratio of around 2,0 for 260/280 and around 2,0- 2,2 for 260/230, were generally accepted as "pure" for the RNA.

Purified RNA was converted to cDNA using kit High Capacity RNA- to cDNA. The reagents provided in the kit are required for reverse transcription of total RNA to single- stranded cDNA. To each sample, following reagents were added to a MicroAmp Fast 8-tube strip 0,1 ml, Open Biosystems:

- 10 ul 2x RT buffer mix (with dNTP's, random octamers and oligo dT-16)
- 1 ul 20 x RT Enzyme mix (with MuLV and RNase inhibitor protein)
- 9 ul RNA sample

Samples were loaded to a 2720 Thermal cycler from Applied Biosystems, and program run for reverse transcription is presented in table 11.

Table 11: Program for reverse transcription of RNA to cDNA with kit from Applied Biosystems.

Step	Temperature	Time
Denaturation	37 °C	60 min
Annealing	95 °C	5 min
Extension	4 °C	∞

3. Experimental methods

cDNA samples were diluted with sterile ion filtered water (SIW) to a final concentration of 1 ng/µl and it was assumed that all RNA was reverse transcribed to cDNA. To determine the fold induction of cytokines produced for the different samples compared to reference control, relative quantification was performed with q-PCR and Taq Man assays.

In a Taq Man based assay, an oligonucleotide probe in a mix with forward and reverse primers specific for the gene of interest is used. The probe has a fluorescent reporter dye on the 5' end, and a quencher dye one the 3' end of the strand. When the probe is intact, the dyes are close to each other, and the fluorescence emitted is low due to fluorescence resonance energy transfer (FRET). When the target gene is present, the primers anneal and the gene of interest is amplified, while the probe anneals downstream to the forward primer site. The probe is cleaved when the Taq DNA polymerase reaches it. The cleavage separates the two dyes on the probe, and the fluorescence signal is increased. In addition the probe is removed from the target stand and the primer extension can continue. Every time the gene is amplified, reporter dyes are cleaved off its probes, increasing the fluorescence intensity proportional to the amount of genes in the sample [3]. A simplified overview of the reaction is shown in figure 5.

Primers/probes specific for the genes and Perfecta qPCR FastMix were used for the reaction mix set-up. The 2 X reaction buffer contains AccuFast Taq DNA polymerase with antibodies that binds to the polymerase, keeping it inactive prior to the PCR denaturation. It also contains optimized concentrations of MgCl2, dNTPs (dATP, dCTP, dGTP, dUTP), UNG, ROX and stabilizers. The reagents were added to a 96 well plate:

- 10 µl Perfecta qPCR FastMix
- 1 µl primer/probe
- 9 µl cDNA with concentration 1ng/µl

PCR plates were spun down and inserted in the StepOnePlus instrument from Applied Biosystems. PCR program steps are presented in table 12. StepOne Software version 2.2 and 2.3 were used to analyze experiments.

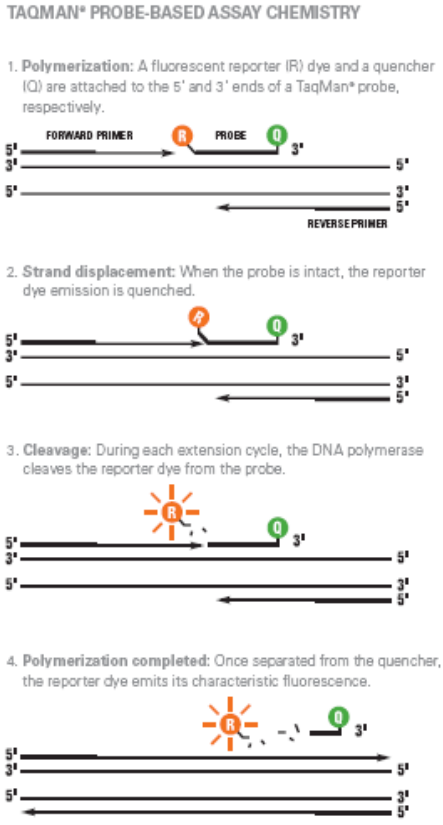

Figure 5: The principle of a TaqMan based assay used in q-PCR to quantify amount of cDNA (RNA) present in the cells. Figure taken from Life Technologies [3].

Table 12: Program run for q-PCR to amplify the genes of interest and quantify them based on emitted fluorescent light from the probes with a Taq Man assay.

Step	Temperature	Time
UNG incubation	45 °C	2 min
Initial denaturation	95 °C	30 s
PCR cycling	95 °C	2 s
	60 °C	20 s x 40 cycles

Samples were analyzed with relative quantification, and cycle threshold (Ct) values from the q-PCR analysis were obtained for each biological replicate. Ct is the number of cycles required for the fluorescent signal to cross the threshold and exceed the background level. The value is also inversely proportional to the amount of target cDNA detected in the sample. Ct values obtained from endogenous control were subtracted from Ct values obtained from cytokine of interest, to obtain delta Ct values. Delta delta Ct values were calculated subtracting the delta Ct value of the reference sample from the delta Ct values of the samples. RQ (relative quantification) values represents fold induction compared to reference sample, and could be calculated by the formula: (2^^-(delta Ct)).

3.11 Plasmid purification

Glycerol stocks of *E.coli* DH5-α with plasmids were frozen down in cryotubes at -80 °C. To purify the plasmids, the bacteria cultures were thawed on ice briefly, and some of the culture was added to LB-medium with antibiotics specific for the plasmids resistance. Cultures were incubated over night in 37 °C under agitation. Following day, plasmids were purified from the confluent bacteria cultures following alternative protocol of PureYield plasmid miniprep from Promega, provided in Appendix 1. Measuring purity and concentrations was performed with NanoDrop ND-1000 as previously described.

3.12 Transient transfection of plasmids with Gene Juice reagent

Transient transfection experiments were performed to assess the transfection efficiency of the U373-CD14 cells, and to determine whether transfection of TLR2 and/or TLR8 gave an upregulation of inflammatory cytokines in response to *M. avium* infection or ligand stimulation. Plasmids were purified as described previously.

When passaging cell cultures, cells were seeded in Costar 6 well plates, Costar 24 well plates and 35 mm glass bottom γ-irradiated tissue cell dishes. To obtain about 50-60 % confluence recommended for optimal transfection, number of cells seeded for each format is presented in table 13. Following day, medium was changed about 1 hour before transfection.

3. Experimental methods

Gene Juice was used as transfection reagent for transient transfection because of its low cytotoxicity, easy usage and earlier reported efficiency for the cell-line in our lab. User protocol TB289 from Novagen was followed for all experiments. Appropriate amount of Optimem or serum-free DMEM medium was added to eppendorf tubes, each tube representing one plasmid. Gene Juice reagent was added dropwise to the medium followed by briefly vortexing, spinning down liquid, and 5 minutes incubation. Plasmid DNA in appropriate concentrations was added to the tube and mixing was performed by carefully pipetting. Tubes were incubated for another 15 minutes. All liquid were added dropwise to the wells/ dishes, and mixed by gently rocking from side to side. Cells were left in incubator for 37 °C, 8 % CO2, and after 24 hours medium was replaced. Further incubation followed for as long as required for the experiment. Corresponding amounts of plasmid, Optimem/ serum-free medium and GeneJuice used for transfection experiments in different formats is presented in table 13, and volumes were scaled up for several wells.

The transfection efficiency of the U373-CD14 cell-line was assessed for different plasmids, concentrations and time-points. Cells were observed by imaging with a confocal microscope or EVOS FL Auto Cell imaging system. Western blotting was preformed for some experiments to determine amount of protein expression. Immunoprecipitation was performed for one experiment to determine amount of transfected proteins pulled out by magnetic Dynabeads.

Table 13: Summary of parameters for different scales of transient transfection with Gene Juice. Amounts are given for one well, and scaled up for several samples.

Plate/ scaled used	Seeded cells day before	Amount of plasmid (ug)	Amount of Optimem/medium (ul)	Amount of GeneJuice (ul)
6 well plate	200 000	1,5	100	4,5
6 well plate/confocal dish	200 000	1	100	3
6 well plate	200 000	0,5	100	1,5
6 well plate	200 000	0,3	100	0,9
24-well plate	70 000	0,4	25	1,2
24-well plate	70 000	0,2	25	0,6

3.13 Western blotting

Western blotting was assessed to separate and identify specific proteins from a complex mixture of proteins, here from cell lysates. The technique allows separation by size with gel electrophoresis, and the protein(s) of interest are transferred to a membrane and visualized by staining with specific primary and secondary antibodies. The thickness of the bands corresponds to amount of protein present, and the amount can also be relatively quantified by comparing it to a stained endogenous control protein [72]. The control protein should be a house- keeping gene, whose concentration levels are not altered to stimulations.

The technique was used to assess levels of protein expression 72 hours post transient transfection of U373-CD14 cells with different concentrations of several plasmids**.**

3.13.1 Protein extraction from cells

After transfection experiments, cells were washed two times with PBS and lysed on ice for 20 minutes with 150 ul 1x lysis buffer, recipe provided in Appendix 2. During the 20 minutes, the plates were rocked every 5 minutes to ensure coverage of whole well. Lysed cells were collected with a cell scraper, transferred to eppendorf tubes, and frozen down at -20°C if not used the same day. To continue the procedure, the eppendorf tubes with lysate were kept on ice for an additional 10 minutes to ensure complete lysis, and vortexed every 5 minutes. Lysate was centrifuged at 10 000 rpm for 20 minutes at 4 °C, and supernatant was collected.

3.13.2 Gel electrophoresis

NuPage Novex 12 % Bis-Tris Gel mini was used for gel electrophoresis. The chambers and gels were assembled and filled with running buffer 1x MOPS as described in the Quick reference leaflet instruction provided with the gels. The chamber was kept in a box with ice during the run to prevent over heating of the system. Fourteen microliter of the protein lysate for each sample was mixed with 7 ul NuPage LDS gel loading buffer 4x containing 0,1 M DTT. Tubes were heated for 10 min at 70 °C to denaturize proteins, and spun down to collect evaporated liquid. Twenty microliter of the lysate was loaded to each well of the gel and See Blue marker and Magic mark were loaded as ladders to determine the protein sizes. Gels were run at settings: 1) 30 minutes, 100 V, 2) 90 minutes, 150 V.

3.13.3 Blotting of proteins to membrane and staining with antibodies

When gel electrophoresis was completed the protein bands were transferred to nitrocellulose membranes, following the dry blotting manual provided by Invitrogen. The blotting was performed at 20 V for 11 minutes in iBlot from Invitrogen, and washed in 1x TBS-T wash buffer (recipe provided in Appendix 2) for 5 minutes under agitation.

Membrane was blocked with 5 % non- fat milk in TBS-T for 1 hour in room temperature under agitation, to prevent unspecific binding of the antibodies.

Primary antibody solution was added to the membrane over night under agitation at 4 °C. Blot was washed in TBS-T, and primary antibody solution for endogenous control Actin was

added to the membrane, and incubated 1 hour in 4°C under agitation. After staining, the membrane was washed 3 times with TBS-T.

Fluorescent secondary antibodies towards primary antibodies were diluted 1:5000 in TBS-T and added to the membrane. The blot was covered in foil and incubated for 1 hour at 4 °C under agitation. The membrane was washed 3 times with TBS-T after ended staining.

The membrane was dried on paper for 30 min under foil to protect flurophores from light exposure. Blots were analyzed with instrument Li-Cor Odyssey Fc and software Image Studio light. Images were acquired for channels 680 nm and 800 nm, and semi-quantification of the bands representing proteins of interest was performed by comparing absorption values to corresponding actin bands absorption values.

3.14 Immunoprecipitation

Immunoprecipitation was performed with magnetic Dynabeads to investigate if transient transfected GFP and GFP-Keap1 in U373-CD14 could be "pulled out" from the cell lysate. The technique allows precipitation of a specific protein antigen out of a solution by using a specific antibody cross-linked to the Dynabeads. With help from a magnet, the proteins can be separated out of the mixture of other proteins, principle shown in figure 6.

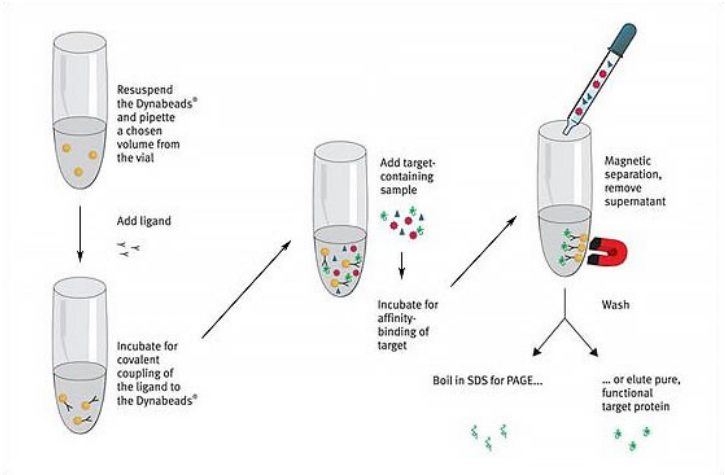

Figure 6: The principle of Immunoprecipitation using magnetic beads. First the ligand of interest, an antibody towards the target protein is cross-linked to the Dynabeads. The sample with the protein-mix is added, and incubated. With help from a magnet, the beads with bound target protein can be removed from the sample, and purified. In the end, the proteins of interest can be eluted. Figure taken from Life Technologies [6].

3.14.1 Protein extraction from cells

After 72 hours transfection, cells were washed two times in PBS and lysed with 200 ul HA-lysis buffer, recipe provided in Appendix 3**.** Cells were kept on ice for 20 minutes, before scraped out of the wells using a cell scraper and transferred to separate eppendorf tubes. Cell lysate was centrifuged at 10 000 rpm for 10 minutes, supernatant was collected in new tubes representing "total lysate input", and frozen down at -20 °C.

3.14.2 Preparation of the Dynabeads and crosslinking of antibodies

Preparation of the magnetic Dynabeads with protein A was performed according to protocol provided by Jennifer Mildenberger, CEMIR. Per sample, 50 ul (1,5 mg) of beads were added to separate eppendorf tubes. Primary antibody GFP ab290 rabbit polyclonal was used as crosslinking antibody for the beads with concentration 1:20 in PBS with 0,02 % Tween-20. Tubes with beads were placed on a magnet to separate the beads from the liquid, liquid was removed, and 100 ul of antibody solution was added to each tube following incubation at room temperature for 1 hour. Using the magnet, supernatant was removed and beads-Ab complex was re-suspended in PBS-Tween two times for washing, and left in PBS-Tween after.

From a fresh stock solution it was prepared 100 mM BS^3 in Conjugation buffer, recipe provided in Appendix 3. The stock solution was diluted to 5 mM by further dilution in same Conjugation buffer. The tubes with Dynabeads-Ab complexes were washed twice in 400 ul Conjugation buffer by using the magnet, and supernatant were discarded. Dynabead-Ab complexes were re-suspended in 250 ul 5 mM BS^3, and incubated at room temperature for 30 min with rotation for cross-linking. Reaction was quenched by adding 12,5 ul Quenching buffer, recipe provided in Appendix 3, and incubated at room temperature for 15 minutes with rotation. Cross-linked Dynabeads were washed three times in 400 ul 0,1 % PBS-Tween by using the magnet, and left in same amount in the end for storage in 4 °C.

3.14.3 Immunoprecipitation, gel electrophoresis and staining

Immunoprecipitation was performed by adding 50 ul of the crosslinked Dynabeads to separate eppendorf tubes, one for each sample. Supernatant was removed with help from magnet, and "total lysate input" from samples were added to each tube, to promote binding of antigen to beads. Twenty microliter of all samples were kept for later loading to gel electrophoresis. Samples were gently re-suspended and left for incubation over night at 4°C on rotator. Following day, samples were placed on the magnet, and supernatant was removed and kept in separate tubes as "unbound sample antigen". The Dynabead-Ab complexes were washed 4 times with 300 ul HA-lysis buffer, and resuspended in 100 ul 0,2 % PBS-T in new eppendorf tubes.

To elute the target antigen from the beads, 4x NuPage LDS gel loading buffer with 0,1 M DTT were mixed 1:1 with HA-lysis buffer (20 ul pr. sample). All samples were placed on the magnet, supernatant removed, and the sample buffer mix was added and mixed by gentle pipetting. Samples were heated for 10 minutes at 80 °C, and supernatant was collected as

"bound target antigen". The "Unbound sample antigen" was mixed in a 2:1 concentration with 4x NuPage LDS gel loading buffer 0,1 M DTT, and 5 % of "total lysate input" mixed in same loading buffer. Tubes were heated for 10 min at 70 °C to denature proteins, and samples were spun down to collect evaporated liquid.

Gel electrophoresis, membrane blotting and analysis were performed as described in Section 3.13.2+3. Primary mouse monoclonal antibody GFP JL-8 was used to stain for GFP at the membrane, to avoid using same primary antibody as for cross-linking to magnet beads.

3.15 Stimulation of U373- CD14, TLR- transfected U373-CD14 cells and stable U373-CD14 cell-lines expressing TLR2 or TLR8

Stimulation of U373-CD14 cells, TLR-transfected U373-CD14 cells and cell-line U373-CD14 stably expressing TLR2 or TLR8 was performed to assess if the response towards *M. avium* were enhanced with the presence of the TLRs. Ligands used for the experiments were LPS, LM, FSL-1, PAM3CSK4 and CL75, and *M. avium* was used at an MOI of 50. At the end of time- points, cells were lysed and q-PCR performed as described previously. Cytokines screened for were TNF-α, IFNβ, IL-8 and IP10, and GAPDH was used as endogenous control.

3.15.1 U373-CD14 cells

When passaging cells, 100 000 cells per well were seeded in 24 well Costar plates the day before experiment start. *M. avium* was prepared as described previously and infected in MOI's of 20 and 50 for time-points 30 min, 2 hours, 4 hours, 6 hours and 8 hours. LPS was used as positive control in concentration 200 ng/ml.

Reference sample used was un-stimulated cells in medium. All experiments were performed in biological triplicates, with two technical duplicates for each sample.

3.15.2 TLR- transfected U373-CD14 cells

Infection of TLR2 and TLR8- transfected U373-CD14 cells were performed to observe if the inflammatory cytokine response was enhanced towards the infection. All transfections were performed as described previous.

Plasmids pcDNA3.1, pcDNA TLR2 WT, pcDNA Cherry TLR2 and pUNO1 TLR8b were used in concentrations of 0,2 or 0,4 ug for 72 hours transfection. Experiments were performed in Costar 24 well plates with set-up like presented in table 14. After 72 hours transfection, the cells were stimulated with the ligands for 4 hours; LPS, LM, FSL-1, PAM3CSK (200 ng/ml) or CL75 (1,5 µl/ml), or infected with 50 MOI *M. avium*. Cells transfected with plasmid pcDNA3.1 were used as reference sample for q-PCR. Experiment was repeated in three biological duplicates for most of the parameters.

Table 14: Experimental overview of transient transfection and co-transfection experiments with TLR2, TLR8 and empty control vector pcDNA3.1 for 72 hours. Different stimulations and infection with 50 MOI of M. avium were performed for 4 hours.

Gene(s) transfected	Ligands						Infection
	LPS	LM	FSL-1	PAM3C SK4	CL75	None	*M. avium* 50 MOI
pcDNA3.1 (none)	•	•	•	•	•	•	•
Untreated cells		•	•	•	•	•	•
Cherry-TLR2		•	•	•	•	•	•
TLR2		•	•	•	•	•	•
TLR8		•	•	•	•	•	•
YFP-TLR8		•	•	•	•	•	•
TLR2 + TLR8		•	•	•	•	•	•
Cherry- TLR2 + TLR8		•	•	•	•	•	•

3.15.3 TLR- transduced U373-CD14 cells

After cells had been stably selected for about a week, cell-lines expressing TLR2 or TLR8 were seeded in Costar 24-well plates, and when about 75 % confluence was reached the stimulation experiment was started. Three biological duplicates were included for all conditions; control sample with untreated cells, and stimulated cells with LM, FSL-1, PAM3CSK4 (200 ng/ml) CL075 (1,5 µl/ml) and 50 MOI of *M. avium* for 4 hours. In addition one control sample with 200 ng/ml LPS stimulation was included. For q-PCR, un-stimulated cells were used as reference sample.

3.16 Designing primers for gateway cloning

Forward and reverse oligo primers with attB1/2 sites for the genes were designed using the Clone Manager 9 software and ordered from Sigma-Aldrich, presented in table 15. The gene sequences in the plasmids are provided in Appendix 4. The primers were quality checked with artificial PCR in Clone Manager 9 together with the sequence of the corresponding plasmid, to see if a product was created. Lengths of primers were optimized in terms of GC content, stability and annealing temperatures. Primers were diluted to a stock of 100 uM, and another working solution of 10 uM.

Table 15: Primers designed for the amplification of genes of interest from target vectors. Purple color represents attB1/2 regions, and red color represents gene-annealing part.

Gene of interest	Forward/ reverse	Primer sequence
GFP-Keap1/ GFP	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTGAGCAAGGGCGAGGAG
GFP-Keap1	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAACAGGTACAGTTCTGCTGG
GFP	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTACTTGTACAGCTCGTCCATGCCG
TLR8	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAAACATGTTCCTTCAG
TLR8	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGTATTGCTTAATGGAATCG
TLR2	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCACATACTTTGTGGATGG
TLR2	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGGACTTTATCGCAGCTCTC

3.17 PCR amplification of genes with designed primers

KOD Xtreme Hot start DNA polymerase kit was used to amplify the gene sequences from the original plasmids, flanking them with attB1/attB2 sites for further gateway cloning. Plasmids were purified from DH5-α *E. Coli* bacteria glycerol stocks as described previously. The technique provides an optimized PCR system for amplification of long or GC rich DNA templates and has a low mutation frequency. To set up the PCR program, New England biolabs NEB Tm calculator was used to calculate the different annealing temperatures for the specific primers. In addition the extension time was optimized to the size of the constructs.

Plasmids used for amplification were pDest GFP, pDest GFP-Keap1, pcDNA YFP-TLR2 and pUNO1 hTLR8b.

The reaction was mixed in tubes as presented in table 16.

Table 16: Reagents, volumes and final concentrations used for set up of KOD Xtreme Hot start DNA polymerase PCR.

Component	Volume	Final concentration
2 x Xtreme Buffer	12,5 µl	1 x
dNTPs (2 mM each)	5 µl	0,4 mM
SIW water	X µl *	
Forward primer (10 µM)	0,7 µl	0,3 µM
Reverse primer (10 µM)	0,7 µl	0,3 µM
Template DNA (5 ng)	0,5 µl	
KOD Xtreme Hot Start DNA polymerase (1 U/ µl)	0,5 µl	0,02 U/ µl
Total reaction volume	25 µl	

* Amount of SIW water was calculated in the end, to fill total volume to 25 µl.

The PCR products were ran on a 1 % agarose gel to separate the products with 1:6 dilution of 6 x loading buffer and 1 kB ladder. Program settings used was 100 V for 40 minutes. Gels were observed and imaged by Bio Doc-H Imaging system from UVP.

For genes GFP and GFP-Keap1, a successful optimized PCR- program used is shown in table 17. For the TLR2 and TLR8 constructs, the reaction volume was doubled to 50 ul. Table 18 shows the PCR program optimized for TLR2 and TLR8.

Table 17: PCR program for amplifying GFP and GFP-Keap1 genes with KOD Xtreme Hot start DNA polymerase

Step	Temperature	Time	
Activation step	94 °C	2 min	
Denature	98 °C	10 s	
Annealing	60 °C, 62 °C, 64 °C, 66 °C	30 s	
Extension	68 °C	3 min	x 25 cycles
Final extension	68 °C	5 min	
Hold	4 °C	∞	

Table 18: PCR program for amplifying the TLR2 sequence with KOD Xtreme Hot start DNA polymerase

Step	Temperature		Time	
	TLR2	TLR8	TLR2	TLR8
Activation	94 °C	94 °C	2 min	2 min
Denature	98 °C	98 °C	10 s	10 s
Annealing	61 °C	53 °C	30 s	30 s
Extension	68 °C	68 °C	2,5 min	3,5 min
x 25 cycles				
Final extension	68 °C		5 min	
Hold	4 °C		∞	

3.18 Purification of genes from PCR products

The gene sequences for GFP, TLR2 and TLR8 were purified directly from the PCR reaction mix with QIAquick PCR purification kit, by following the manual provided by purchaser. Product from annealing temperature 64 °C was used for GFP. Ten microliters 3 M sodium acetate, provided in the kit, was added to adjust the pH during the purification, and 50 ul of elution buffer was used to elute DNA from the column.

After running PCR products on a gel to separate them by size, the bands for the GFP-Keap1 constructs were cut out from the gel under UV light, added into same tube and purified with QIAquick Gel extraction kit, by following the manual provided by purchaser. Ten microliters of 3 M sodium acetate was added to adjust the pH, and sterile water was used to elute the DNA from the column.

Measuring purity and concentrations were performed with NanoDrop as described previously.

3.19 Gateway cloning

Gateway cloning was used as a tool to create lentivectors containing TLR2, TLR8 or GFP-Keap1, and expression vectors with myc- tagged GFP- Keap1, for use in U373-CD14 cells or THP1 cells. Gateway cloning is a cloning method based upon the lambda bacteriophage that infects *E.coli* and mediates swapping of genes through recombination of specific sites as part of the infection cycle. Two recombination sites consisting of specific DNA sequences; attL and attR, attB and attP, recombine uniquely by exchanging strands with the help from recombination enzymes. This natural occurring system has been used to generate different variants of the att- sites that recombine specifically with each other. Each of the sites has a 7-base pair nucleotide sequence, called the core sequence (O), and two flanking arms, e.g. attB1 has B, O and B'. Different engineered attB(1-6) sites would have different variants of the O sequence, creating specific recombination with its attP(1-6) sites. The same applies for attL and attR regions used in the gateway cloning system [73].The principle can be used to move genes, flanked by the att-sites, between plasmids. The start point is to choose the gene of interest, design specific forward and reverse primers flanked by attB1/attB2 sites, and use PCR to extend the primers and amplify the gene. A donor vector with attP1 and attP2 sites is chosen for gene insert, and contains selectable markers, the *ccdB* gene and antibiotic selection gene. The *ccdB* gene kills the cells by inducing gyrase- mediated double- stranded DNA breakage.

In the **BP reaction**, an enzyme-mix called BP Clonase can mediate the recombination that swaps the gene of interest into the donor vector, and generates an entry vector with attL1 and attL2 sites, shown in figure 7. The by-product is the *ccdB* gene, flanked by attR1 and attR2 sites. This entry vector can now be transformed into competent *E.coli* strain DH5- α, and positive clones can be selected based on antibiotic resistance of the entry plasmid. All growing colonies would be assumed to contain the gene of interest because it does not contain the toxic *ccdB* gene and since it is growing in the antibiotic selective media.

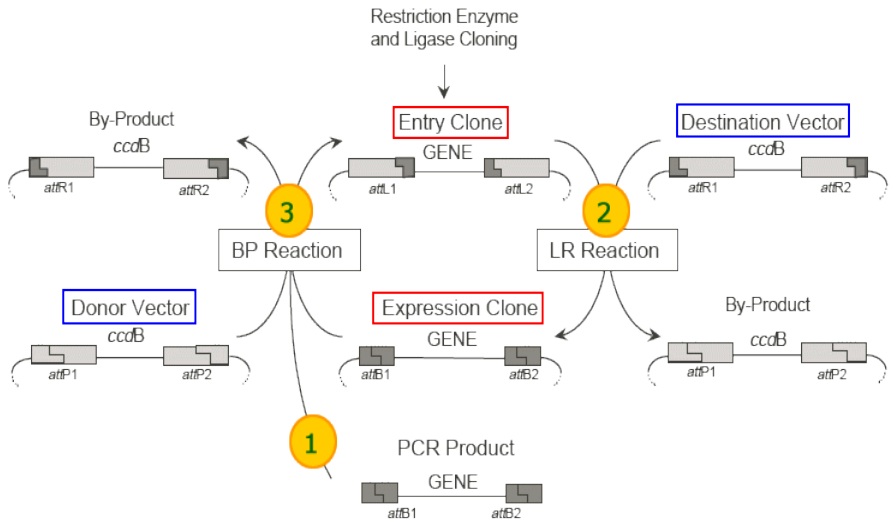

Figure 7: An overview of gateway cloning, BP and LR reactions, from PCR product to expression clone with gene of interest. Picture taken from BIOINFORX [2]

The **LR reaction**, is carried out by the same principle and the gene flanked by attL1 and attL2 sites can recombine specifically with a destination vector containing the *ccdB* gene flanked by attR1 and attR2 sites. The specific recombination and swapping of the genes occurs by adding the enzyme-mix LR Clonase, and creates the expression clone with the gene of interest flanked by attB1 and attB2 regions. The by-product would be the *ccdB* gene flanked by attP1 and attP2 regions, like a reversed BP reaction [73]. The specific antibiotic resistance gene in the expression vector can be used to select positive clones when transforming the LR reaction into the competent DH5- α *E.coli* strain, and plating on selective plates.

3.19.1 Entry vector generation with genes of interest, and DH5-α transformation

The BP reaction was performed using Gateway BP Clonase II Enzyme mix for attB flanked constructs GFP, GFP-Keap1, TLR2 and TLR8.

In all reaction tubes it was added:

- 100- 150 ng DNA sequence with attB1/attB2 sites
- 1 ul pDONR221 vector (150 ng)
- Up to 8 ul with TE buffer, pH= 8,0.

Both positive control with pEXP7-tet fragment with attB1/attB2 sites and negative control without DNA constructs were provided in each set-up.

After thawing the BP Clonase II Enzyme mix briefly on ice and vortexing twice, 2 µl was added to each tube. The reactions were incubated over night at 25°C and the next day terminated by adding 1 ul Proteinase K.

For transformation of the vector into bacteria, 25 ul of the competent OneShot DH5- α was used for each reaction. The bacteria was thawed on ice, 1 ul of the BP reaction was added to the tube and left on ice for 30 minutes. To heat-shock the cells the tubes were put on 42 °C incubation for 45 seconds. Tubes were left on ice to rest for 2 minutes, and 250 ul prewarmed SOC medium was added to the tubes. The bacteria were gently resuspended with a pipette and the transformation incubation was performed for 1,5- 2 hours under agitation at 37 °C.

One hundred microliter of the transformed bacteria were plated on 50 ug/ml kanamycin-selective LB plates and left in 37 °C incubation over night.

Next day, separate bacterial colonies from the plates of pENTR221 GFP, GFP-Keap1, TLR2 and TLR8 were re-streaked on new plates, added into 12 ml falcon tubes with 3 ml LB medium and 50 ug/ml kanamycin, and left in 37 °C incubation over night under agitation. Plates with grown colonies were left in fridge at 4°C.

The bacteria cultures with entry plasmids were purified as described previous.

3.19.2 Expression vector generation from entry vectors, and DH5-α transformation

Gateway cloning was performed using Gateway LR Clonase II plus Enzyme mix for all constructs, to obtain expression vectors from entry vectors with genes of interest; GFP, GFP-Keap1, TLR2, and TLR8. In all reaction tubes it was added:

- 100 ng pENTR221 plasmid
- 150 ng destination vector
- Up to 8 ul with TE buffer, pH= 8,0.

Negative controls without attL1/attL2 flanked DNA constructs were provided in each set-up.

After thawing the LR Clonase II plus Enzyme mix briefly on ice and vortexing twice, 2 ul was added to each tube. The reactions were incubated over night at 25°C and the next day terminated by adding 1 ul Proteinase K.

For transformation of the vector into DH5-α bacteria, same procedure was followed as described previously for the BP reaction. One hundred microliter of the LR reaction were plated on 100 ug/ml ampicillin- selective LB plates and left in 37 °C incubation over night.

Next day, separate bacteria colonies from the plates were re-streaked on new plates and added into 12 ml falcon tubes with 3 ml LB medium and 100 ug/ml ampicillin, and left in 37 °C incubation over night under agitation. Plates with colonies were left at 4°C.

LR reactions were performed for pENTR221 GFP-Keap1 and GFP plasmids with expression vectors pLenti CMV Puro DEST w118-1 and pDest-myc, and for pENTR221 TLR2 and TLR8 plasmids with expression vector pLenti CMV Puro Dest w118-1.

3.20 Restriction enzyme digestion for pENTR221 GFP-Keap1 and GFP plasmids

Restriction enzyme digestion was performed for GFP and GFP-Keap1 recombined plasmids to assess the presence of the genes. As the GFP-Keap1 sequence initially contained an attB1 site between the two genes, it was wanted to assure the presence of both genes in the plasmids. A strategy for restriction enzyme digestion was made for the pENTR221 plasmids with genes of interest with help from Marte Singsås Dragset, CEMIR, using the Clone Manager 9 software. Restriction enzymes EcoRV and ApaLI were chosen.

An amount of 500 ng of the pENTR221 plasmids and 300 ng of the pDONR221 plasmid were used in the reactions with 1 µg of each restriction enzyme and 2,5 µl 10 xNEBuffer 2 + 100 µg/ml BSA, in total 25 µl reaction volume. Reactions were incubated over night in 37 °C. Buffer 2 efficiency for enzyme ApaLI was 100 % and 75 % for EcoRV.

The day after, a 1 % agarose gel was made and all samples were ran on gel to separate the digestion products by size, as previously described.

3.21 Sequencing of pENTR221 plasmids

To be sure that the constructs were correctly inserted into the plasmids and that the reading frame was precise, some entry plasmids were chosen to send for sequencing to GATC in Germany. All plasmids chosen were sent in two different tubes, one for forward sequencing with M13 forward primer, and one for reverse sequencing with M13 reverse primer. Each tube contained 5 pmol of the M13 primer diluted in 5 µl SIW. Plasmids were added in concentration 100 ng/ul diluted in 5 ul SIW. Obtained data were analyzed with Clone Manager 9, and sequences were aligned with the gene sequences of interest and predicted sequences of the pENTR221 plasmids with inserted genes to look for similarities.

pENTR221 plasmids of GFP-Keap1, GFP, TLR2 and TLR8 that appeared to be positive for genes of interest, were chosen to sequence. In addition, the chosen pENTR221 of GFP-Keap1 were completely sequenced with an additional designed forward primer: TGTACGCCTCCACTGAGTGC.

3.22 GoTaq Green PCR to qualify presence of interesting genes

As a second way of qualifying the presence of the genes of interest in addition to sequencing, GoTaq Green PCR was performed for both pENTR221 plasmids and expression plasmids either before or after sequencing. The PCR reaction was performed either for bacteria colonies or for purified plasmids. The reaction set-up is provided in table 19, and the PCR programs in table 20. The master mix contains bacteria derived *Taq* DNA polymerase, dNTPs, $MgCL_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. The mix also contains both a blue and yellow dye, that allows direct loading to an agarose gel. The primers used were specific for the gene of interest that was screened for.

Table 19: Reagents and volumes used to set up the reaction for GoTaq Green PCR

Component	Volume
GoTaqGreen 2x MM	5 µl
Forward primer (10 µM)	0,5 µl
Reverse primer (10 µM)	0,5 µl
Template DNA (100 ng) or bacteria culture	X *
SIW	X **
Total reaction volume	10 µl

* About 100 ng of the template DNA was used in the reaction, and a small amount of bacteria colonies were just dipped into the reaction mix.
** SIW was added up to final volume 10 ul

All tubes were spun down, and the PCR program was run as described in the provided protocol from the manufacturer. Annealing temperatures specific to the primers and extension time specific to construct length was adapted to each reaction, presented in table 20. After the PCR reaction, products were run on a 1% agarose gel as previously described.

Colonies of pENTR221 GFP-Keap1 from BP reaction, and colonies of expression clones for GFP-Keap1 and GFP were used directly in the PCR tubes with the reagents to screen for positive clones. In addition GoTaq Green was performed for purified expression clones of GFP and GFP-Keap1 to screen for the presence of both GFP and Keap1. Positive controls for GFP or GFP-Keap1 were provided in reaction set up.

Go Taq Green was performed for purified pENTR221 plasmids from the BP reaction for both TLR2 and TLR8 before sequencing. In addition purified expression clone plasmids for TLR2 and TLR8 were screened after LR reaction for the presence of the genes of interest. Positive controls for TLR2 and TLR8 were provided in reaction set up.

Table 20: GoTaq Green PCR program used for GFP, GFP-Keap1, TLR2 and TLR8 specific primers

Step	Temperature			Time		
	GFP/ GFP-Keap1	TLR2	TLR8	GFP/ GFP-Keap1	TLR2	TLR8
Activation	95 °C	95 °C	95 °C	2 min	2 min	2 min
Denature	95 °C	95 °C	95 °C	45 s	45 s	45 s
Annealing	64 °C	61 °C	53 °C	45 s	45 s	45 s
Extension	72 °C	72 °C	72 °C	3 min	2,5 min	3,5 min
x 30 cycles						
Final extension	72 °C			5 min		
Hold	4 °C			∞		

3.23 Cryopreservation of colonies in glycerol stocks

To be able to purify more of the plasmids made by gateway cloning later, it was created glycerol stocks for all the bacteria cultures with successful cloning. New bacteria cultures were started from the colonies on the plates representing the successful cloned pENTR221 plasmids, and the expression clones. Next day when bacteria cultures were confluent 60 % autoclaved glycerol was added to a final concentration of 15 % glycerol, to protect the cells from formation of ice crystals. The glycerol stocks were frozen down at -80 °C with appropriate antibiotic in the medium to make sure that the bacteria in the culture contained the plasmid with the gene of interest.

3.24 Immunofluorescence staining for screening of GFP and Keap1 in cloned pDest- myc plasmid

Immunofluorescence is a technique that uses fluorescent- labeled antibodies to detect specific target antigens in cells through microscope imaging. Indirect immunofluorescence was used, with a primary antibody towards target protein and a fluorescent secondary antibody towards the Fc region of the primary antibody [74].

Screening of gene GFP and immunofluorescent staining of Keap1 in gateway cloned vector pDest myc-GFP-Keap1 was performed to quality check the presence of the genes with an additional method, and to verify the protein localization and function.

The vector was transfected with Gene Juice as described previously, into U373-CD14 cells in different concentrations**.** Untreated cells were provided as a negative control for the experiment. Set-up of the transfection in the different glass bottom dishes is presented in table 21. After transfection for 72 hours, some of the cells were infected with *M. avium* dsRed, to observe association of the bacteria with Keap1, and functionality of the Keap1 protein according to literature. Bacteria were prepared and cells were fixed as described previously.

Table 21: Experimental set-up for transfection with pDest-myc-GFP-Keap1 in different concentrations, immunofluorescence staining for Keap1 and infection with 100 MOI M. avium for 4 hours. Every glass dishes with cells were treated differently as presented.

Confocal dish #	Transfected pDest-myc-GFP-Keap1	Concentration of plasmid (ug)	Immunofluorescence staining of Keap1	M. avium infection 100 MOI 4 hours
1	-	1	+	-
2	+	1	-	+
3	+	1	+	+
4	+	1	+	-
5	-	1	-	-
6	+	0,5	-	-
7	+	1,5	-	-
8	-	-	-	-

For cells that were permeabilized and immunofluorescence stained for Keap1, staining was performed directly after fixation.

After fixation, cells were treated with 1 ml of Quenching buffer for 10 minutes to reduce autofluorescence. Liquid was replaced by 1 ml PBS/ Saponin/ A+ serum antibody buffer to permeabilize cells, and left for another 10 minutes. Cells were washed three times with 1 ml PBS/Saponin. One milliliter blocking buffer was added and left in dishes at room temperature

for 1,5 hours to increase the specificity of the antibody. An antibody solution was made with 1:100 concentration of primary antibody for Keap1 in antibody buffer to a final concentration of 3,7 ng/ul. The solution was added to the cells and left over night in 4°C to stain for protein of interest. Next day, cells were washed three times with the antibody buffer. Secondary antibody, Alexa Fluor dye 647 was diluted 1:1000 in the antibody buffer to a final concentration of 2 ug/ml. Solution was added to the cells and left in 4°C for 40 minutes to ensure binding of antibodies to the Fc region of primary antibody. Cells were again washed three times with 1 ml antibody buffer, and two times with PBS/ Saponin. The cells were left in 1 ml PBS and imaged with confocal microscopy as described previously.

3.25 Establishment of stable cell-lines expressing TLR2 or TLR8 by lentiviral transduction of U373-CD14

The use of lentiviruses to insert specific genes of interest into the host cell genome is a fast and efficient way to modulate gene expression, and can be used for both dividing and non-dividing cells. To create the virus particles, different vectors constructed by using HIV-1 provirus are used. Foreign envelope protein from vesicular stomatitis virus G (VSV-G) is used for protein coat of the virus because of its broad tropism, creating a pseudotyped virus particle. The pseudotyped virus has more potential target cells, and is more efficient due to its binding mechanism. The use of 3rd generation vectors makes the process safer because the essential viral genes from HIV-1 are separated into three packaging vectors and the fourth plasmid carries the transgene of interest. For 2nd generation vectors, the HIV-1 proteins are collected in only two plasmids, instead of three. Unless recombination occurs between all the vectors, the virus cannot replicate and reproduce after the initial infection, hence the more vectors the viral genes are divided on the higher safety it provides.

First packaging plasmid pMD.G encodes the pseudotyped VSV-G protein coat with an amphitropic nature. Second packaging plasmid pRSV/REV encodes protein REV which is essential for the nuclear transport of the proviral RNA. The unspliced and partially spliced viral mRNAs from HIV-1 have REV- responsive elements (RRE) that can bind to REV for transportation. Third packaging plasmid pMDLg/pRRE contains Gag and Pol polyprotein, which encodes structural proteins and important integration enzymes like reverse transcriptase, HIV protease and integrase [7]. The transgene that is cloned into the expression vector by Gateway cloning encodes the protein of interest. How the different plasmids are assembled to create the virus particles is presented in figure 8.

3. Experimental methods

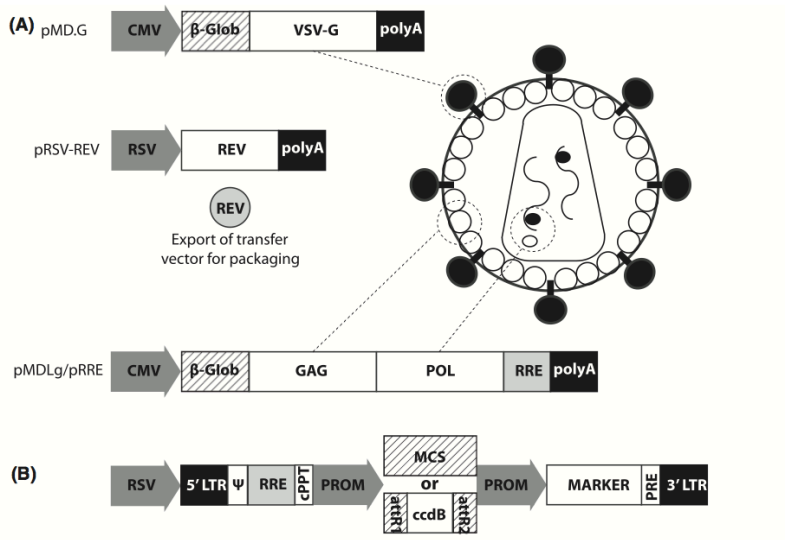

Figure 8: An overview of how third- generation lentiviral vectors are used to assemble the virus particles. The essential virus genes are split between three separate packaging plasmids shown in A) pMD.G, pRSV/REV and pMDLg/pRRE. The general structure of a transfer vector for the expression of a certain gene created by gateway cloning is presented in B). The LTR sequence are involved in transcription of the expression cassette and integration within the host genome. Figure taken from Shearer et al, Experimental design for stable genetic manipulation in mammalian cell-lines: Lentivirus and alternatives, 2015 [7].

To assemble the viral particle, an easily transfected cell-line that is suitable for lentiviral production, is transiently transfected with all the packaging plasmids and the expression clone. Viral particles produced are secreted into cell supernatant, and are used to transduce the cells of interest for expression of the transgene.

Lentiviral transduction was performed for the U373-CD14 cell-line to stably integrate TLR2 and TLR8 in separate cells, into the host genome.

3.25.1 Producing lentiviruses in HEK 293T cells

HEK 293T cells were seeded the day before experiment start, 300 000 cells per well in two Costar 6 well plates. Next day, when cells had reached about 60 % confluence, the transfection of HEK 293T cells with Gene Juice was performed after changing medium on the cells. The protocol for Gene Juice transfection is described previously, and amount of reagents used for transfection of one well is presented in table 22.

Table 22: Set- up for 1 well of transfection with amount of lentiviral plasmids, packaging plasmids, serum-free medium and Gene Juice.

Plate format	Serum-free medium (ul)	GeneJuice (ul)	Plasmids	Amount added (ug)
Costar 6 well- plate	666	20	pMDLg/pRRE,	2,25
			pRSV/REV	2,25
			pMD2.G,	1,5
			pLenti CMV Puro Dest w118-1 cloned with either GFP-Keap1, TLR2 or TLR8	0,6

Protocol for amounts of different plasmids for making the viruses for 3rd generation viruses were kindly provided by Sagar Darvekar, NTNU. The lentiviral vectors pLenti CMV Puro w118-1 was previously cloned with the gateway system, and contained genes of interest; GFP- Keap1, TLR2 or TLR8. Two wells were transfected for each transgene. In addition a well with only cells was used as a negative control. Since the TLR2 and TLR8 containing vectors did not have fluorescence, the GFP-Keap1 transduced cells served as controls for fluorescence and transfection efficiency.

After 24 and 48 hours, cells were observed with fluorescent microscope Zeiss Axiovert 100 M. The viruses were harvested after 48 and 72 hours from all wells by pipetting off and filtering the viral supernatant thorugh a 0,2 um syringe filter to remove the loosened cells. Virus supernatant were frozen down in -80°C, separate aliquots for each well. New complete medium was added to the remaining cells in the wells carefully after 48 hours, and cells were discarded after 72 hours harvesting.

3.25.2 Puromycin dosage response studies for U373-CD14

To determine at what concentration of Puromycin that would make it possible to select for only positive cells containing the plasmids with puromycin resistance gene after transduction with lentiviruses, a puromycin dosage response study was conducted.

Cells were seeded the day before experiment start in a Costar 24 well plate, about 50 000 cells/well. The day after, Puromycin was added to the wells in different concentrations in duplicates:

Untreated 0 ug/ml	0,5 ug/ml	2 ug/ml
0,1 ug/ml	1 ug/ml	3 ug/ml
0,25 ug/ml	1,5 ug/ml	5 ug/ml

In addition all conditions were tested with and without G418 added to the medium. Ideally the selection would take place under presence of both G418 and puromycin, since the cells with CD14 plasmid also would have to be selected for.

Cells were observed with light microscopy (Primovert, Zeiss) after 24 hours, and surviving cells were passaged to new Costar 6 well plates. After 48 and 72 hours cells were observed again. After 96 hours, cells were passaged for second time. New observations were performed at 120 and 144 hours to observe the cell viability.

3.25.3 Transducing U373-CD14 cells with the viral supernatant

The day before experiment, early passage number U373-CD14 cells were seeded in two Costar 6 well plates, 300 000 cells per well in complete medium. Following day, the cells were 45 % confluent, and it was decided to proceed with transduction even though cells could have been slightly more confluent.

Viral supernatants harvested from 48 hours after transfection, were used to transduce cells. Half a milliliter of viral supernatant mixed with 0,5 ml serum-free DMEM and a concentration of 8 ug/ml Polybrene was used. After mixing, 1 ml was added dropwise to the wells. Two wells were transduced for each pLenti CMV Puro Dest w118-1 plasmid, GFP-Keap1, TLR2 and TLR8. A control well of only cells was used to monitor the cell viability.

After 48 hours of virus transduction, the cells were observed in fluorescent microscope Zeiss Axiovert 100 M, to look for emitted fluorescence of the GFP-Keap1 transduced cells. Supernatant was removed, cells were washed in PBS to remove the viruses, and cells were passaged into T25 flasks as described previously. Two flasks were used for each transduction plasmid, and one flask was used for control cells without transduction. Selection with 1,2 ug/ml Puromycin was performed by adding antibiotics in the growth medium.

After 3 days in the T25 flasks, cells were imaged to control viability and morphology with light microscope (Primovert, Zeiss), and with EVOS FL Auto Cell Imaging system as described previous, to look at the GFP fluorescence. All cells were passaged into T75 flasks and left for selection 1 week before experiments were conducted.

3.26 Statistical analysis

To determine if the fold- induction responses from q-PCR analysis were significant towards ligands or infection with *M. avium* compared to reference samples, unpaired two-tailed t-tests were performed with Graph Pad Prism software version 6. For all analysis a p- value of 0,05 with a corresponding confidence interval of 95 % were used.

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4.1 U373-CD14 and THP1 cells were capable of phagocytosing *M. avium* with different efficiencies

A macrophage cell-line with the ability to phagocytose *M. avium* was aimed to establish for further experiments, and the purpose of the experiment was to observe the phagocytic efficiency of two different cell-lines, THP1 and U373-CD14. Imaging experiments with confocal microscopy were performed with different MOI's and different time points. For THP1 cells 10 MOI, 20 MOI and 50 MOI were assessed for 4 hours infection, while for U373-CD14 cells 10 MOI was assessed for 4 and 6 hours, 50 MOI for 4 and 8 hours, and 100 MOI for 4 hours infection. We aimed to find optimal infection parameters to get as high possible frequency of cells infected on an average. When assessing interaction experiments later, it would be beneficial that all cells are infected on an average, as pathways upon infection would be detailed. We observed that both cell-lines had the ability to phagocytose *M. avium*, but with different efficiency.

We concluded by counting infected cells per image that a MOI of 100 for 4 hours was optimal for the U373-CD14 cell-line, and a MOI of 20 over 4 hours was optimal for the THP1 cells to get as high frequency of cells possible infected with M. avium, without overloading with too many bacteria pr cell. Example images of U373-CD14 cells are presented in figure 9, and images for THP1 presented in figure 10. A MOI of 10 and 50 for 4 hours infection was assessed for both cell-lines, and by comparing images we concluded that THP1 were more efficient for phagocytosing *M. avium* than U373-CD14 by counting number of bacteria present in each cell and number of cells infected. As both cell-lines were able to phagocytose *M. avium* they were both further candidates for a model cell-line. To be sure that bacteria were present inside cells, Z-stack were created through the cells and assembled in presented images with Image J software.

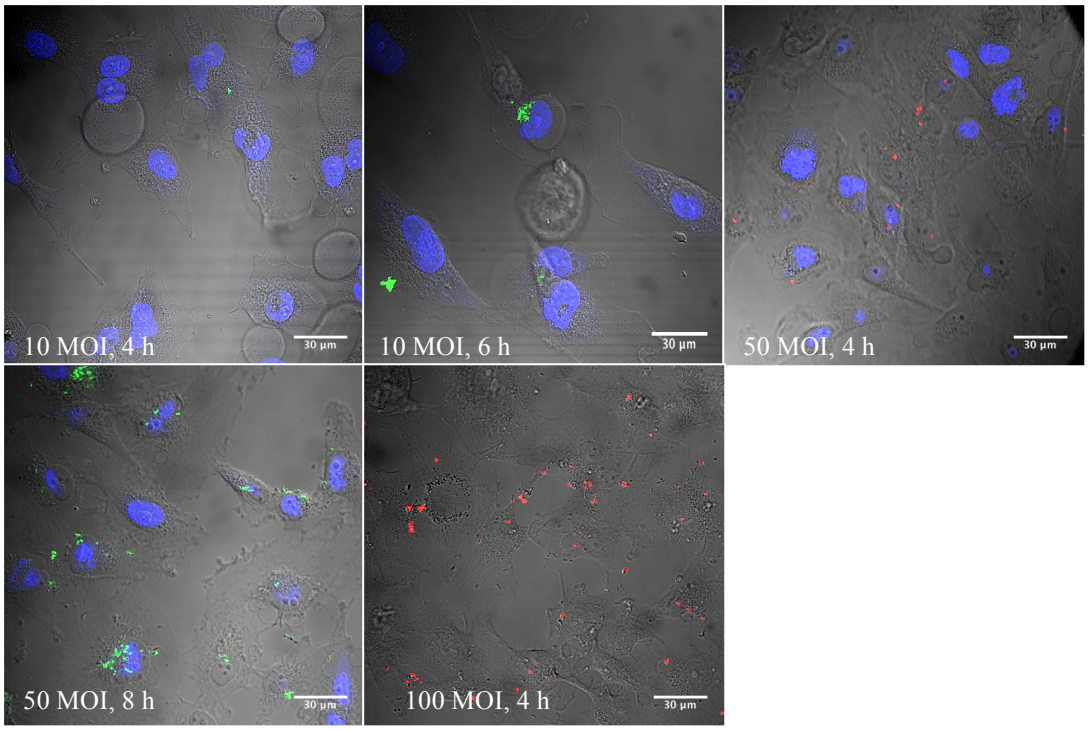


Figure 9: Infection of U373-CD14 cells with 10 MOI for 2 and 6 hours, 50 MOI for 4 and 8 hours, and 100 MOI for 4 hours. Imaging of fixed cells was performed with confocal microscopy, 63x objective. M. avium strain 104 expressing fluorescent dsRed or CFP was used for the experiments, and nucleus stain Draq5 was used for some experiments.

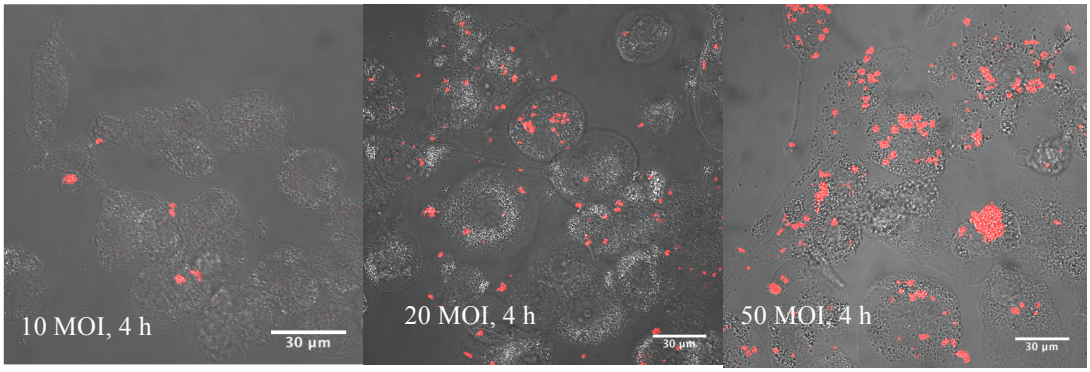


Figure 10: Infection of PMA- differentiated THP1 cells after 3 days with 10 MOI, 4 hours; 20 MOI, 4 hours, and 50 MOI, 4 hours. Fixed cells were imaged with confocal microscopy, 63x objective. The M. avium strain 104 expressing fluorescent dsRed was used for the experiments.

4.2 U373-CD14 cells responded poorly to infection with *M. avium*

The THP-1 cell-line is already known to up-regulate inflammatory cytokines upon mycobacteria infections from previous studies [75, 76]. However, since these cells are tricky to transfect, it would be more beneficial to use the U373-CD14 cells as a tool in interaction studies. To determine if the cell-line U373-CD14 initiated a response to *M. avium* in terms of upregulation of inflammatory cytokines, a stimulation and infection experiment was performed. We wanted to achieve an inflammatory response similar to human primary macrophages, to be able to detail further the Keap1 regulation of IKKβ and TBK1 upon *M. avium* infection in this model. As the inflammatory cytokines would be the "read-out" from interaction and infection experiments, they would have to be produced in detectable levels. We chose to screen for cytokines TNF-α, IL-8, IFNβ and IP10, as they were expressed as a result of activation of the kinases IKKβ and TBK1.

Samples were analyzed by q-PCR. The fold induction and relative quantification (RQ) was assessed to measure levels of the cytokines for different stimuli, compared to un-stimulated reference sample. All results were calculated from 3 biological replicates. Standard deviation values were calculated for the three biological duplicates from three independent experiments, and are presented in figure 11. Statistical analysis was performed for all cytokines independently, for each time-point to determine if the cytokine responses were significant, either by down-regulation or up-regulation. All significance was calculated compared to reference sample, un-stimulated sample with fold induction 1.

LPS was used as positive control stimulation for the stably transfected CD14 receptor and Toll-like receptor 4. Overall, we observed that the U373-CD14 cells gave very low or no responses towards *M. avium* for all cytokines measured, compared to the positive control, presented in figure 11.A.

In figure 11.A the cytokine response to 200 ng/ml LPS stimulation is presented for time-points 30 minutes, 2 hours, 4 hours, 6 hours and 8 hours. The cytokines TNF-α and IFNβ show highest upregulation after 2 and 4 hours, while IL-8 and IP10 responses increased with higher time exposure, giving highest fold induction response after 8 hours infection.

In figure 11.B the cytokine response to 20 MOI of *M. avium* is presented, and in figure 11.C the response towards 50 MOI of M. avium is presented for same time- points.

From 30 minutes and up to 4 hours, *M. avium* did not induce significant difference in cytokine levels for **TNF-α**. After 6 hours it was observed a significant increase for 50 MOI infections, and after 8 hours for both MOI's 20 and 50 (RQ around 2,5).

For all time points, **IFNβ** levels were significantly down-regulated as a response to *M. avium*, except for 2 hours, 50 MOI infections that was not calculated significant.

For 30 minutes, 4 hours, and 8 hours, **IL-8** levels were significantly up-regulated as a response to 20 MOI *M. avium* infections (RQ 2), while not significant for 50 MOI infections.

For 2 hours infection the levels were up- regulated for both MOI's of infection, and for 6 hours infection the response was not significant.

The levels of **IP10** after 30 minutes of *M. avium* infection were not significant compared to reference sample. For both 2 and 4 hours the levels were down-regulated for both MOI's, and for 6 and 8 hours the levels were down-regulated only for 20 MOI while not significant for 50 MOI of infections.

For all time- points of infection the trend was the same for the regulation of all cytokines and both 20 MOI and 50 MOI displayed the same trend. Both regulation of IFNβ and IP10 was either not significant or down regulated, while regulation of TNF-α or IL-8 was either not significant or up regulated. For 50 MOI, the cytokines TNF-α and IL-8 were slightly increased, especially after 6 hours stimulation, but the RQ remained lower than 6. Even though the responses were calculated as significant, the fold- inductions were still low compared to positive controls with LPS stimulation, and we presumed that the low RQ values were not of any biological relevance as they were all below RQ 4 (except TNF-α response for 50 MOI infection, 6 hours with a high standard deviation). The results indicate that U373-CD14 cells might be deficient in components needed for an optimal response towards infection with *M. avium*. Search in literature and previous findings in our group pointed to the lack of TLR2 and TLR8 expression as a possible reason for the low responses [77].

Figures showing the regulation of cytokines for the different stimulations based on the different time-points are presented in Appendix V.

4. Results

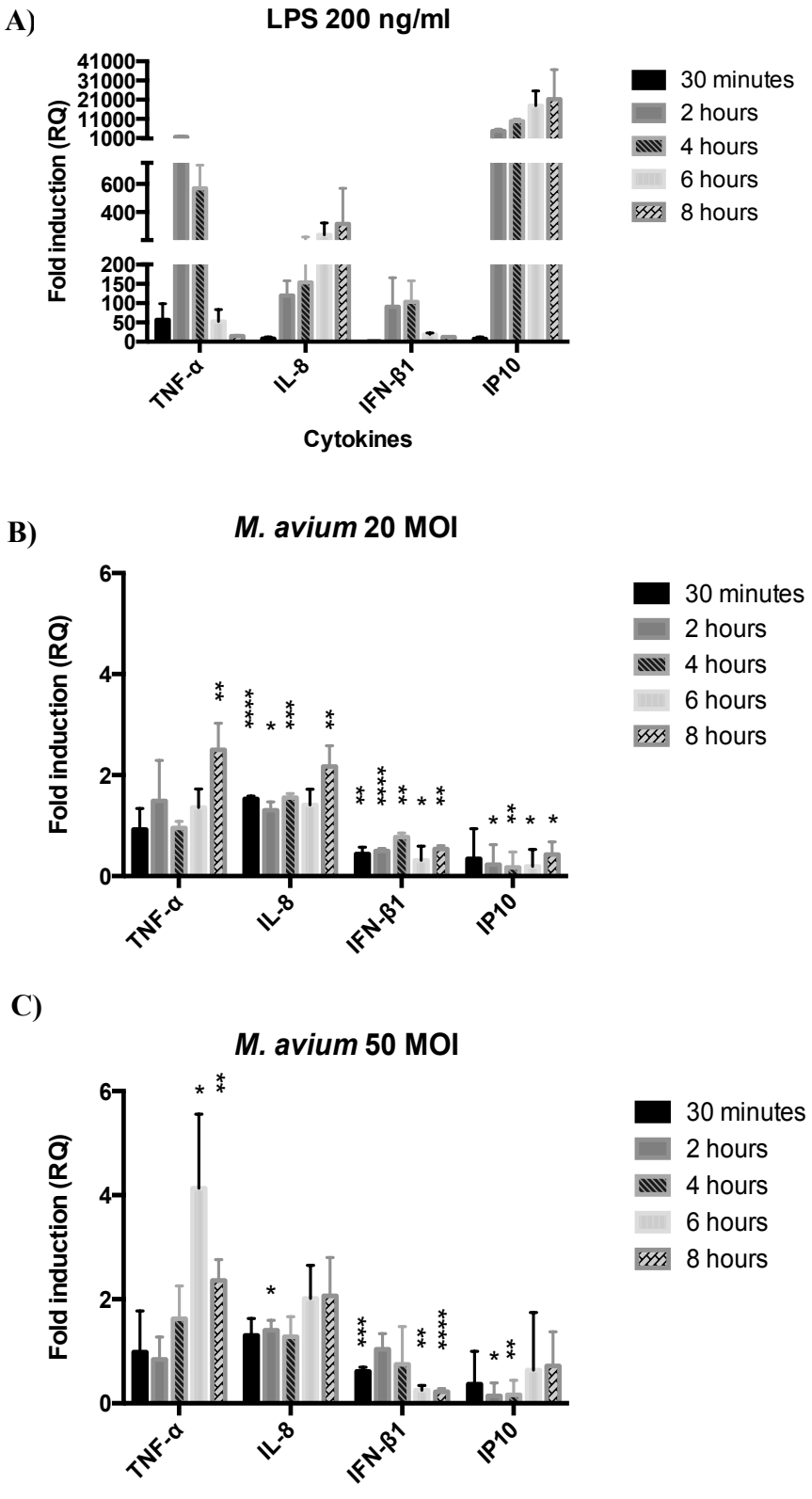


*Figure 11: The cytokine responses for the cell-line U373-CD14 after stimulation for 4 hours with 200 ng/ml LPS (control) presented in (A), 20 MOI infection of M. avium (B), and 50 MOI infection of M. avium (C). Cytokines screened for were TNF-α, IFNβ, IL-8 and IP10, and GAPDH was used as endogenous control. Fold inductions and relative quantifications (RQ) of cytokines are presented compared to reference sample, un-stimulated cells with RQ 1. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures.*

4.3 U373-CD14 cells were transiently transfected with different efficiencies for various expression plasmids.

The transfection efficiency of the U373-CD14 cell-line was investigated to look at expression of various proteins at different time-points and concentrations. The efficiency was assessed for TLR containing plasmids, and for plasmids with GFP and GFP-Keap1, to find a concentration and time-point to proceed with in further experiments. Plasmids with and without fluorescent tags were investigated.

4.3.1 Transfection of U373-CD14 with 1 ug GFP-Keap1 for 72 hours displayed highest expression of fluorescent proteins

Using Gene Juice transfection reagent, expression plasmids pDest GFP and GFP-Keap1 at concentrations of 1 ug were transfected into U373-CD14 cells in a 6 well plate format over different time-points, and cells were imaged with EVOS FL Auto Cell Imaging systems. Figure 12 represents 24, 48 and 72 hours transfection images for both plasmids. As the images were obtained with different light exposures, we could not quantify the fluorescence from the EVOS images, even though this would be ideal to compare the different time points and concentrations. Because of this, the qualification of protein expression was preformed visually by counting fluorescent cells. Images were obtained from random places in the wells, which could also affect the results.

Twenty-four hours post transfection, about 12 % of the cells were transfected with and showed protein expression of GFP, and about 20 % of the cells were transfected with GFP-Keap1. We observed 48 hours post transfection that about 26 % of cells were transfected with GFP, and about 8 % of cells were transfected with GFP- Keap1. After 72 hours, about 35- 40 % of cells were transfected with GFP, and about 33 % of cells were transfected with GFP-Keap1. The transfected cells and un-transfected cells for GFP 72 hours post transfection were hard to distinguish, as the background fluorescence in the image was high. Cells were generally counted as transfected if green fluorescence was observed.

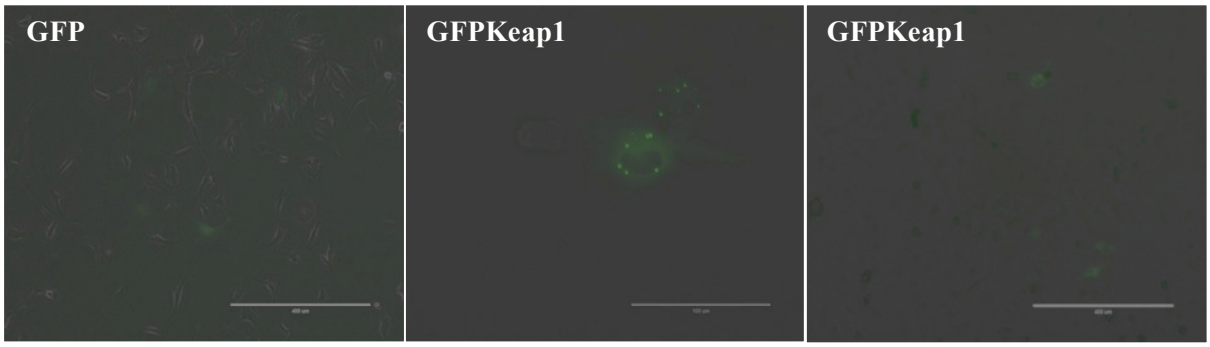
We also observed cells 72 hours post transfection from another biological experiment using a confocal microscope to observe the protein expression in each cell. A negative control without transfection was included, and we observed that cells emitted green auto-fluorescence. The background auto-fluorescence was not as prominent in all cells as the emitted fluorescence from GFP flurophores, but it was still more difficult to distinguish the signals. Images are presented in figure 13A and B. The images from confocal microscopy presented dots of green emitted fluorescence, which was thought to present clusters of GFP-Keap1 proteins.

To quantify the expression of proteins with two different transfection concentrations, the plasmids pDest GFP and pDest GFP-Keap1 were transiently transfected into U373-CD14 cells with Gene Juice reagent in concentrations 0,5 ug and 1 ug for 72 hours. Western blotting was performed to assess the expressed protein sizes and amounts. The developed membranes are presented in figure 14.A. The top panel represents the Actin gene that was used as an endogenous control. The Actin protein has a size of 42 kDa, which matches the size compared

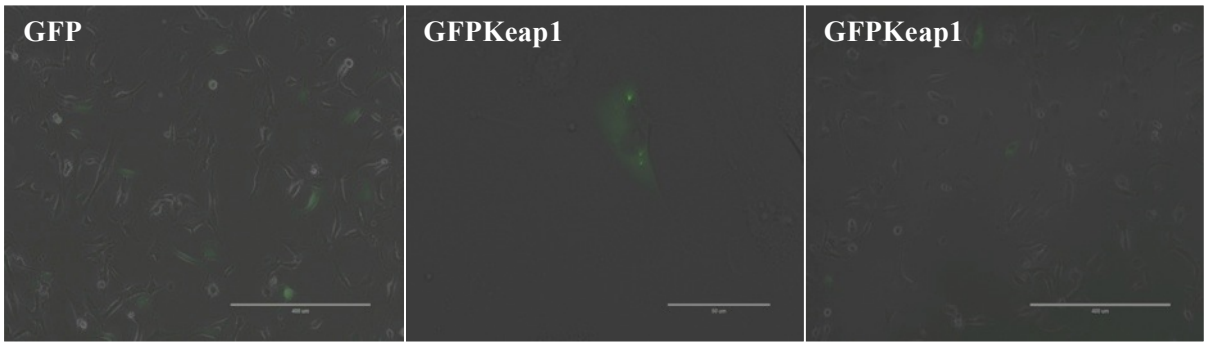
to the ladder. The bands in the middle panel represents 1 ug and 0,5 ug transfected GFP-Keap1, respectively. The total size of GFP-Keap1 can be observed around 96 kDa (GFP 26,9 kDa and Keap1 69,6 kDa). The four bands in the lowest panel (lane 2, 3, 7, and 9) represent transfection of 1 ug and 0,5 ug protein GFP, respectively, with a size 26,9 kDa that matches the ladder sizes.

Three biological replicates were analyzed for 1 ug plasmid concentration for both plasmids, and one biological sample for 0,5 ug plasmid concentration for both plasmids. A relative quantification of the protein expression with Actin as endogenous control was performed with Image Studio Light. Absorbance signals from fluorescent antibodies towards primary antibody for proteins of interest were normalized to same absorbance signals from Actin in the same well, to remove variation of different loading amount to the wells and providing a relative quantification value for the amount of proteins present. The calculations are provided in figure 14.B.

24 hours:



48 hours:



72 hours:

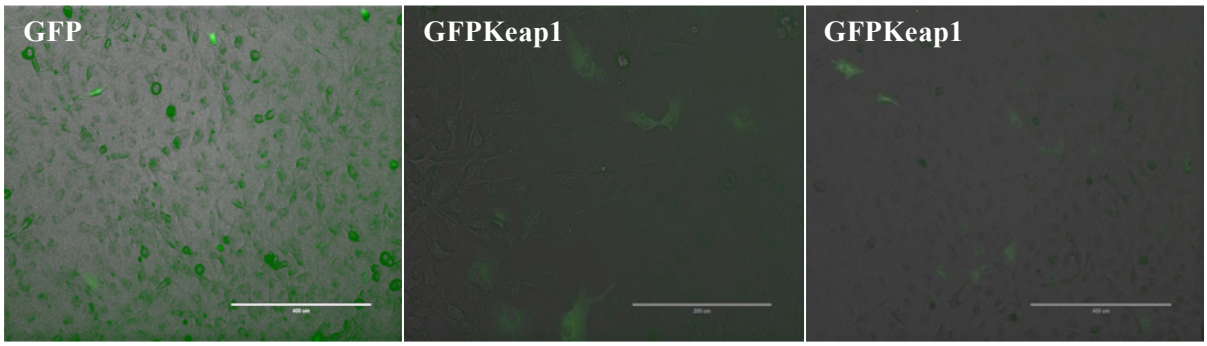


Figure 12: Gene Juice transient transfection with 1 ug of expression plasmids pDest GFP and GFP-Keap1, images obtained after 24, 48 and 72 hours respectively, with EVOS microscopy, different objectives. Images were obtained from random places in the well. Scale bars for all GFP images: 400 µm, GFP-Keap1 images in the middle: 100, 60, and 200 µm respectively, and GFP-Keap1 images at the right side 400 µm. Images were obtained from Costar 6-well plates.

4. Results

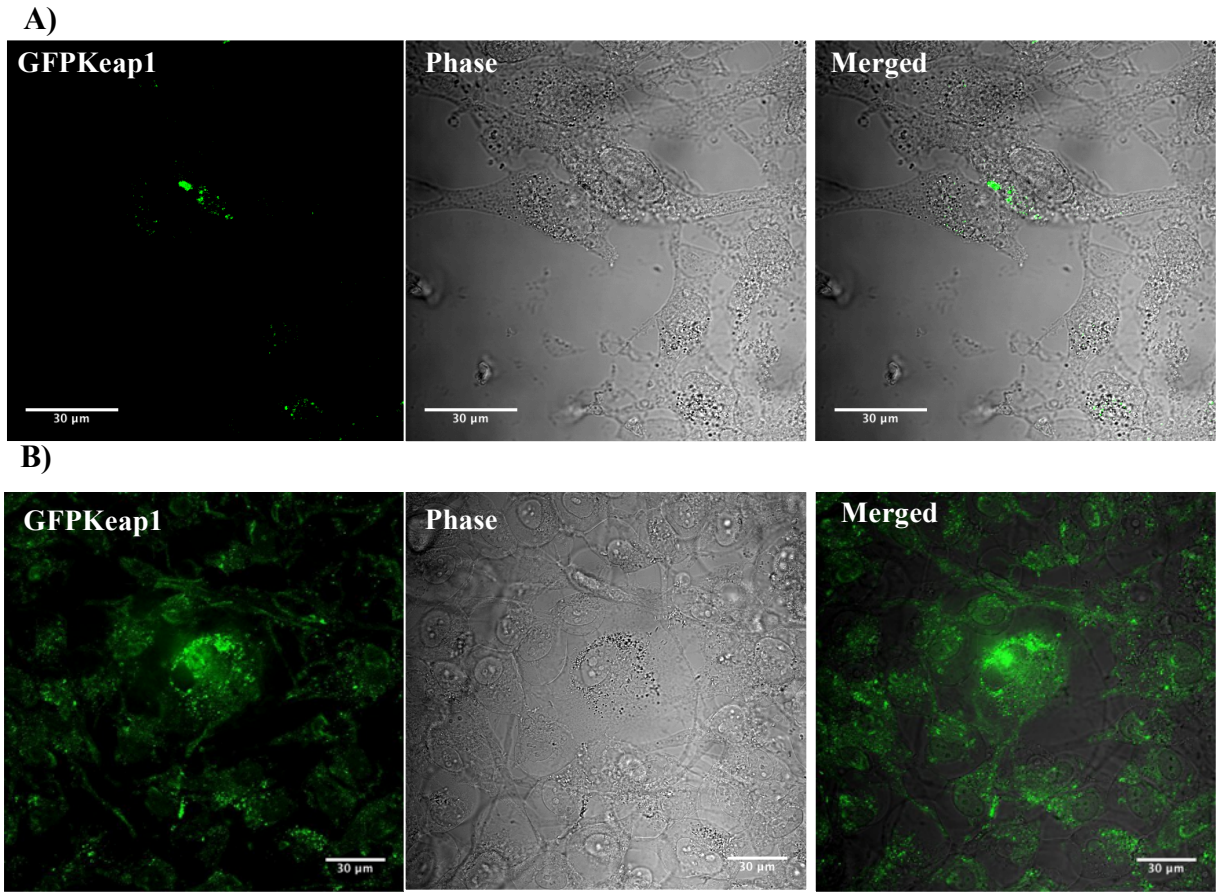


Figure 13: A) Un-transfected control cells. Cells were observed with confocal microscopy and a 63x objective. B) U373-CD14 cells 72 hours post transfection with 1 ug GFP-Keap1. Left images represent the GFP channel, middle images phase transmission, and right images merged channels. Scale bars: 30 µm.

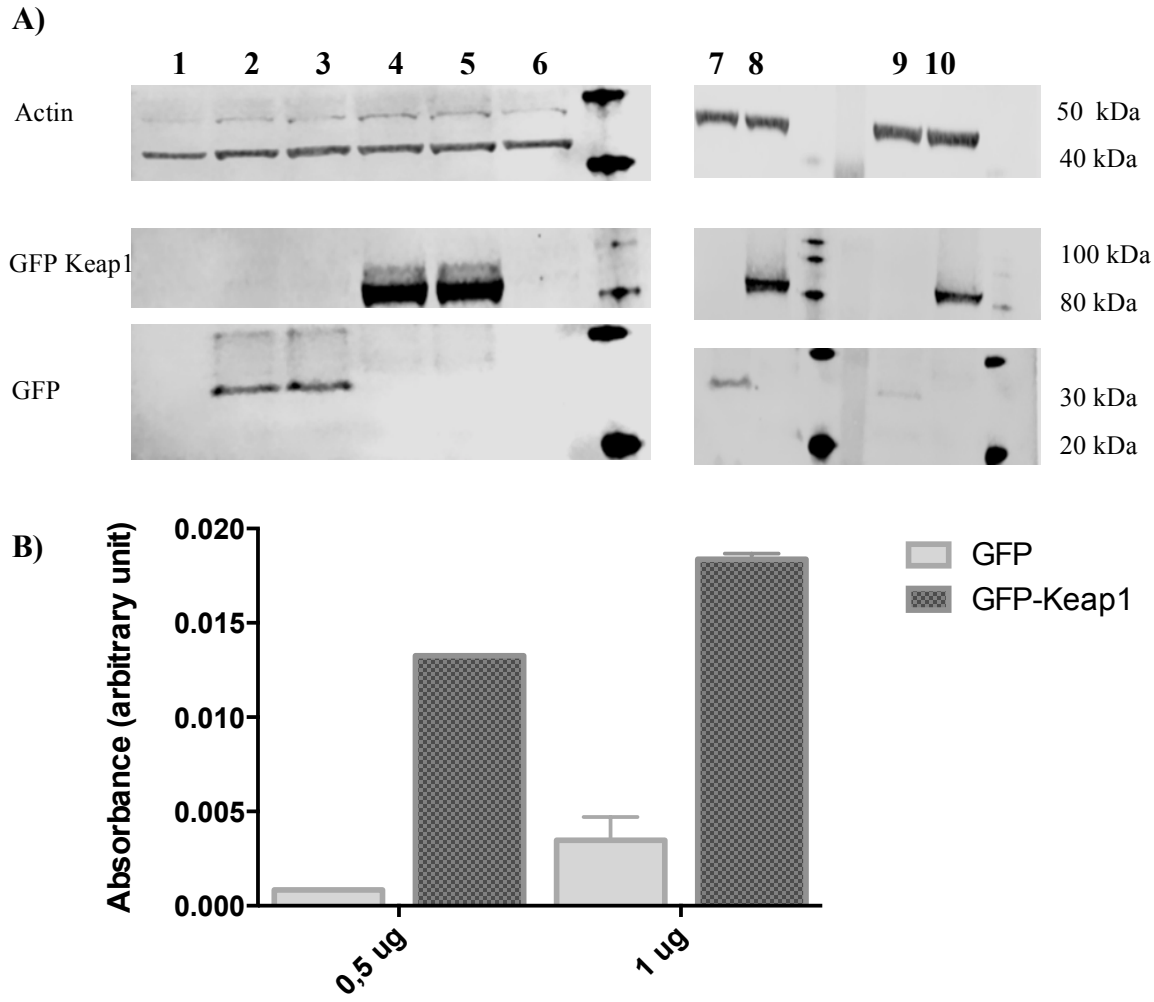


Figure 14: U373-CD14 cells transfected 72 hours with 1 ug GFP (lane 2, 3, 7), 0,5 ug GFP (lane 9) size 26,9 kDa and 1 ug GFP-Keap1 (lane 4, 5, 8), 0,5 ug GFP-Keap1 (lane 10) size 96 kDa. Control samples (lane 1 and 6) are un-transfected cells, and ladder used for comparing sizes was Magic marker. Protein amounts and sizes are displayed by separation on a NuPage 4-12 % gel (presented in A), and staining with appropriate antibodies to detect fluorescent signal. Endogenous control Actin with size 42 kDa was used for relative quantification of protein amounts, presented in B. The absorbance signals from fluorescent antibodies towards genes of interest were normalized to corresponding signals from Actin. Three biological replicates for 1 ug concentration were analyzed for both plasmids, and one sample for 0,5 ug.

We concluded that 72 hours post transfection with 1 ug pDest GFP and GFP-Keap1 gave the most efficient protein expression in the cells. In addition GFP-Keap1 transfected cells gave a higher protein expression than GFP transfected cells for both concentrations, based on relative quantification with Actin as a control.

Overall, we concluded that the transfection efficiency of the U373-CD14 cells were highest after 72 hours as over 30- 40 % of cells emitted green fluorescence observed visually by confocal and EVOS microscopy, and that 1 ug concentration gave a higher protein expression than 0,5 ug based on relative quantification with Western blotting.

4.3.2 Transfection of U373-CD14 with 0,4 ug Cherry TLR2 for 48 hours and longer displayed highest expression of fluorescent proteins

Using Gene Juice transfection reagent, 0,4 ug of expression plasmid pcDNA Cherry and 0,2 and 0,4 ug of Cherry-TLR2 were transfected into U373-CD14 cells in a 24 well plate format over different time-points 24, 48, and 72 hours, and cells were imaged with EVOS FL Auto Cell Imaging systems. Figure 15 represents images for transfection with the two plasmids, concentrations and all time points. Same plasmid backbone with different insert genes was studied to assess the transfection efficiency of the different genes. Toll-like receptor 2 was chosen as candidate gene due to future planned experiments depending on an efficient transfection and expression of the receptor. As the images were obtained with different light exposures, we could not quantify the fluorescence from EVOS images, even though this would be ideal to compare the different time points and concentrations. Because of this, the qualification of protein expression was preformed visually by counting amount of fluorescent cells for each condition. Images were obtained from random places in the wells, which could also affect the results.

We observed two different phenotypes of transfected cells; TLR2 Cherry were only expressed certain areas in the cells, e.g. in the cell membrane and some places in the cytosol, while the expression of Cherry was observed distributed in the whole cell. The expression of TLR2 Cherry was observed in clusters about one big spot per cell, and not spread evenly throughout the membrane as expected. Closer examination with confocal would have to be preformed to confirm this, as confocal provides a higher resolution for images than EVOS microscope.

For 24 hours transfection with 0,2 ug TLR2 Cherry, no cells were counted as transfected because only weak fluorescence were detected and it was hard to distinguish cells. For 48 hours, about 34 % of the cells were counted as transfected, but the expression for each cell was generally low concluded by the low emitted light. After 72 hours, about 33 % of the cells were counted as transfected, and the expression was assumed to be higher for each cell visually observed by the amount of emitted fluorescence.

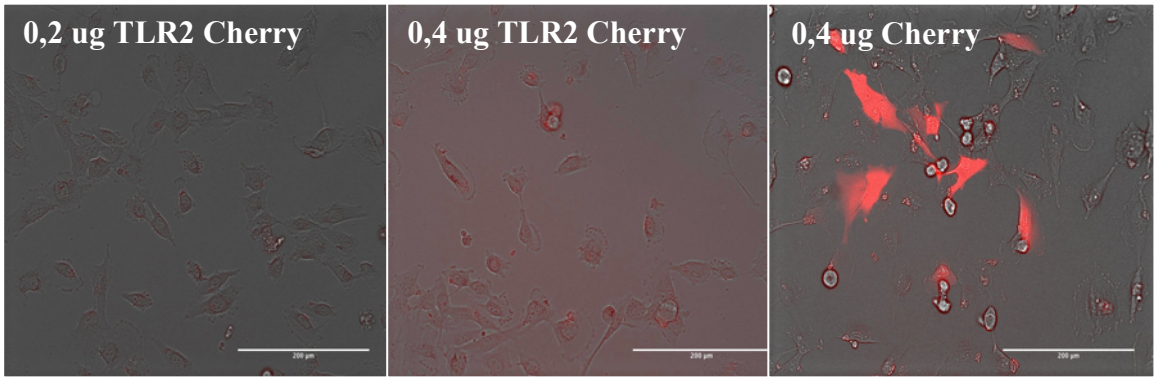
For cells transfected with 0,4 ug TLR2 Cherry, about 25 % of the cells were counted as transfected after 24 hours, 64 % after 48 hours, and 34 % after 72 hours. The amount of fluorescence from each cell was generally observed as higher than for 0,2 ug concentration, especially for 48 hours.

For cells transfected with Cherry, about 32 % of the cells were counted as transfected after 24 hours, 36 % after 48 hours, and 46 % after 72 hours. The amount of fluorescence for each cell was higher than for TLR2 Cherry transfections.

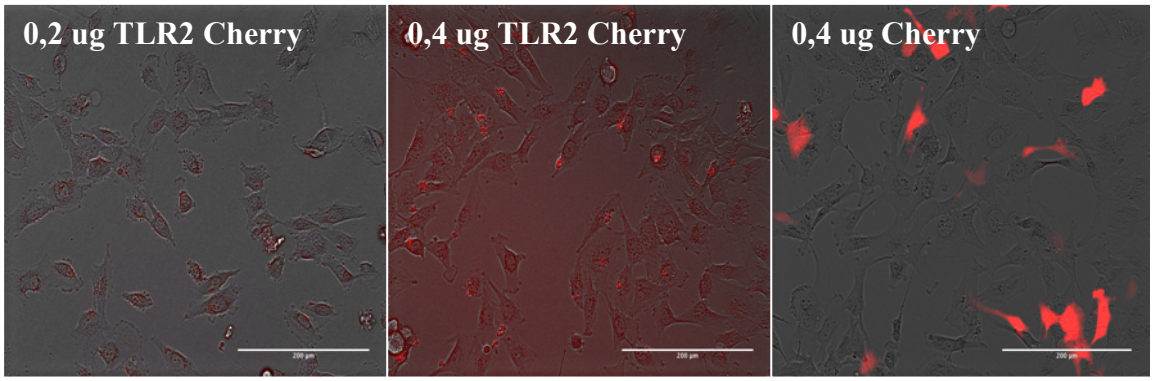
We concluded that a transfection concentration of 0,4 ug plasmid gave higher expression of the receptor in each cell than 0,2 ug based on the amount of emitted fluorescence, and a higher amount of transfected cells on an average. We also observed that 48 and 72 hours post transfection gave highest transfection efficiency, and that transfection with the two different genes gave two different phenotypes of expression in the U373-CD14 cells.

4. Results

24 hours:



48 hours:



72 hours:

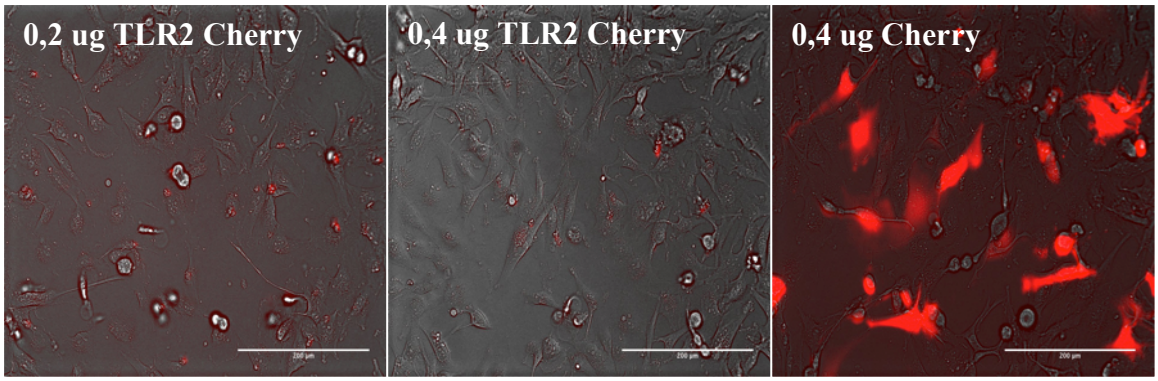


Figure 15: U373-CD14 cells imaged 24, 48, and 72 hours post transfection with EVOS FL Auto cell imaging system and a 20x objective. Left images represent transfection with 0,2 ug pcDNA Cherry TLR2, middle images 0,4 ug pcDNA Cherry TLR2, and right images 0,4 ug pcDNA Cherry. All images were taken from random places in the wells. Scale bars: 200 µm.

4.3.4 U373-CD14 cells transfected with 1 ug plasmid gave highest protein expression for both TLR8 and TLR2, 72 hours post transfection

To assess the protein expression of the wild type TLR2 and TLR8 genes in plasmids 72 hours post transfection, different concentrations of plasmids were transfected into U373-CD14 cells. We observed with imaging that the amount of protein expression could vary for the cells with different genes and plasmids; hence the protein expression was wanted to assess with Western blotting. Concentrations used for transfection were 300 ng, 500 ng, 1 ug and 1,5 ug plasmid for both pcDNA TLR2 and pUNO1 TLR8b. The protein expression is presented in figure 16.A. As a control sample, empty plasmid pcDNA3.1 was transfected into the cells. The top panel represents staining for endogenous control Actin at 42 kDa, middle panel represents protein amounts of TLR8, and lowest panel represents protein amounts of TLR2.

We did not observe any bands for TLR2 at transfection concentrations below 500 ng. Same observation was done for TLR8 at transfection concentrations 300 ng, and it was presumed that the concentrations were not sufficient for a detectable protein expression in the cells. The cells transfected with pcDNA3.1 did not show expression of TLR2 or TLR8, which reflects the findings of U373-CD14 cells not possessing the receptors endogenously [77]. We observed bands for TLR2 proteins for 1 and 1,5 ug plasmid transfection at expected protein size 95 kDa, and bands were detected for TLR8 proteins at size 140 kDa for 500 ng, 1 ug, and 1,5 ug. The size of 140 kDa might represent un-cleaved TLR8 in the cells (Jørgen Stenvik, personal communication). Relative quantification with Actin as loading control was performed for both proteins to determine the amount of protein expression. Absorbance signals from fluorescent proteins towards primary antibodies for proteins of interest were normalized to corresponding signals from Actin in the same lanes, to remove variation of different loading amount to the wells and providing a relative quantification for the amount of proteins present. The calculations are provided in figure 16.B.

Two biological replicates were analyzed for each concentration of both plasmids. Based on the relative quantification of the protein expression, the absorbance for protein amounts with 1 ug plasmid transfection was highest for both proteins, and therefore chosen to proceed with in further experiments. In general, the protein expression was low for all concentrations and both plasmids, compared to expression of GFP-Keap1, shown in figure 14.B. We observed a large variation of absorption between the two replicates of 1 ug transfection with TLR2. No positive control cells were included in the experiment, but the antibodies towards TLR2 and TLR8 were used previously in our group, and were reported to be functional. Ideally control cells with known expression of TLR2 and TLR8 would have been used for positive controls.

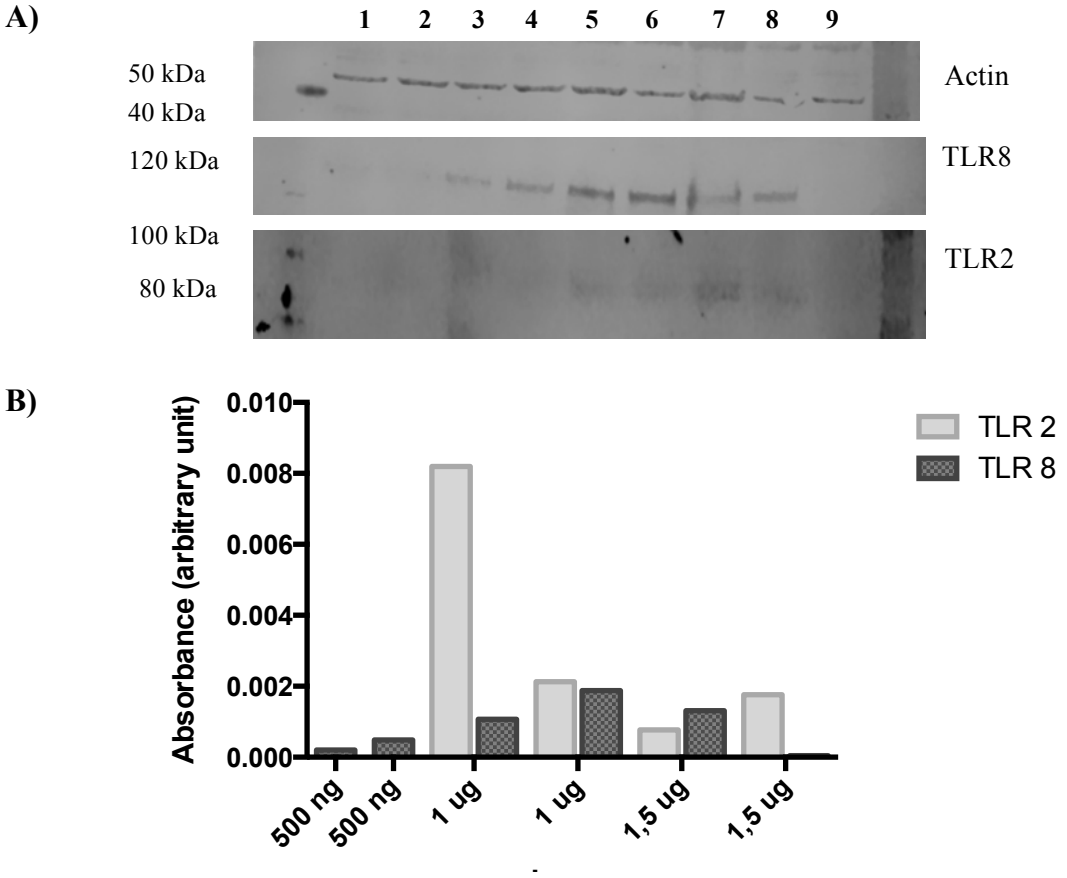


Figure 16: U373-CD14 cells transfected 72 hours with TLR2 shown in size 95 kDa and TLR8 shown in size 140 kDa. Lane 1+2 represents transfection with 300 ng, lane 3+4 with 500 ng, lane 5+6 with 1 ug, lane 7+8 with 1,5 ug, and lane 9 with 1 ug pcDNA3.1 control plasmid. Protein sizes were compared to ladder Magic marker. Protein expression of the plasmids is shown by separation on a NuPage 4-12 % gel (A), and staining with appropriate antibodies to detect fluorescent signals. Endogenous control Actin with size 42 kDa was used for relative quantification of protein amounts, presented in B. The absorbance signals from fluorescent antibodies towards genes of interest were normalized to corresponding signals from Actin. Two biological replicates were analyzed for each concentration for both plasmids.

Collectively, we concluded from all the transient transfection experiments that 1 ug (or 0,4 ug in scaled- down format) concentration gave highest frequency of fluorescent cells and highest protein expression. We also observed that 48 and 72 hours incubation gave the highest number of cells expressing fluorescence (generally over 30 %), based on visual qualification of fluorescence between the different time-points. The protein expression after 72 hours varied a lot from plasmid to plasmid.

4.4 Keap1 expression was confirmed in transfected U373-CD14 cells after immunoprecipitation

Immunoprecipitation was performed to assess the efficiency of the assay for pulling out transfected constructs with Keap1 and later be able to determine the binding partners of the protein. Transfection of U373-CD14 cells with 1 ug GFP and 1 ug GFP-Keap1 was performed in duplicates, and one sample for each plasmid transfection were infected 72 hours post transfection with 50 MOI *M. avium* for 4 hours, presented in figure 17. The infection was performed to observe interactions induced or abolished by *M. avium* infection. Magnetic beads with bound antibody towards GFP were used to pull out the GFP and GFP-Keap1 proteins from the cell lysate. We loaded 5 % of total input lysate, bound GFP and GFP-Keap1 (antigens) to beads, both uninfected and infected, to the wells to separate proteins. In addition the remaining lysate after incubation with magnetic beads were loaded to establish the efficiency of the beads.

Antibodies towards GFP and Keap1 were used to determine the presence of proteins at the membrane. A different GFP antibody than used for cross-linking to the magnetic beads (GFP ab290 rabbit polyclonal, Abcam) was used to stain for GFP antigens (GFP JL-8 mouse monoclonal, Living Colors) on the membrane to avoid cross-linking.

The membrane with remaining antigens after incubation with magnet beads (unbound antigens) is not presented because it did not contain any visible bands. We concluded that all present antigens had bound to the magnetic beads based on this observation. We presumed that too low percentage of the total protein lysate were loaded, because no bands was observed for the GFP transfected cells and only weak bands for GFP-Keap1 transfected cells. We further observed that GFP did not bind efficiently to the beads, but this could be due to the low transfection efficiency as no bands were detected from the total lysate. In contrast, GFP-Keap1 was able to bind to the beads and efficiently get “pulled-out”, as visualized by the appearance of strong bands at correct size. More bound GFP-Keap1 was observed than total amount of GFP-Keap1 in the lysate, which confirms that the percentage of total lysate loaded was too low. The observed protein sizes of GFP and GFP-Keap1 corresponded to right protein sizes at 26,9 kDa and 96 kDa, respectively. We observed a band with different protein size positive for both GFP and Keap1 antibodies at around 220 kDa which could represent inefficiently denaturized Keap1 dimers. Another band at 70 kDa positive for Keap1 antibody was observed, and could represent endogenous Keap1 with size 69,6 kDa that had dimerized to the transfected Keap1 and was pulled out simultaneously.

All bands from cells that were infected seemed weaker than un-infected cells, but no relative quantification could be calculated because endogenous control of a house- keeping gene was not included in the staining. In addition the expression was only preformed one time, and further evidence is needed to draw this conclusion.

We concluded that immunoprecipitation was an efficient assay to preform pull-down experiments with for the future detailing of Keap1’s interaction partners, as GFP-Keap1 was efficiently bound to the magnetic beads and purified from the cell lysate. By the low amount

of GFP present in the cell lysate, it was though that the transfection was not efficient enough to pull out the present GFP, hence the bands could not be visualized.

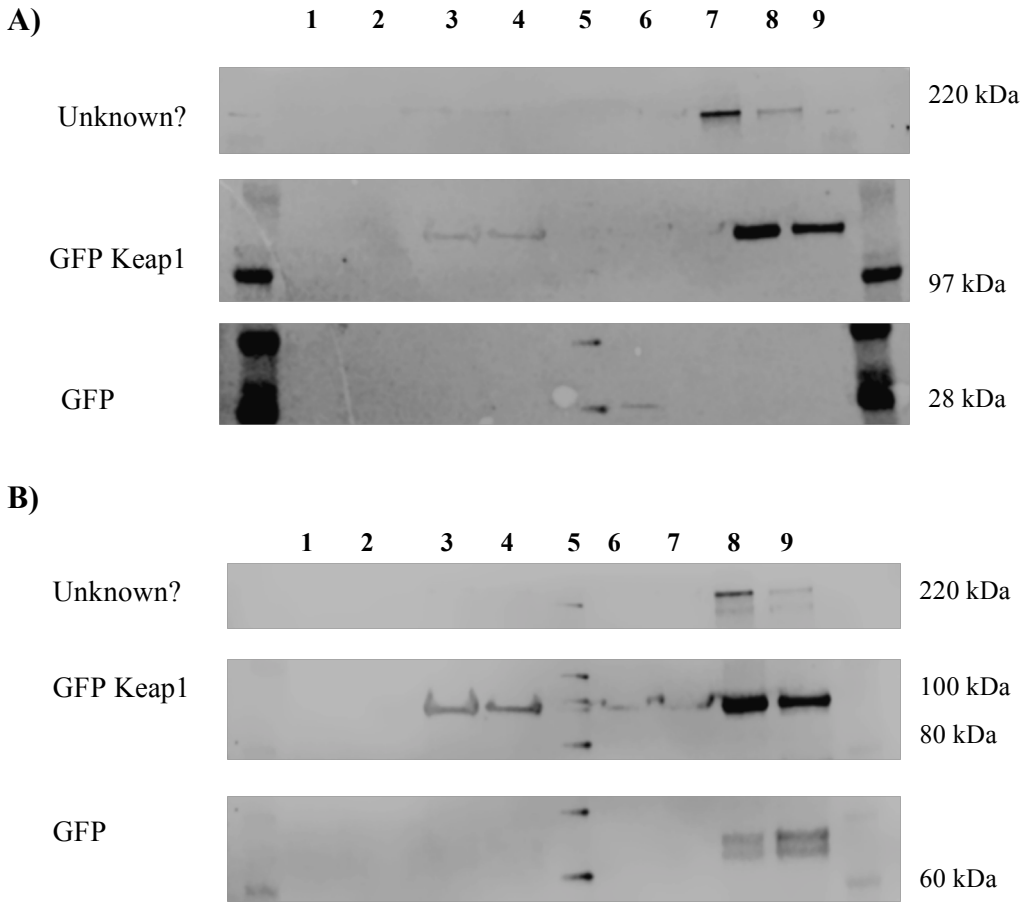


Figure 17: Figure A represents channel for GFP and figure B channel for Keap1- staining of samples loaded. Lane 1: 5 % of total protein lysate input GFP transfection, 2: 5 % of total protein lysate input GFP+ 50 MOI infection 4 hours, 3: 5 % of total protein lysate input GFP-Keap1 transfection, 4: 5 % of total protein lysate input GFP-Keap1 transfection+ 50 MOI infection 4 hours, 5: Magic marker ladder, 6: Bound GFP to beads, 7: Bound GFP to beads+ 50 MOI infection 4 hours, 8: Bound GFP-Keap1 to beads, 9: Bound GFP-Keap1 to beads+ 50 MOI infection 4 hours.

4.4 Transiently transfected U373-CD14 cells with TLR2 or TLR8 displayed an inflammatory response to *M. avium* infection

As we discovered that the cell-line U373-CD14 lacked expression of endogenous TLR2 and TLR8, and that the response towards *M. avium* was low for chosen inflammatory cytokines, we hypothesized that transient transfection of TLR2 or TLR8 could enhance the response. TLR2 or TLR8 plasmids with and without fluorescence were co-transfected and transfected into U373-CD14 cells. Cells were stimulated with known TLR2- and TLR8 ligands as a positive control for expression and functionality of the receptors, and infected with *M. avium* at 50 MOI. LPS stimulation of pcDNA3.1 control plasmid- transfected cells was assessed to observe if the response to LPS was affected by transfection by comparing to un-transfected cells, and as a positive control in the experiments. Un-transfected cells were stimulated with all ligands to compare the responses.

All cell lysates were analyzed with q-PCR for mRNA expression of the genes. Cells transfected with empty pcDNA3.1 vectors were used as reference sample to control for possible increased background inflammation activity resulting from the transfection itself.

4.4.1 Cytokine production from LPS stimulation gave evidence of increased background of cytokine levels

The LPS response was compaired for un-transfected U373-CD14 cells and for pcDNA3.1 transfected U373-CD14 cells, to determine if the different conditions gave altered induction of inflammatory cytokines. The graphs in figure 18 show that the transcription of all cytokines (TNF-α, IL-8, IFNβ, and IP10) was higher for LPS stimulated un-transfected cells than for transfected cells, compared to reference samples that were not stimulated. We observed that the difference was highest for IL-8 and IP10 cytokine levels.

By studying the delta Ct values of each cytokine for both conditions of un-stimulated cells that would represent the basal background levels, it was observed that the values were either slightly or highly increased for un-transfected cells compared to transfected ones, as presented in table 23. The Ct values of endogenous control GAPDH levels were stable for all conditions. The values were obtained from biologically separate experiments.

The change in fold induction was calculated for the pcDNA3.1- transfected cells with un-transfected cells as a reference sample. A decrease in the Ct value of 1 corresponds to a two-fold induction, RQ response. We presumed that the decreased delta Ct values for transfected cells with pcDNA3.1 empty vectors could explain the lower cytokine response to LPS. The values for IL-8 and IP10 specifically were dissimilar between the two conditions and as observed for the high change in calculated RQ values, this could be explained by increased "background" activity as a result of the transfection and variable background levels between samples. The high background could vary between biologically different samples and hence subtract increased cytokine levels caused by the stimulations and not transfection.

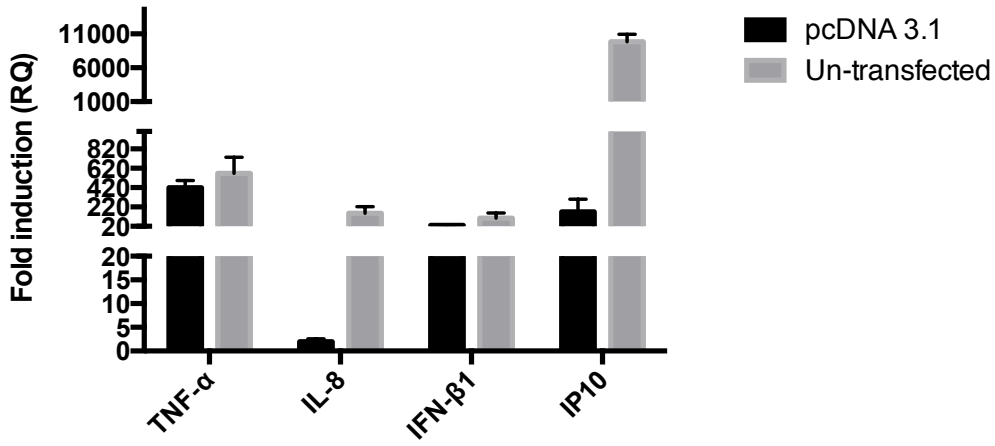


Figure 18: Response towards 4 hours stimulation with LPS concentration 200 ng/ml for un-transfected cells and pcDNA3.1 transfected cells. Cytokines screened for with q-PCR were TNF-α, IL-8, IFNβ, and IP10 and GAPDH was used as endogenous control. Reference samples were un-stimulated un-transfected cells and un-stimulated pcDNA3.1 transfected cells with RQ 1.

Table 23: Delta Ct values for un-stimulated un-transfected cells compared to Ct values for un-stimulated transfected cells, and RQ calculated for transfected cells based on un-transfected cells as reference sample.

	Un- transfected cells, un-stimulated	**Transfected cells pcDNA3.1, un-stimulated**	
Gene	Delta Ct	Delta Ct	RQ
TNF-α	17	13	16
IL-8	5	-5	1024
IFNβ	13	12	2
IP10	15	10	32

4.4.2 Stimulation with TLR2-ligands up-regulated inflammatory responses in TLR2 and TLR8 transfected U373-CD14 cells

Stimulation with TLR2- ligands LM, FSL-1, and PAM3CSK4 for transfected U373-CD14 cells was preformed to assess the levels of inflammatory cytokines. We expected an enhanced inflammatory response for the TLR2-transfected cells if the TLR2 receptor was functional and present.

Un-transfected cells and cells co-transfected with both TLR2 Cherry and TLR8 wild-type were not screened for expression of IP10.

The results obtained from 4 hours stimulation with LM, FSL-1, and PAM3CSK are presented in figure 19. By comparing the response for reference sample pcDNA3.1 and un-transfected cells, we could observe a significant down-regulation in the response for un-transfected cells, except for IFNβ for PAM3CSK4. Both TLR2 wild-type and TLR2 cherry plasmids were used for transfection. The responses for TLR2 wild-type were only significantly up-regulated for TNF-α (RQ 2-3) for FSL-1 stimulation, while TLR2 Cherry transfected cells gave a significant up-regulation of IFNβ (RQ 5-10) and IP10 (RQ 10-20) for all TLR2-ligands and TNF- α up-regulation (RQ 15-20) for FSL-1 stimulation. For TLR8 wild type- transfected cells, significant up-regulation of IFNβ (RQ 15-20) was observed for all TLR2- ligand stimulations, and IP10 up-regulation (RQ 5) for FSL-1 and PAM3CSK4 stimulations. We did not expect an inflammatory response for the TLR8 transfected cells towards TLR2-ligand stimulations. TLR8-YFP-transfected cells did not respond with significant up-regulation to any of the stimulations.

Cells co-transfected with TLR2 wild- type and TLR8 wild- type displayed a significant up-regulation of IFNβ (RQ 3-4) to LM stimulation. Cells co-transfected with TLR2 Cherry and TLR8 wild- type did not display a significant up-regulation of the inflammatory cytokines for neither of the stimulations. We expected a higher up-regulation of cytokines for the co-transfected cells, especially cells transfected with TLR2 Cherry based on the up-regulation of inflammation for the cells transfected with TLR2 Cherry alone.

Standard deviations were calculated and statistical analysis was performed for samples with three biological replicates, and presented in figure 19.

4. Results

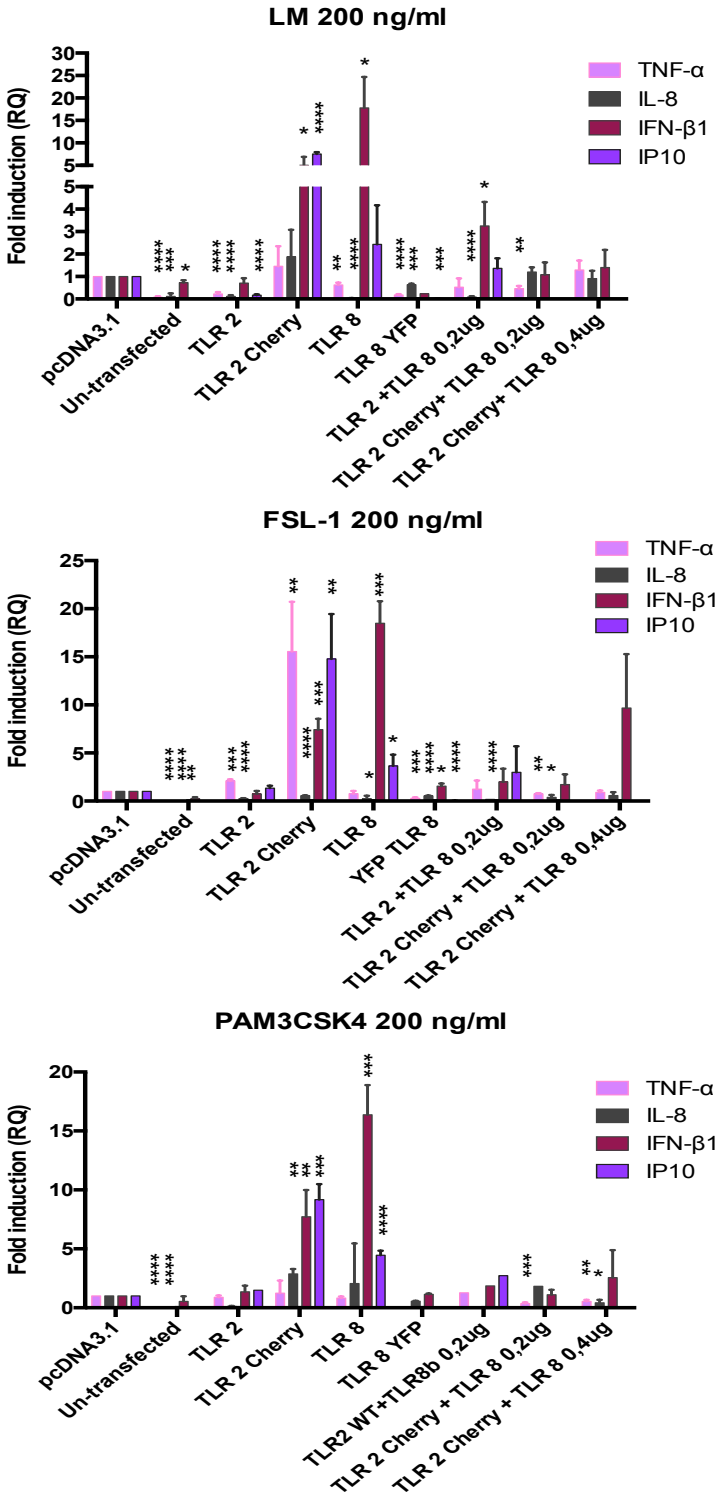


*Figure 19: Un-transfected and transfected U373-CD14 cells with different plasmids for TLR2 and TLR8 wild-type or fluorescence-fused genes presented. Figures are displaying up or down-regulation as a response to 4 hours stimulation with LM, FSL-1, or PAM3CSK4 (TLR2 ligands) for genes TNF-α, IL-8, IFNβ, and IP10. Fold induction is presented compared to reference sample cells transfected with pcDNA3.1. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures. Statistical analysis not performed for: TLR8- YFP transfection for IFNβ for LM stimulation; un-transfected cells for IP10 for both FSL-1 and PAM3CSK4; TLR2 wild-type for IP10 for PAM3CSK4; TLR8 YFP for all cytokines for PAM3CSK4; TLR2 wild-type+ TLR8 for all cytokines for PAM3CSK4; TLR2 Cherry+ TLR8 0,2 for IP10 for all stimulations (and IL-8 in addition for PAM3CSK4); TLR2 Cherry+ TLR8 0,4 for IP10 for all stimulations.*

4.4.3 Stimulation with TLR8-ligand up-regulated inflammatory responses in TLR8 wild-type transfected cells

Stimulation with CL075 was performed for transfected cells as an up-regulated inflammatory response was expected if the TLR8 receptor was present and functional.

Un-transfected cells and cells co-transfected with both TLR2 Cherry and TLR8 wild-type were not screened for IP10.

The result from 4 hours stimulation of all transfection parameters with CL075 is presented in figure 20. By comparing responses for reference sample pcDNA3.1 and un-transfected cells, we could observe a down-regulation in the response for un-transfected cells. The cells transfected with TLR8 wild-type responded with a significant up-regulation (RQ 5-17) of all screened cytokines to the CL075 ligand, while cells transfected with TLR8-YFP did not show any response to the ligand. The cells co-transfected with both TLR2 and TLR8 displayed significant up-regulation of IFNβ (RQ 2) for combination with wild-type TLR2, and up-regulation of TNF-α for combination with 0,4 ug TLR2 Cherry (RQ 2). We expected a higher up-regulation of cytokines for cells co-transfected with both TLRs. Especially for cells transfected with 0,4 ug of both plasmids, as 0,4 ug were used for transfection in cells with separate plasmids TLR8, and responded with higher up-regulation.

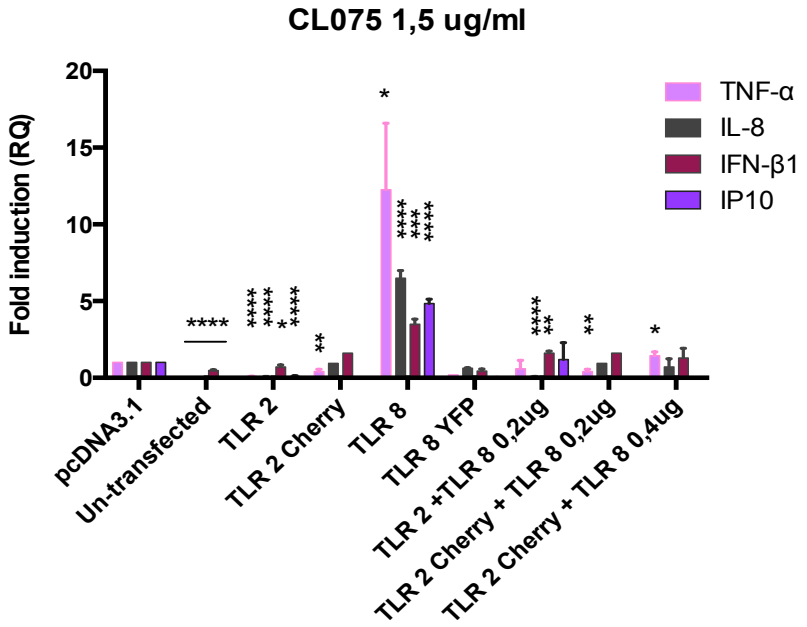


*Figure 20: Un-transfected and transfected U373-CD14 cells with different plasmids for TLR2 and TLR8 wild-type or fluorescence-fused genes presented. Figures are displaying up-regulation as a response to 4 hours stimulation with CL075 (TLR8 ligand) for genes TNF-α, IL-8, IFNβ, and IP10. Fold induction is presented compared to reference sample cells transfected with pcDNA3.1. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures. Statistical analysis not performed for: TLR2 Cherry transfected cells for IL-8, IFNβ and IP10; TLR8 YFP for all cytokines,; TLR2 Cherry+ TLR8 0,2 for IL-8, IFNβ, and IP10; TLR2 Cherry + TLR8 0,4 for IP10.*

4.4.4 Infection with 50 MOI *M. avium* up-regulated inflammatory responses in cells transfected with TLR2 and TLR8

Infection with 50 MOI *M. avium* for 4 hours was performed for transfected cells as we expected an enhanced inflammatory response for cells co-transfected or transfected with TLR2 and TLR8 compared to the U373-CD14 un-transfected cells. Un-transfected cells and cells co-transfected with both TLR2 Cherry and TLR8 wild-type were not screened for IP10.

The result for cytokine production of infected cells is presented in figure 21. By comparing responses for reference sample pcDNA3.1 and un-transfected cells, we could observe a down-regulation in the responses for un-transfected cells. Cells transfected with TLR2 Cherry displayed the highest up-regulation of inflammatory cytokines with a fold induction between 10-30 compared to reference sample for all screened cytokines, as a response towards infection. The up-regulation was calculated significant for TNF-α, IFNβ and IP10. Cells transfected with TLR2 wild-type, displayed significant up-regulation of TNF-α, IFNβ and IP10 (RQ 3-5). TLR8 wild-type transfected cells had significantly higher expression of TNF-α, IFNβ and IP10 towards infection (RQ 5-7), compared to reference sample.

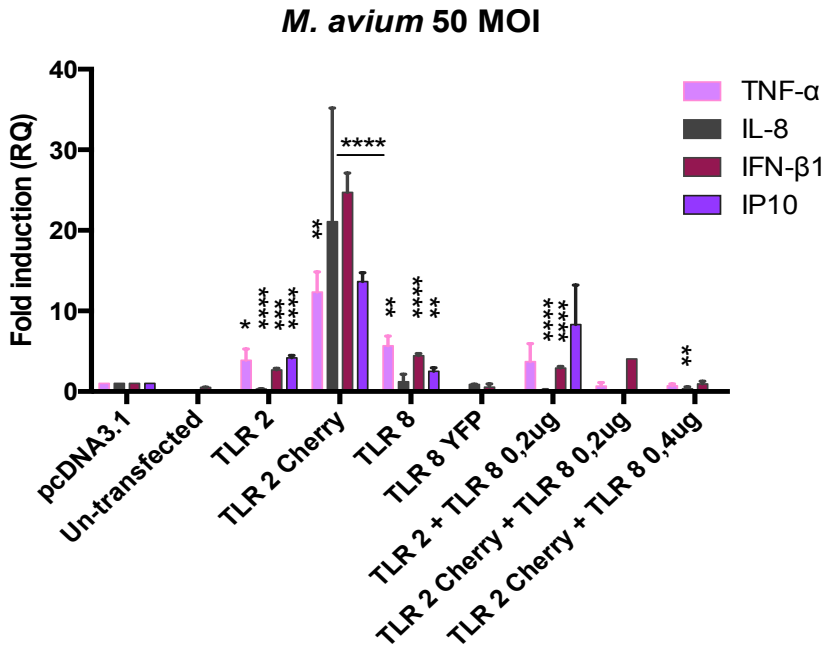


*Figure 21: Un-transfected and transfected U373-CD14 cells with different plasmids for TLR2 and TLR8 wild-type or fluorescence-fused genes presented. Figures are displaying up-regulation as a response to infection 4 hours with 50 MOI M. avium, for genes TNF-α, IL-8, IFNβ, and IP10. Fold induction is presented compared to reference sample cells transfected with pcDNA3.1. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures. Statistical analysis not performed for: Un-transfected cells, all cytokines; TLR8 YFP, all cytokines; TLR2 Cherry + TLR8 0,2, all cytokines; TLR2 Cherry+ TLR8 0,4, IP10.*

It was expected a higher up-regulation of cytokines for cells co-transfected with both TLRs, especially the cells with TLR2 Cherry as they displayed higher response alone. The only significant up-regulation detected for the co-transfected cells was from TLR2 wild-type + TLR8 transfection for IFNβ cytokines towards infection, with RQ below 5.

4.4.5 Transfection with pUNO1 vector up-regulated cytokines IL-8 and IFNβ as a background

The previously observed results for up-regulation of cytokines in transfected cells, pcDNA3.1-empty vector transfected cells as reference sample was believed to cover the background response activity towards transfection. Since the pUNO1 TLR8 transfected cells displayed an up-regulation of IL-8, IFNβ, and IP10 for the TLR2 ligands, we hypothesized that the pUNO1 vector could initiate the response itself, and that the response was not caused by TLR8 activation.

For the experiment, transfected cells with TLR2 Cherry, and co-transfected cells with TLR2 Cherry and TLR8 wild-type were not screened for IP10. In addition, the vector with TLR8 YFP was not included, as the plasmid did not initiate a response for any of the previous experiments. Results are presented in figure 22 with calculated statistical analysis for samples with more than 3 biological replicates.

We observed that the pUNO1 TLR8 transfected cells gave a significant high up-regulation of IFNβ as a response to the transfection itself (RQ 25), and that cells co-transfected with TLR2 Cherry and TLR8 wild-type gave an up-regulation of cytokines IL-8 and IFNβ (RQ 5), compared to cells transfected with pcDNA3.1 empty vectors. The Cherry TLR2 plasmid transfected cells gave a slight significant up-regulation of IFNβ with RQ 1,5. As the TLR2 plasmid transfected cells did not induce a response, it was believed that the co-transfection with TLR2 and TLR8 initiated a response alone because of the presence of the TLR8 vector.

To observe all the responses towards the ligand stimulations and infection with *M. avium* for the different parameters without including background activity responses towards the vectors, all values were normalized and presented in figure 39, Appendix VI. All values for the stimulation responses were divided on the mean value of the response towards the vectors alone. Normalized values for cytokine IP10 was not obtained for cells transfected with TLR2 Cherry, TLR2 Cherry + TLR8 wild- type, as they were not screened for response towards the vectors alone. In addition it was not calculated a normalized response for TLR2 Cherry, cytokine IP10 for stimulation CL075 as the value was not obtained in earlier experiments. Standard deviation and statistical analysis was performed for samples with 3 biological replicates.

When we normalized for background responses, results presented in figure 39.B, Appendix VI, the previously observed up-regulation of IFNβ for TLR8- transfected cells to TLR2-ligands was lost, while IP10 responses were not. We observed that only cells transfected with TLR2 wild-type or Cherry gave a high up-regulation with RQ 15- 90 of inflammatory cytokines towards infection, and that TLR8 transfected cells had a low up-regulation for cytokines TNF-α and IP10 (RQ 5-10). TLR2 Cherry alone gave an enhanced response

towards infection compared to co-transfected cells with both TLR2 and TLR8. We did not expect this, at least not for concentration 0,4 ug of both plasmids, as it was the same amount of plasmid for TLR2 Cherry alone. Some values for vector responses, like transfected cells with the TLR2 vectors and co-transfected cells with TLR2 wild-type + TLR8, were lower than reference sample RQ 1, and normalizing to these values resulted in an increased response towards the stimuli.

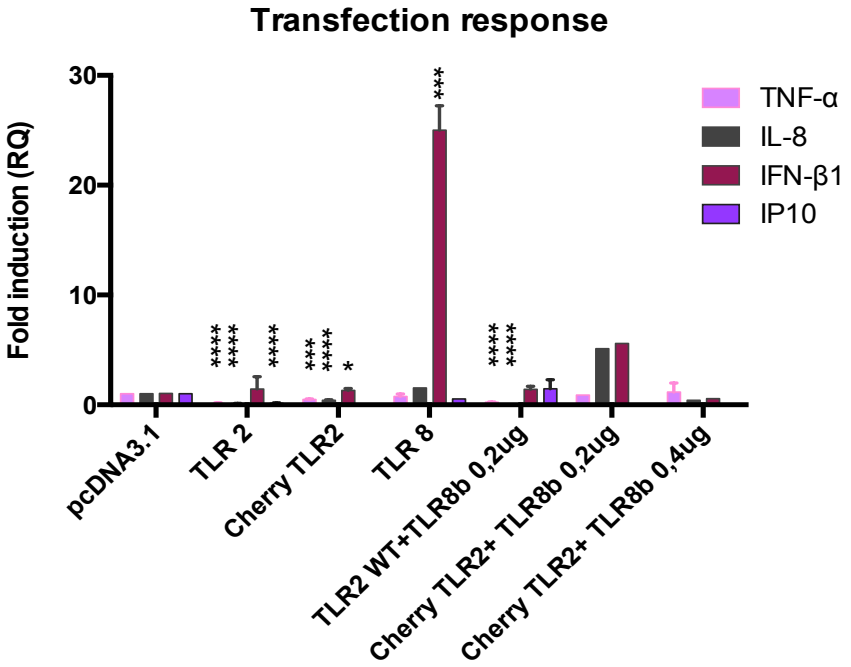


*Figure 22: Un-transfected and transfected U373-CD14 cells with different plasmids for TLR2 and TLR8 wild-type or fluorescence-fused genes presented. Figures are displaying up-regulation as a response to the plasmid itself after 4 hours, for genes TNF-α, IL-8, IFNβ, and IP10. Fold induction is presented compared to reference sample; cells transfected with pcDNA3.1. . Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures. Statistical analysis not performed for: pUNO1 TLR8b IL-8 and IP10, Cherry TLR2+ TLR8 0,2 all cytokines, and Cherry TLR2+ TLR8 0,4 IL-8, IFNβ, and IP10 because of only one biological replicate.*

We concluded after the transient transfection and co-transfection of cells with the different plasmids with TLR2 and TLR8 and stimulations, that the function of the TLR2 and TLR8 receptors was not that efficient because the cytokine expression was variable. However, the transfection with TLR2 Cherry seemed to work at least partially as cells responded with an up-regulation of the cytokines towards TLR2-ligands and did not respond to the TLR8-ligand. In addition, the cells responded to infection with *M. avium*. TLR2 wild-type transfected cells gave various results, as they did not respond to TLR2- ligands that efficiently (fold inductions below 3 compared to reference sample), but with an up-regulation towards infection for all cytokines except IL-8 with RQ around 5. The TLR8 wild-type- transfected cells responded to

TLR2- ligands with up-regulation of cytokines IFNβ and IP10, which was not expected. The up-regulation of IFNβ could be explained by the up-regulated response towards the vector itself, and the response was diminished when normalizing values. The response for co-transfected cells with TLR2 and TLR8 gave lower responses for ligand stimulations and *M. avium* infection, compared to cells with separate transfections in same concentrations. We did not expect this, especially not for the combination with TLR2 Cherry, as it was assumed to work efficiently. The low but significant up-regulation of cytokines observed for some of the stimulations, were thought not to be of biological relevance.

4.5 Lentiviral -and myc-tagged expression vectors with Keap1, TLR2 and TLR8 were successfully made with gateway cloning

To make a stable cell-line of U373-CD14 expressing TLR2 or TLR8, we designed lentiviral vectors by gateway cloning. In addition, lentiviral vectors expressing GFP-Keap1 was created for the THP1 cells, in case the cell-lines U373-CD14 TLR2/8 did not respond to *M. avium* infection. The lentiviral vector with GFP-Keap1 could be used for imaging purposes of intracellular localization because of the GFP- tag and might also work for interaction studies if the GFP tag do not interfere with any of the protein's interaction sites.

In addition to lentiviral vectors, pDest-myc vectors with GFP and GFP-Keap1 was designed for pull-down experiments in the U373-CD14 TLR2/8 cell-line if they turned out to respond to *M. avium* infection.

All plasmids successfully designed with gateway cloning are presented in table 24.

Table 24: Lentiviral and myc-tagged vectors created successfully with gateway cloning, purposed for use in either cell-line U373-CD14 or THP1.

Cell-line	U373-CD14		THP1	
Lentiviral vectors	pLenti CMV Puro Dest w118-1 TLR2	pLenti CMV Puro Dest w118-1 TLR8	pLenti CMV Puro Dest w118-1 GFP	pLenti CMV Puro Dest w118-1 GFP-Keap1
Myc-tagged vectors	pDest-myc-GFP	pDest-myc-GFP-Keap1		

4.5.1 Gene sequences for GFP, GFP-Keap1, TLR2 and TLR8 were successfully amplified with attB regions

To purify the genes of interest from the donor vectors, specific primers were designed for the genes TLR2, TLR8, GFP and GFP-Keap1 both forward and reverse with attB/2 sites flanking them. By using KOD Xtreme Hot start DNA polymerase; the genes were successfully amplified from the vectors. Different PCR programs specific for the primers were designed, and the PCR products are presented in figure 23A+B. The products were linked to attB1/2 sites to further use them in gateway cloning BP reaction after amplification and purification.

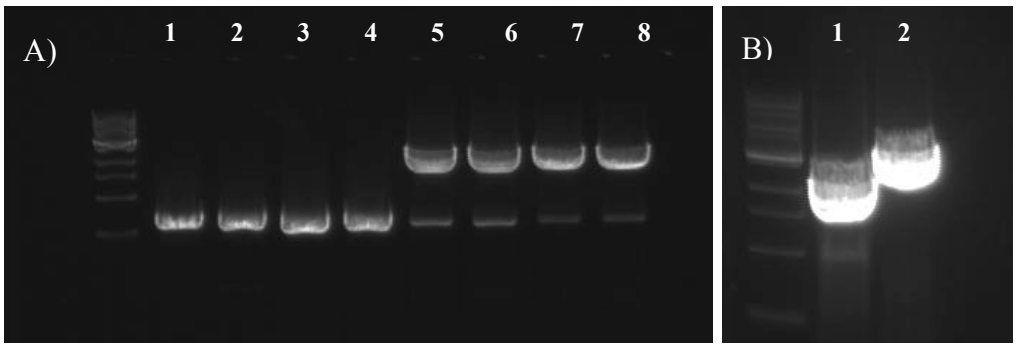


Figure 23: Figure A represents the PCR products from optimized program for GFP and GFP-Keap1 constructs. Lane 1: GFP 60°C, 2: GFP 62°C, 3: GFP 64°C, 4: GFP 66°C, 5: GFP-Keap1 60°C, 6: GFP-Keap1 62°C, 7: GFP-Keap1 64°C and 8: GFP-Keap1 66°C. Figure B represents the PCR products for TLR2 and TLR8. Lane 1: TLR2 , 2: TLR8 construct. Ladder used was 1 kB and program run for gel was 100 V, 40 minutes.

For figure 23A, for GFP and GFP-Keap1, the products in lane 1 to 4 for GFP were at correct sizes around 717 bp. The products for construct GFP-Keap1 in lanes 5 to 8, displayed a promising size of around 2588 bp (1871 bp for Keap1 + 717 bp for GFP), as the bands were observed between the ladder products of 2000 bp and 3000 bp. The gel electrophoresis could have been run at 90 V instead of 100 V to be able to get a better separation of the ladder and easier distinguish between the band sizes. In addition, an additional product was observed in lanes 5 to 8, with similar size as for GFP. The band represents a side product and could be either GFP alone or another sequence of similar size. To only include the correct band-sizes the top bands were cut out from the gel and purified for the GFP-Keap1 constructs.

For figure 23.B, the TLR2 product seemed to be at correct size around 2360 bp, close to ladder product 2000 bp. TLR8 construct also seemed to be at correct size around 3126 bp, close to ladder product 3000 bp. It was a bit hard to determine exactly where the bands were present in the lane because of large amount of constructs present in the loading.

The sizes of the products were based on the size of the genes from the donor vectors pDest GFP, pDest GFP-Keap1, pcDNA YFP-TLR2 and pUNO1 TLR8b, gene sequences provided in Appendix IV.

4.5.2 The product sizes of the restriction enzyme digestion gave evidence of positive entry plasmids for GFP-Keap1

As the BP reaction with GFP and GFP-Keap1 gave positive colonies of bacteria on kanamycin resistance plates, it was known that the bacteria clones contained the pDONR221 plasmid. But we still did not have complete evidence of correct gene recombination, it was only assumed to be correct as the bacteria survived and the *ccdB* gene was thought to be lost. The pDest GFP-Keap1 donor plasmid used for amplification of GFP-Keap1, possessed an attB1 site between the genes GFP and Keap1. As the swapping of the construct would be of the region between the attB1 and attB2 sites, it was not sure if both genes would be swapped together or if only the last part with Keap1 would be swapped alone. Because of this issue, additional screening of the pENTRY221 GFP-Keap1 plasmids was important, to assess if both genes were present. A restriction enzyme digestion was performed for pENTR221 plasmids with GFP and GFP-Keap1, and controls of pDONR221 were digested with same enzymes. The results are presented in figure 24.

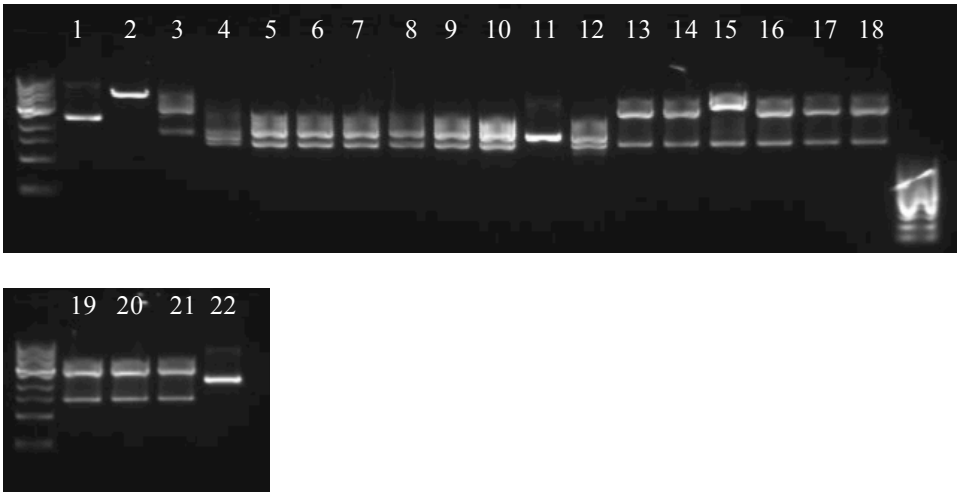


Figure 24: Products of restriction enzyme digestion of pENTR221 plasmids with either GFP or GFP-Keap1, and control pDONR221. Restriction enzymes used were ApaLI and EcoRV. Ladder used in first lane is 1 kB ladder. Lane 1: pDONR221 uncut, 2: pDONR221 cut with EcoRV, 3: pDONR221 cut with ApaLI, 4: pDONR221 double cut, 5: pENTR221 GFP nr.1 double cut, 6: pENTR221 GFP nr.2 double cut, 7: pENTR221 GFP nr.3 double cut, 8: pENTR221 GFP nr.4 double cut, 9: pENTR221 GFP nr.5 double cut, 10: pENTR221 GFP nr.6 double cut, 11: pENTR221 GFP nr.2 uncut, 12: pENTR221 GFP-Keap1 nr.1 double cut, 13: pENTR221 GFP-Keap1 nr.2 double cut, 14: pENTR221 GFP-Keap1 nr.3 double cut, 15: pENTR221 GFP-Keap1 nr.4 double cut, 16: pENTR221 GFP-Keap1 nr.5 double cut, 17: pENTR221 GFP-Keap1 nr. 6 double cut, 18: pENTR221 GFP-Keap1 nr.7 double cut, 19: pENTR221 GFP-Keap1 nr.8 double cut, 20: pENTR221 GFP-Keap1 nr.9 double cut, 21: pENTR221 GFP-Keap1 nr.10 double cut, 22: pENTR221 GFP-Keap1 nr.8 uncut.

4. Results

The predicted product sizes were calculated for the two restriction enzyme digestions and for double digestion, and presented below.

pENTR221 GFP-Keap1

Expected products if digested with both enzymes and correct cloning: 3759 bp+ 1443 bp.

Expected products if incorrect cloning: 3759 bp + 726 bp.

pENTR221 GFP

Expected bands if digested with both enzymes and correct cloning: 1821 bp + 1443 bp.

Do not expect wrong cloning because only one gene was recombined.

pDONR221 (control)

Expected bands if digested with EcoRV: 4762 bp.

Expected bands if digested with ApaLI: 3057 bp + 1705 bp.

Expected bands if digested with both enzymes: 1614 bp + 1443 bp + 1705 bp.

We observed that pENTR221 GFP plasmids (lanes 5-11) had the right sizes and it was concluded that the GFP gene was present. We observed that some of the pENTR221 GFP-Keap1 plasmids (lane 12-22) had correct sizes and were successful, except clone number 1. We concluded that the GFP and Keap1 gene was present in the plasmids. We also observed that clone nr. 4 had a slightly bigger top band than the rest of the clones.

4.5.3 Go taq green PCR screening with specific primers, gave evidence of positive entry clones for all genes

Go taq green PCR was performed to screen for bacteria colonies that contained the GFP gene in plasmids pENTR221 GFP-Keap1. Bacteria colonies were used directly in the PCR tubes, and the result is presented in figure 25. The plasmid pENTR221 clone nr 4, which possessed a slightly bigger product after the restriction enzyme digestion, was chosen to include.

By observing the products on the gel and comparing product sizes to the ladder, it seemed like products in lanes 11, 12, 13, 17, 18, 19 and 20 possessed the GFP gene, while products in lanes 15 and 16 were slightly positive.

Go Taq Green PCR was preformed for the GFP-Keap1 plasmids as sequencing did not give any positive clones with GFP the first time. Go Taq Green PCR was not performed for the GFP plasmids because all clones turned out to be positive with sequencing.

Go Taq Green PCR was also performed for purified plasmids pENTR221 TLR2 and TLR8, four clones for each, to choose positive clones for sequencing. The product sizes from the reaction is presented in figure 26.

A TLR2 control plasmid was left out from the Go Taq Green PCR program, and we chose to use the attB flanked TLR2 construct as a control directly. For TLR8, both a control plasmid pUNO1 TLR8b was used as a control in the Go Taq Green PCR reaction and the attB flanked TLR8 construct was used as an addition control. Based on the results, it seemed like all the cloned entry vectors were positive for the gene TLR2 or TLR8.

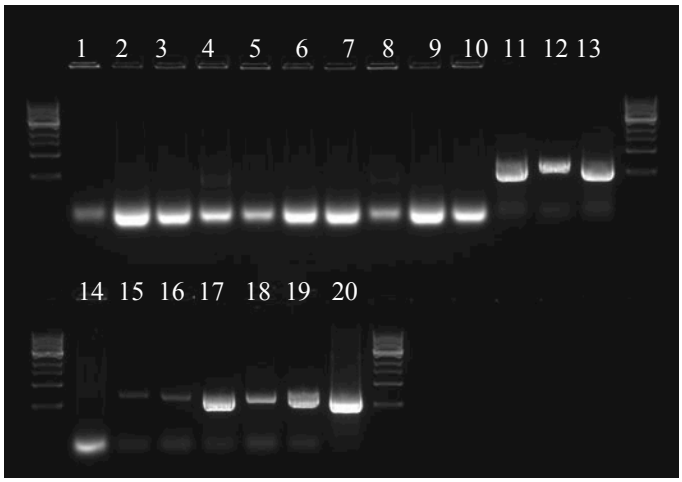


Figure 25: Products from Go Taq Green PCR with specific primers of GFP and PCR program with specific annealing temperatures for the primers. 19 newly picked colonies after BP reaction and transformation of pENTR221 GFP-Keap1 were screened directly for the presence of GFP presented in lanes 1-19. A 1kB ladder was used to look at the sizes of the products. Lane 20 represents purified pENTR221 GFP-Keap1 clone 4.

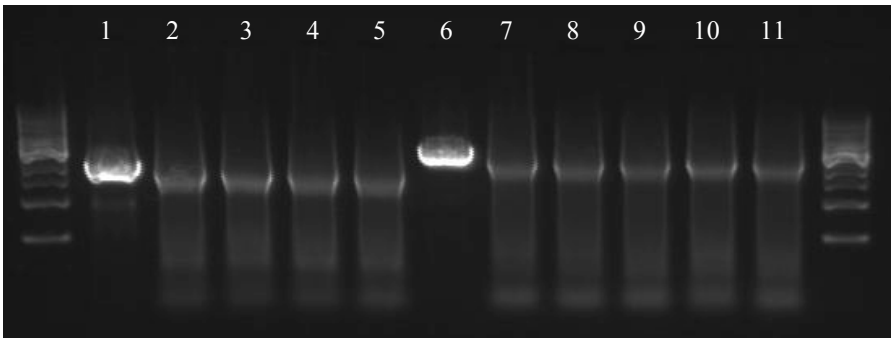


Figure 26: Go Taq Green PCR products presented from pENTR221 clones with TLR2 or TLR8. A ladder of 1 kB was used to assess the sizes of the products. Lane 1: attB flanked TLR2, 2: pENTR221 TLR2 clone 1, 3: pENTR221 TLR2 clone 2, 4: pENTR221 TLR2 clone 3, 5: pENTR221 TLR2 clone 3, 6: attB flanked TLR8, 7: control vector pUNO1 TLR8b with same PCR program, 8: pENTR221 TLR8 clone 1, 9: pENTR221 TLR8 clone 2, 10: pENTR221 TLR8 clone 3, 11: pENTR221 TLR8 clone 4.

4.5.4 Sequencing confirmed successful cloning of entry plasmids

Sequencing of pENTR221 plasmids with inserted genes was performed to quality-check the reading frames and the presence of the genes of interest. After sending chosen plasmids for pENTR221 GFP, GFP-Keap1, TLR2 and TLR8 to sequencing, the results were analyzed in software Clone Manager 9. The gene sequences returned from the sequencing were aligned with the correct gene sequences and with the predicted sequence of the pENTR221 vector with inserted genes.

All plasmids with GFP inserted were positive for GFP, and the similarity with the gene at alignment varied from 98- 100% for different plasmids. Plasmid pENTR221 GFP clone nr 3 was chosen because it gave a 99 % match with the sequence from the forward primer, and a 100 % match with the sequence from the reverse primer.

From all plasmids sent for sequencing with predicted presence of GFP-Keap1, only one of them were positive for the GFP gene, clone number 4. This clone was earlier observed to have a slightly bigger band in the restriction enzyme digestion products, which could have indicated the difference between them even though it did not match with the predicted product sizes calculated. Clone number 4 was chosen to proceed with, and had a 99 % similarity with the GFP-Keap1 gene, both for forward and reverse sequencing. In addition, the middle part of the plasmid was sequenced to fully map the GFP-Keap1 gene sequence and check the reading frame.

For the TLR2 and TLR8 entry plasmids, the alignments gave a range of similarity from 97 % to 99 %. The pENTRY221 TLR2 clone nr 2 and 3 were chosen to proceed with based on 98 and 99 % similarity to the original sequence and plasmid. The pENTRY221 TLR8 clone nr 1 and 3 were chosen to proceed with based on 99 % similarity to the original sequence and plasmid.

4.5.6 Go taq green PCR screening with specific primers, gave evidence of positive expression clones for all genes

After LR reaction with gateway cloning was performed for GFP and GFP-Keap1 containing plasmids, some bacteria colonies were screened for the presence of GFP to be sure that the expression clones contained the gene of interest, result presented in figure 27.

By comparing the result from the positive control plasmid to the expression clones, it seemed like most of the picked colonies did not contain the GFP gene and only some products were weakly positive. We purposed that the reason for this could be either too much or too little bacteria culture added to the PCR tubes. We presumed that the lower band in the lanes represented the primers as they were not able to efficiently amplify the gene sequence because of overload or too low amount of plasmids.

We chose two colonies for each expression plasmids purified; pLenti CMV Puro Dest w118-1 GFP-Keap1, pLenti CMV Puro Dest w118-1 GFP, pDest-myc-GFP-Keap1 and pDest-myc-GFP, and they were again screened for both GFP and Keap1, presented in figure 28.

Instead of adding the control plasmid pENTR221 GFP-Keap1 nr 4, another wrong control plasmid was added with only Keap1. Since the forward primer was specific for GFP-Keap1, the gene was not amplified efficient as observed in lane 14, figure 28. For the rest of the samples, it seemed like all the purified plasmids were positive for GFP. In addition the plasmids with cloned GFP-Keap1 seemed positive for Keap1, even though bands were weak. Qualifications were based on the product sizes compared to the ladder.

After LR reaction for constructs with TLR2 and TLR8, purified pLenti CMV Puro Dest w118-1 TLR2/TLR8 vectors were screened for the presence of the gene of interest to be sure that the gene swapping had occurred in an correct way. Results from the screening are presented in figure 29.

Based on the PCR products and comparing to positive controls that were sequenced, we concluded that the expression clones were positive for TLR2 or TLR8. Some bands were weaker than other, and plasmids with stronger bands were chosen to use for further experiments.

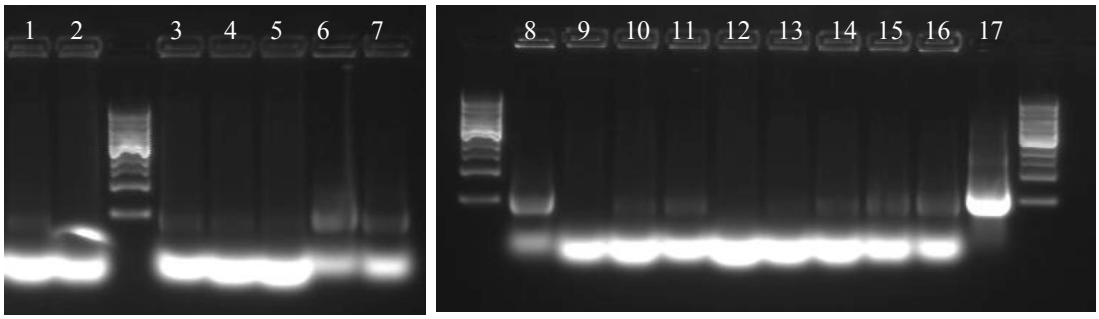


Figure 27: Screening of expression clones in bacteria culture for the presence of the GFP sequence. Lanes 1-4: pLenti CMV Puro Dest w118-1 GFP-Keap1 clone 1-4, lanes 5-8: pLenti CMV Puro Dest w118-1 GFP clone 1-4, lanes 9-12: pDest-myc-GFP-Keap1 clone 1-4, lanes 13-16: pDest-myc-GFP clone 1-4, lane 17: positive control pENTR221 GFP clone nr.3

4. Results

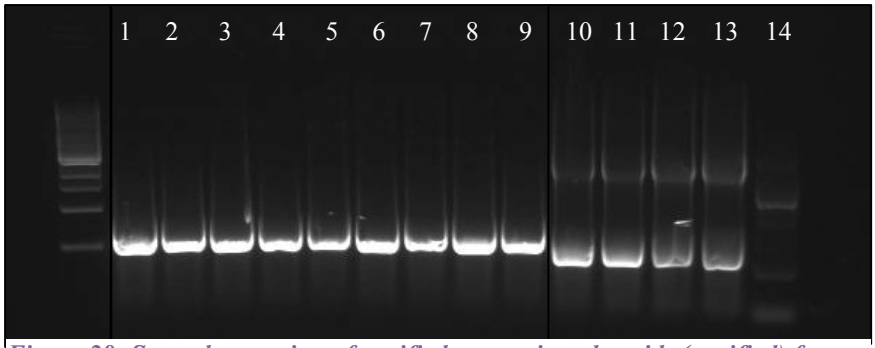


Figure 28: Second screening of purified expression plasmids (purified) for GFP and Keap1 genes. Screened for GFP:Lanes 1+2: pLenti CMV Puro Dest w118-1 plasmid nr. 1 and 3, lanes 3+4: pLenti CMV Puro Dest w118-1 GFP plasmid nr. 3 and 4, lanesl 5+6: pDest-myc-GFP-Keap1 plasmid nr. 2 and 3, lanes 7+8: pDest-myc-GFP plasmid nr. 2 and 4, lane 9: control plasmid pENTR221 GFP nr.3. Screened for Keap1: lane 10 +11: pLenti CMV Puro Dest w118-1 GFP-Keap1 plasmid nr. 1 and 3, lanes 12+13: pDest-myc-GFP-Keap1 plasmid nr. 2 and 3, lane 14: control plasmid wrong

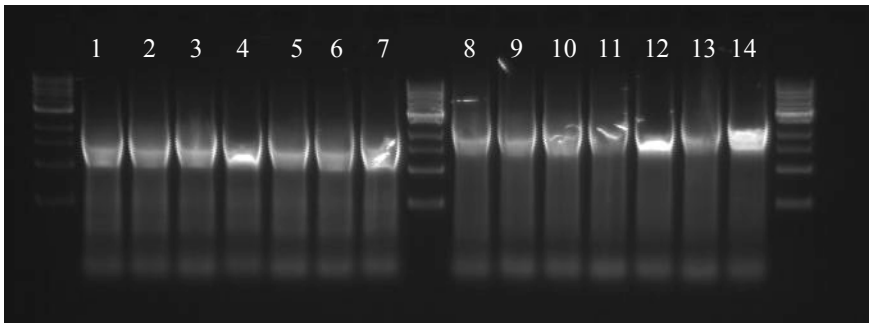


Figure 29: Screening for gene TLR2 or TLR8 of purified pLenti CMV Puro Dest w118-1 plasmids from LR reaction with GO Taq Green PCR. A ladder size of 1kB was used to quantify the sizes of the PCR products. Lane 1: pENTR221 TLR2 plasmid nr.3 positive control, 2: pLenti CMV Puro Dest w118-1 TLR2 plasmid nr.2.1, 3: pLenti CMV Puro Dest w118-1 TLR2 plasmid nr.2.2, 4: pLenti CMV Puro Dest w118-1 TLR2 plasmid nr. 2.3, 5:pLenti CMV Puro Dest w118-1 TLR2 plasmid nr. 3.1, 6: pLenti CMV Puro Dest w118-1 TLR2 plasmid nr. 3.2, 7: pLenti CMV Puro Dest w118-1 TLR2 plasmid nr. 3.3, 8: pENTR221 TLR8 plasmid nr. 1 positive control, 9: pLenti CMV Puro Dest w118-1 TLR8 plasmid nr. 1.1, 10: pLenti CMV Puro Dest w118-1 TLR8 plasmid nr.1.2, 11: pLenti CMV Puro Dest w118-1 TLR8 plasmid nr.1.3, 12: pLenti CMV Puro Dest w118-1 TLR8 plasmid nr.3.1, 13: pLenti CMV Puro Dest w118-1 TLR8 plasmid nr.3.2, 14: pLenti CMV Puro Dest w118-1 TLR8 plasmid nr.3.3

4.6 Imaging displayed that the GFP gene was present in pDest myc-GFP-Keap1 cloned vectors

To determine the presence of the GFP gene in the cloned plasmid pDest-myc-GFP-Keap1 with an additional method than PCR screening, we chose to do confocal imaging of transfected U373-CD14 cells. Previous cloning showed challenges with cloning both the GFP and the Keap1 gene as one transcript because of the presence of an attB1 recombination site between the genes. Because of this issue, we first chose to look for the presence of GFP in the plasmids. Only the pDest-myc-GFP-Keap1 cloned vector could be tested with regular transfection, because the others would require virus transduction for protein expression in the cells. Three different concentrations were used for transfection, 0,5, 1, and 1,5 ug, and the results are presented in figure 30. Because only some few images were obtained for each condition, and images were taken at random places in the wells, quantification could not be preformed to determine the presence of GFP. But the presence of GFP could be qualified visually by observing images assembled with software Image J.

We observed two different phenotypes of transfected cells for all concentrations; some had GFP expressed in a dot-like manner while other cells had a high expression distributed in the whole cell. By observing all images from each concentration it gave evidence of more cells with a distributed phenotype for higher concentrations. We concluded that the GFP gene was present in the cloned vector by the observed green fluorescence from the transfected cells. Not all cells were transfected efficiently, but previous results from transient transfection experiments for the U373-CD14 cells gave evidence of “normal” transfection efficiency with the cloned plasmid.

4. Results

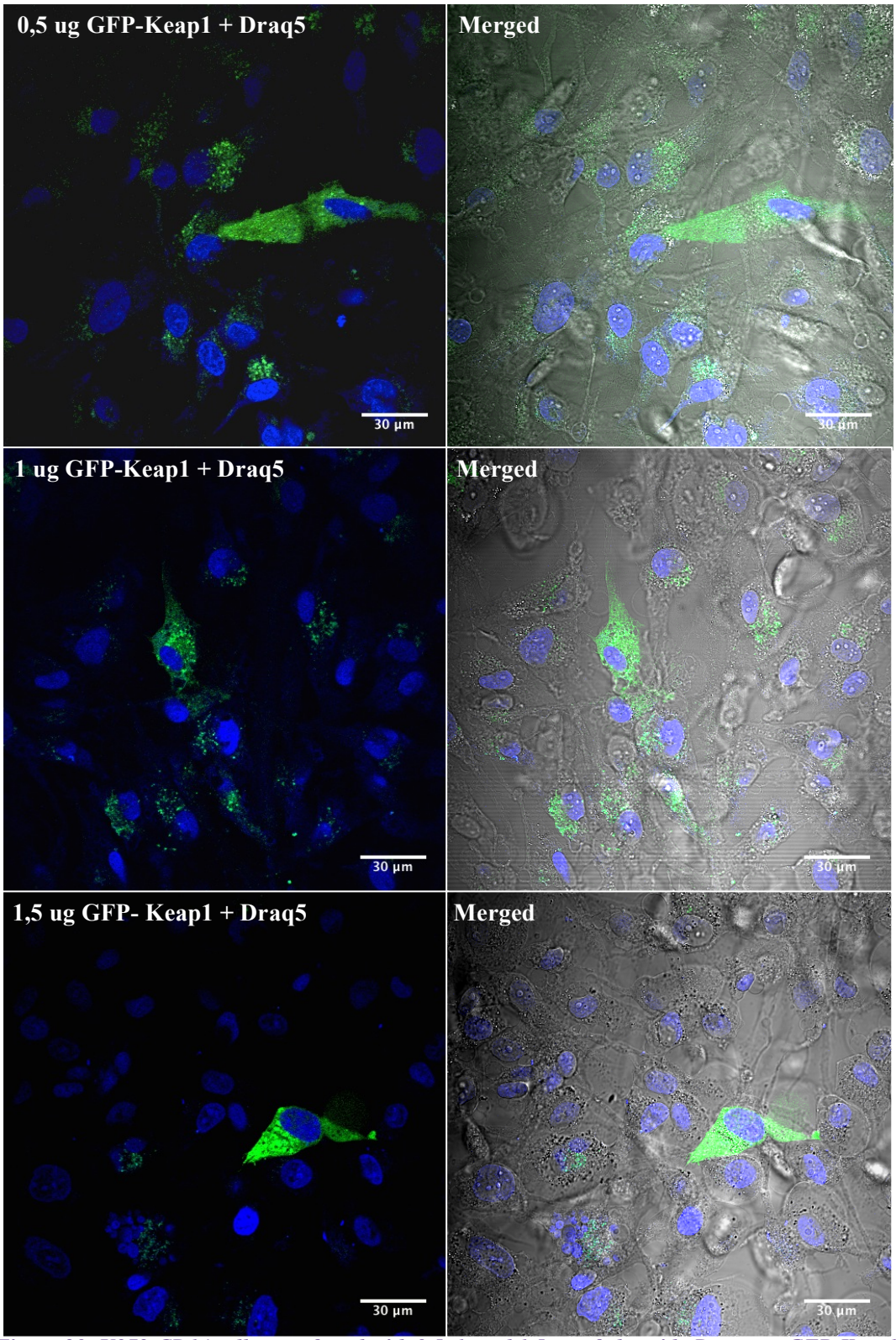


Figure 30: U373-CD14 cells transfected with 0,5, 1, and 1,5 ug of plasmid pDest-myc-GFP-Keap1 for 72 hours. Images to the left show the cell nucleus in blue with Draq5 staining, and GFP+Keap1 expression in cells. Images to the right show phase contrast of cells and fluorescent channels merged. Scale bars: 30 µm

4.7 Imaging displayed that cells transfected with pDest myc-GFP-Keap1 had some associating GFP and Keap1 proteins

To qualify if both GFP and Keap1 expression could be observed by transfection of U373-CD14 cells, cells were fixed and Keap1 was stained for using immunofluorescence staining with primary antibody towards Keap1, and secondary fluorescent antibody towards the Fc region of the primary antibody. Both GFP fluorescence and Keap1 antibody fluorescence were observed separately in order to qualify if the proteins were associated with each other. It was observed more Keap1 than GFP expression in the cells, which was expected due to additional endogenous amounts of Keap1 in the cells. The results presented in figure 31 and 32, are a result of randomly taken images of transfected cells in glass dishes from two biological independent experiments. We did not quantify the association of the proteins, as only a few images were obtained for each sample. To determine if GFP and Keap1 actually associated, we used a synchronization function in the Image J software to be able to follow the same spots for the images representing fluorescence from the protein and antibody at all time, and qualification was preformed visually.

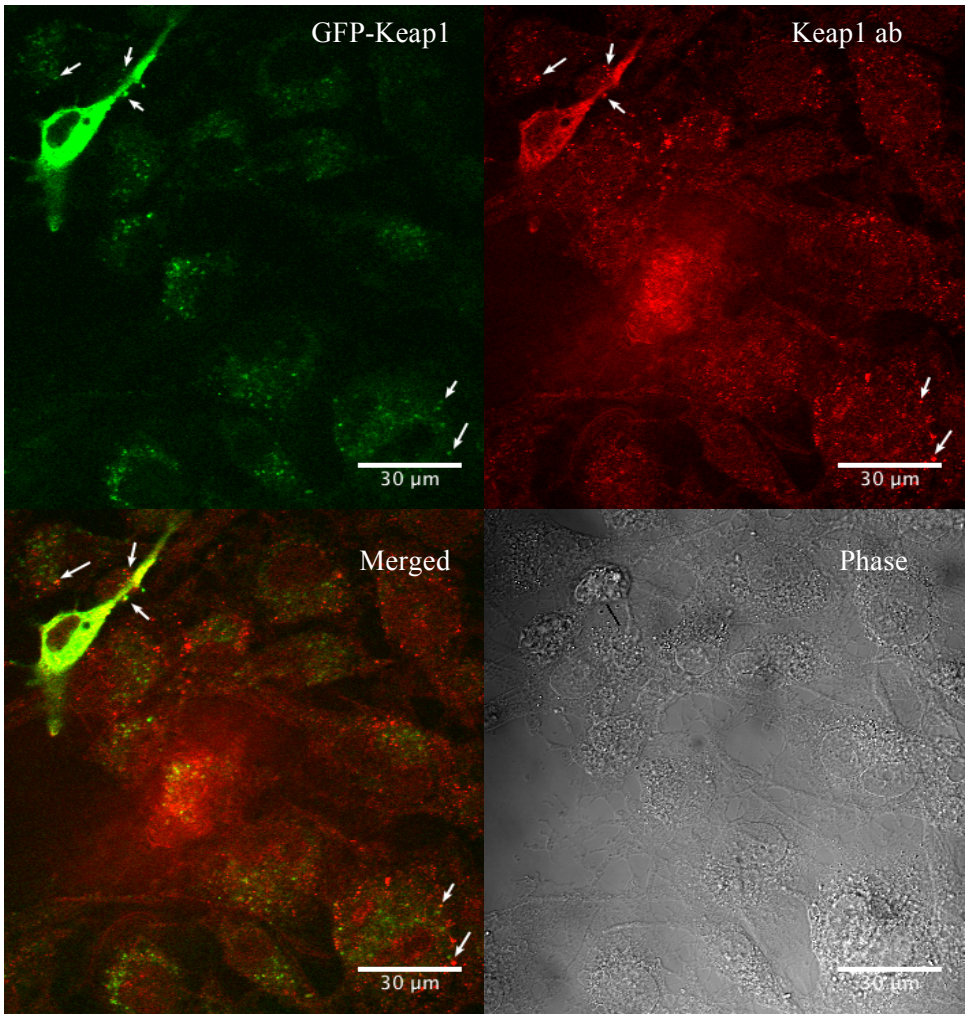


Figure 31: U373-CD14 transfected cells with 1 ug pDest-myc-GFP-Keap1 after 72 hours. Cells were observed with 63x objective on confocal microscopy. The images represent emission of GFP fluorescence, emission of immunofluorescence stained Keap1 with Alexa Fluor dye 647, and merged channels. Arrows pointing at dots represents GFP and Keap1 association. Scale bars: 30 µm

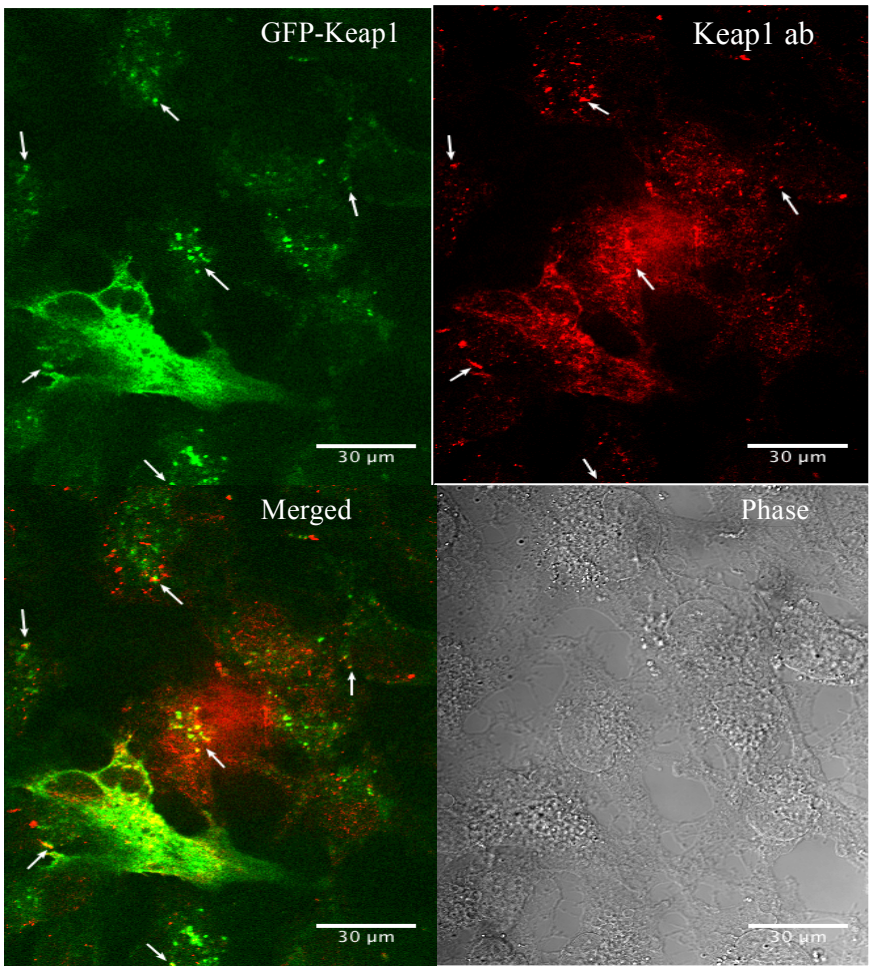


Figure 32: U373-CD14 transfected cells with 1 ug pDest-myc-GFP-Keap1 after 72 hours. Cells were observed with 63x objective on confocal microscopy. The images represent emission of GFP fluorescence, emission of immunofluorescence stained Keap1 with Alexa Fluor dye 647, and merged channels. Arrows pointing at dots represents GFP and Keap1 association. Scale bars: 30 µm.

The dots were believed to represent protein clusters of the target proteins. Arrows were applied in images were association was observed. We observed two phenotypes of transfected cells as seen previously; some cells expressed GFP and Keap1 diffused in the whole cytoplasm, while others only as dots. To be sure that the associations did not represent crossover of fluorescence emission through the channels, the emission of one fluorophore was detected through the filter combination reserved for the second fluorophore, and vice versa. Even though it was found association for some dots with GFP and Keap1 in the cells, the majority of the expression dots were not associated. Some of the green fluorescence could represent emitted auto-fluorescence, and some of the red dots could represent stained endogenous Keap1 in the cells. Also we did not know if the observed association was present due to coincidence, as it was only observed for some cells. Collectively, we concluded that since association was observed some places in the cells, Keap1 was believed to be present in the plasmids, especially since Go Taq Green PCR had confirmed the presence with specific Keap1 primers.

4.8 Imaging of endogenously stained Keap1, gave evidence of lower expression of Keap1 than for cells transfected with pDest myc-GFP-Keap1

To determine if the transfection of plasmids with Keap1 into U373-CD14 cells actually presented an overexpression, cells stained for endogenously Keap1 were observed by confocal microscopy. Image presented in figure 33 was obtained from a random place in the glass dish of the U373-CD14 cells, and represents endogenously stained Keap1 with Alexa Fluor dye 647 antibody. Compared to figures 31 and 32 that represents stained Keap1 both endogenously and over- expressed by transfection, we could clearly see a difference in the expression amount. Cells with transfected Keap1 gave a higher amount of stained Keap1 compared to un-transfected cells. Images were assembled with Image J. The evidence for the presence of Keap1 in the cloned expression plasmids was further supported by the observations of less Keap1 in un-transfected cells.

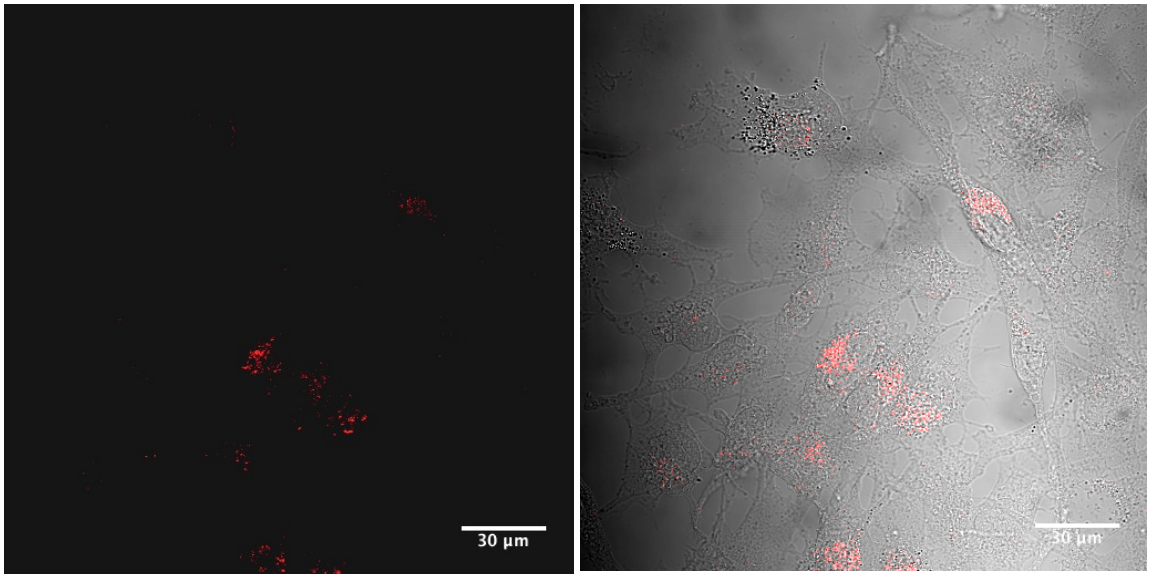


Figure 33: U373-CD14 stained for endogenous expression of Keap1 with immunofluorescence staining with primary antibody towards Keap1 and secondary fluorescent antibody Alexa Fluor dye 647. Image to the left represents channel for Alexa Fluor dye 647, and image to the right represents merged phase transmitted channel and fluorescent channel. Cells were observed with confocal microscopy and objective 63x from a random place in the glass dish. Scale bars: 30 µm.

4.9 Transfection with pDest myc-GFP-Keap1 and infection of U373-CD14 cells, displayed evidence of association between Keap1 and *M. avium*

Based on previous observations of GFP and Keap1 association in some transfected cells, it was aimed to determine if the native function of Keap1 was preserved even though it was fused at its N-terminal end with a GFP protein. In previous studies by our laboratory [65], we found that Keap1 was recruited to *M. avium* containing phagosomes 4 hours post infection. By transfecting the U373-CD14 cells with pDest-myc-GFP-Keap1 plasmids, and infecting cells with *M. avium* for 4 hours, we aimed to replicate these findings if the proteins were functional. Images were obtained from one glass dish at random places with infected cells stained for Keap1, to explore the association of Keap1 with *M. avium*. Because the bacteria would be internalized into phagosomes, it was not presumed that they would co-localize, but only be associated with each other, as Keap1 would position itself upon the phagosome. Images obtained with confocal microscopy were assembled with Image J, and results are presented in figures 34 and 35. To determine if dots with emitted light from GFP fluorescence, dots with emitted light from Alexa Fluor dye 647 towards Keap1 primary antibody, and dots with emitted dsRed fluorescent light from *M. avium* associated, it was used a synchronization function in the Image J software to be able to follow the same spots for the images obtained from the different channels at all time. The association was not quantified, only visualized and determined by counting number of *M. avium* bacteria associating with GFP and Keap1.

In figure 35 and 36, 30 % and 28 % of the *M. avium* bacteria respectively, were observed to associate with GFP and Keap1, presumed expressed from the plasmids transfected into the cells. We could probably have expected a higher percentage if the transient transfection efficiency was higher. In addition, some of the bacteria were only observed to associate with endogenously expressed Keap1, but these associations were not considered, as the purpose was to determine the functionality of the GFP- fused Keap1 protein in the cloned plasmids.

The observed association with *M. avium* could represent an evidence of correct Keap1 folding and native function in the cells. However, since images were taken from random places in the wells, it's important to consider the opportunity of the associations being a result of co-incidences. Several images of cells displaying the same trend would have to be collected and quantification preformed for complete evidence. It was not obtained any images from cells with un-transfected cells, stained for endogenous Keap1 and infection with *M. avium*. It was expected that for transfected cells, there would be more association with bacteria compared to cells with only endogenous Keap1 present, as it would be a higher number of Keap1 proteins in the cells. This relation could in addition be explored to create further evidence of the functionality of the fused GFP-Keap1 protein. In addition to pointed association dots, it was observed that GFP and Keap1 associated several other places without bacteria, which was a further evidence of presence of Keap1 in the plasmids.

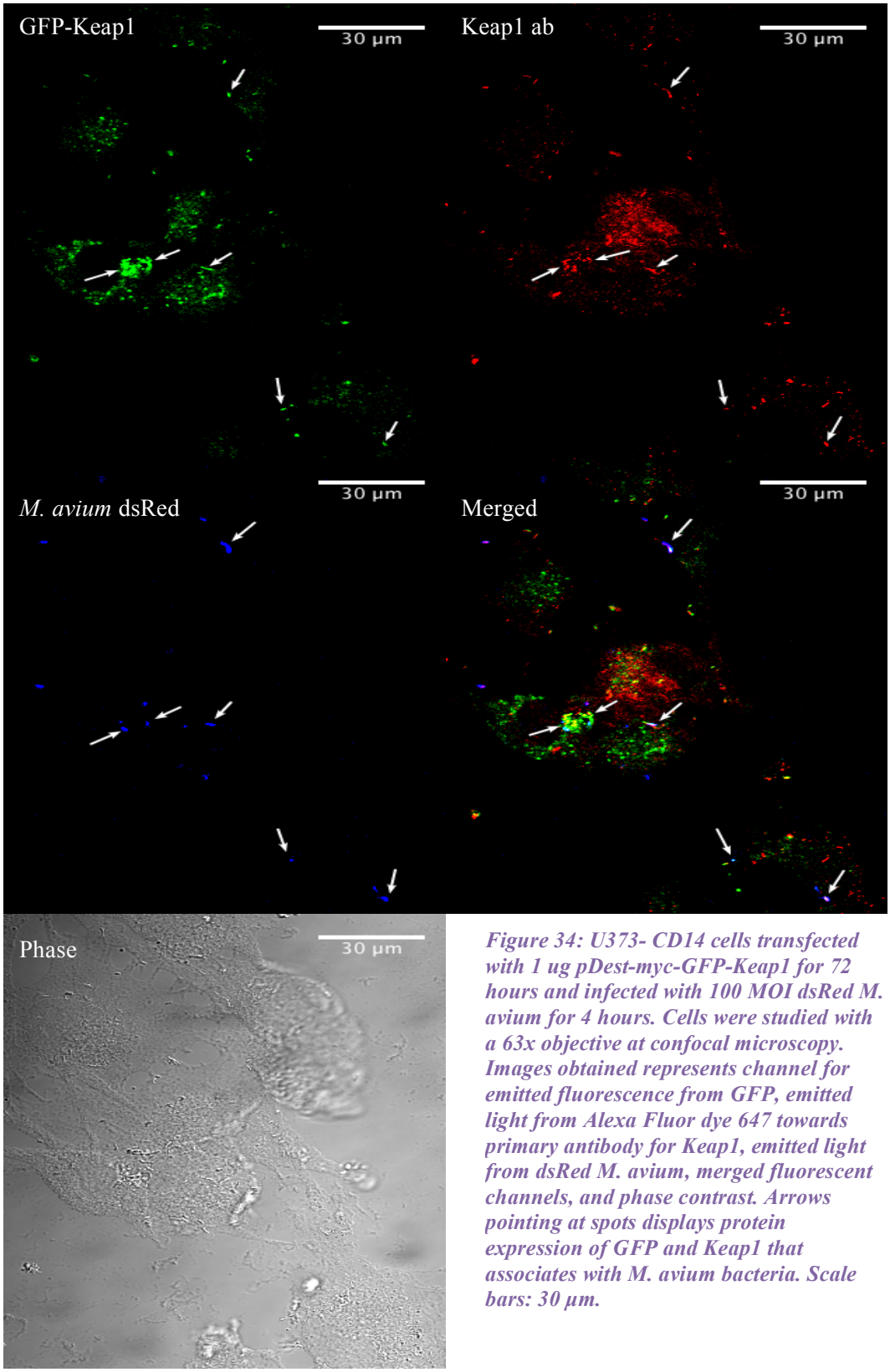


Figure 34: U373- CD14 cells transfected with 1 ug pDest-myc-GFP-Keap1 for 72 hours and infected with 100 MOI dsRed M. avium for 4 hours. Cells were studied with a 63x objective at confocal microscopy. Images obtained represents channel for emitted fluorescence from GFP, emitted light from Alexa Fluor dye 647 towards primary antibody for Keap1, emitted light from dsRed M. avium, merged fluorescent channels, and phase contrast. Arrows pointing at spots displays protein expression of GFP and Keap1 that associates with M. avium bacteria. Scale bars: 30 µm.

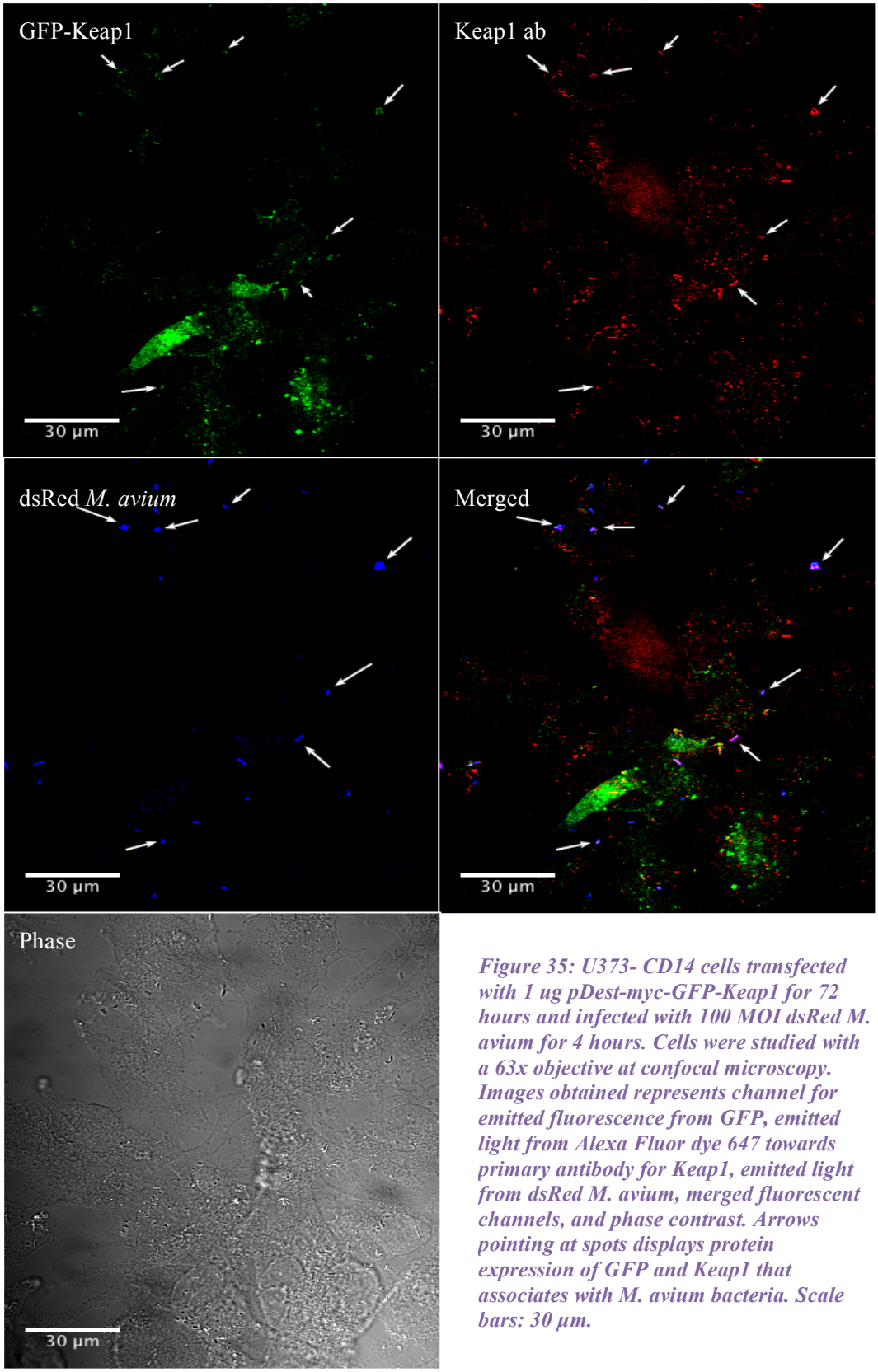


Figure 35: U373- CD14 cells transfected with 1 ug pDest-myc-GFP-Keap1 for 72 hours and infected with 100 MOI dsRed M. avium for 4 hours. Cells were studied with a 63x objective at confocal microscopy. Images obtained represents channel for emitted fluorescence from GFP, emitted light from Alexa Fluor dye 647 towards primary antibody for Keap1, emitted light from dsRed M. avium, merged fluorescent channels, and phase contrast. Arrows pointing at spots displays protein expression of GFP and Keap1 that associates with M. avium bacteria. Scale bars: 30 µm.

4.10 The cell-line U373-CD14 was stably expressing GFP-Keap1 48 hours post transduction with lentiviruses

After lentiviral transduction of cell-line U373-CD14 with lentivectors containing GFP-Keap1, TLR2 or TLR8, cells were observed in fluorescent microscope to determine the efficiency of transduction. Only GFP-Keap1 transduced cells could be observed for fluorescence, and was used as a control for the other cells with TLR2 and TLR8 without fluorescent tags. It was assumed that the efficiency of transduction would be the same, as the same concentrations of reagents and viruses were used for the experiments. Forty- eight hours post transfection cells in Costar 6 well plates were observed for green fluorescence. We observed that all cells were expressing GFP with high efficiency.

From optimization studies with different Puromycin concentrations for un-transduced U373-CD14 cells we observed that a concentration of 1,2 ug/ml Puromycin slowly killed the cells over a week with three passages. This concentration was chosen to use for the transduced cells, to select for cell stably expressing the expression clone plasmids of interest with Puromycin resistance and GFP-Keap1, TLR2 or TLR8.

To all transduced cells we added Puromycin in a concentration of 1,2 ug/ml 48 hours post transduction into the growing medium, to be sure that the cells would continue to keep the plasmids and not shed them. After culturing cells for about 5 days in T-25 flasks in Puromycin containing medium, cells could be moved from the virus laboratory and observed with EVOS FL Auto microscopy. Images was obtained for cells transduced with GFP-Keap1, and are presented in figure 36.

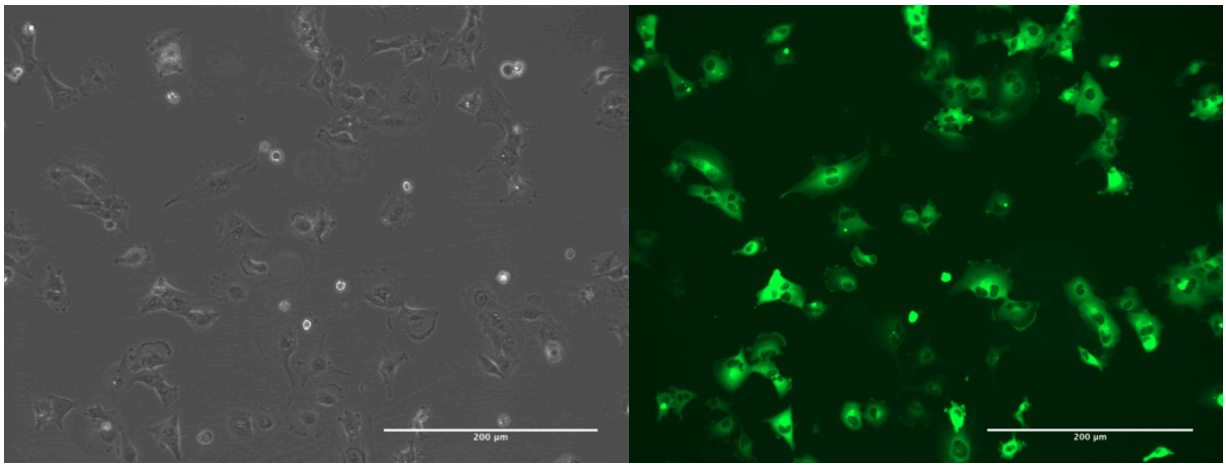


Figure 36: U373-CD14 cells 5 days post transduction with lentiviruses containing plasmids expressing GFP-Keap1. Cells were observed in a T-25 flask with EVOS microscopy and 20x objective.

We observed that all cells were expressing GFP-Keap1 with a high overexpression as the cells were whole- green, and even dead cells floating contained the plasmid of interest. We also observed that the GFP-Keap1 containing cells did not tolerate the Puromycin as well as the cells with TLR2 and TLR8, as there were dead cells floating around in the medium with GFP fluorescence. Based on this observation, the GFP-Keap1 cells were cultured in 1 ug/ml

Puromycin instead, while the TLR2 and TLR8 containing cells were kept in 1,2 ug/ml Puromycin for further selection over 3 days before using cells for experiments.

4.11 Stimulation and infection of cell-lines U373-CD14 TLR2 and TLR8 displayed a high up-regulation of inflammatory cytokines

As the transfection of U373-CD14 cells with plasmids containing TLR2 and TLR8 displayed an up-regulation of inflammatory cytokines as a response towards *M. avium* infection, we hypothesized that a stable expressing cell-line of TLR2 and TLR8 would give increased, reproducible, and stable responses. As the purpose of the cell model was cytokine read-out from infection with *M. avium* after different alterations, it was beneficial to have cells in experiments where all cells included expressed the receptor of interest.

Cells selected for a week in medium with Puromycin, were used for experiments and three biological replicates were used for each condition stimuli. Only one control sample for LPS stimulations were included to observe if the response in up-regulation of inflammatory cytokines was changed from LPS response in U373-CD14 cells and transfected U373-CD14 cells. Cell lysates was analyzed with q-PCR to look at the level of gene expression of cytokines. The response towards LPS, ligands LM, FSL-1, PAM3CSK4, CL075 and infection with 50 MOI *M. avium* for cell-lines U373- CD14 TLR2/ TLR8 for 4 hours, are presented in figure 37.

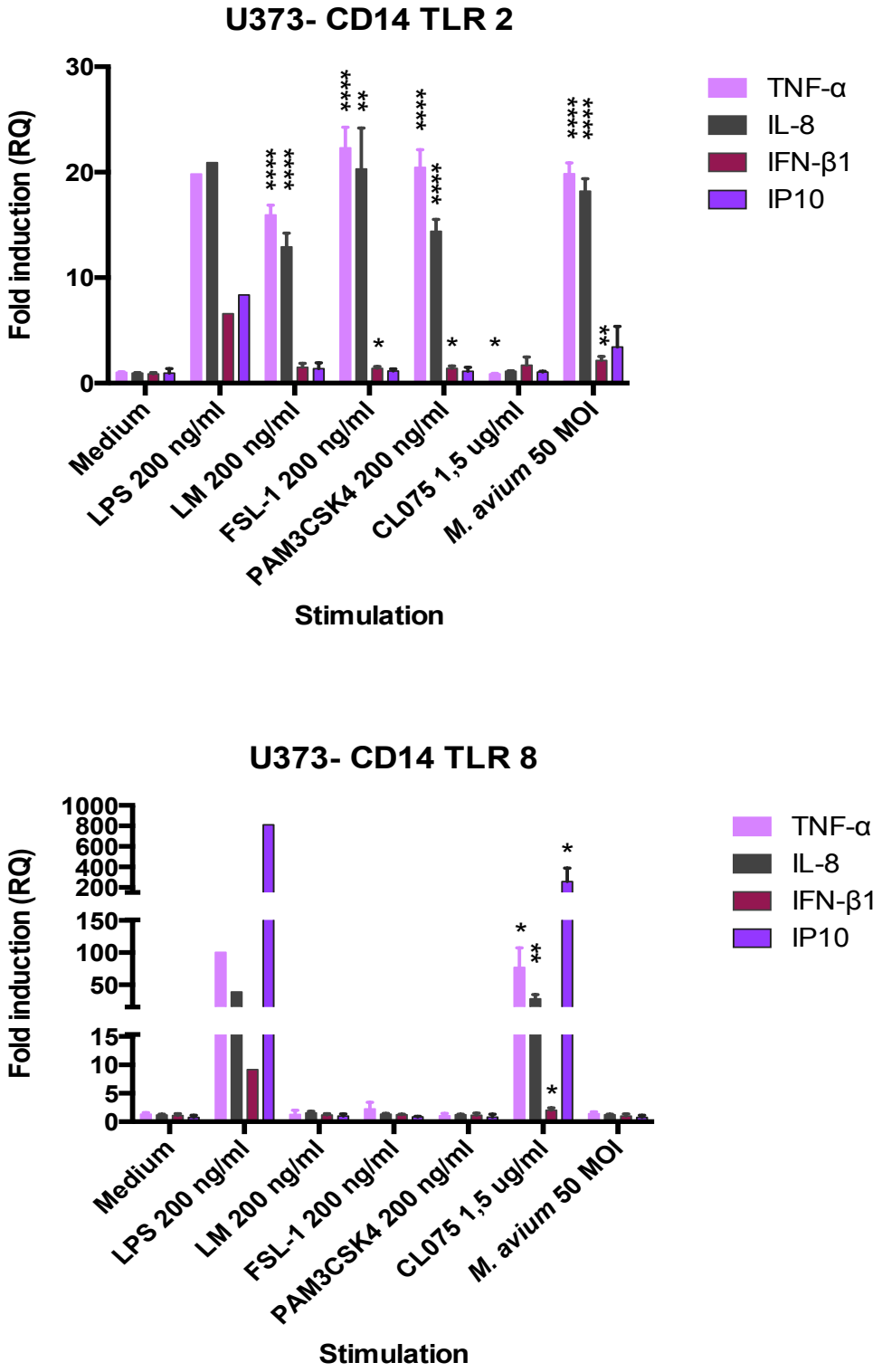


*Figure 37: Cell-lines U373- CD14 TLR2/ TLR8 stimulated with LPS, LM, FSL-1, PAM3CSK4, CL075 and infection with 50 MOI M. avium for 4 hours. Results are presented as fold induction RQ compared to reference samples, un-stimulated cells. Samples were analyzed with q-PCR and cytokines screened for was TNF-α, IL-8, IFNβ, IP, and GAPDH was used as endogenous control. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures. Statistical analysis not performed for: LPS stimulated samples for all cytokines.*

4. Results

The LPS response was determined for the transduced cells to se if the transduction itself changed the basal level of inflammatory cytokine response compared to earlier results from un-transfected U373-CD14 cells and transient transfected U373-CD14 cells with pcDNA3.1. The LPS response was determined as lower compared to un-transfected/transduced cells, and the stable cell-line expressing TLR2 gave in general lower responses than the TLR8 expressing cell-line. By comparing delta Ct values for reference samples of untreated cells and the stable cell-lines, presented in table XX, we show that the transduction itself induced an inflammatory expression in the cells, in particular for IP10, which was removed as a background.

Table 25: Delta Ct values for untreated reference sample compared to Ct values for transduced cell-lines with TLR2 and TLR8 reference samples, and calculation of RQ

	Un- transfected/ transduced cells, un-stimulated	**Transduced cells TLR2, un-stimulated**		**Transduced cells TLR8, un-stimulated**	
Gene	Delta Ct	Delta Ct	RQ	Delta Ct	RQ
TNF-α	17	14	8	15	4
IL-8	5	3	4	3	4
IFNβ	13	12	2	11	4
IP10	15	8	128	5	1024

The U373-CD14 cell-line stably expressing TLR2 responded with up-regulation of all inflammatory cytokines screened for to stimulation with LPS. To stimulation with TLR2 ligands LM, FSL-1, and PAM3CSK4, the cytokines TNF-α and IL-8 were significantly up-regulated with an RQ around 20 compared to reference sample, while no significant up-regulation was observed for IP10. For LM it was not observed a significant response of IFNβ, while for FSL-1 and PAM3CSK, it was slightly significant. To stimulation with TLR8-ligand CL075, no up-regulation of inflammatory cytokines was observed. For infection with 50 MOI of *M. avium*, it was observed a significant up-regulation of all inflammatory cytokines screened for except for IP10. For IP10 an average fold induction of 3,5 was observed, but a high standard deviation could be the reason for no calculated significance. The cell- line gave a high measurable and stable up-regulation to infection with *M. avium*, and the goal of making a responding cell-line was achieved.

The U373-CD14 cell-line stably expressing TLR8 responded with up-regulation of all inflammatory cytokines screened for to stimulation with LPS. Towards ligand CL075 stimulation the up-regulation of TNF-α, IL-8, and IP10 could be observed with fold induction values over 50 compared to reference sample, and all cytokines were significantly up-regulated. Up-regulation of IFNβ was observed at a low level (fold induction of 1,7 compared to reference sample). The cell-line did not show any significant up-regulation of the screened

cytokines towards stimulation with the TLR2- ligands LM, FSL-1, or PAM3CSK4, and no significant up-regulation was observed for infection with 50 MOI of *M. avium*. These findings showed that the cell-line with all TLR receptors present except TLR2 (or TLR8) were not able to respond to the bacteria in terms of up-regulation of chosen cytokines. When the cell-line possessed all TLRs except TLR8, it was able to respond with a high up-regulation of chosen inflammatory cytokines, which highlighted the importance of TLR2 in *M. avium* signaling. In addition the presence and functions of TLR2 and TLR8 were validated, as they responded to own ligands and not to ligand(s) for the other receptor.

5. Discussion

In this project, a cell-line and expression plasmids were created as tools to further detail the involvement of Keap1 in inflammatory signaling, particularly the NF-κB and IRF pathways, upon infection with *M. avium*. Previous findings in our group were preformed in primary human macrophages from PBMCs, and indicated a role of Keap1 in regulating IKKβ and TBK1 upon infection, and the interaction with IKKβ is previously shown [62]. The limitations with primary cells in relation to transfection, protein interaction studies and donor variations, further addressed the need for a macrophage-like model cell-line. A model cell-line could be transfected with full- length Keap1 or deletion constructs of Keap1 to further detail the interactions and regulations of IKKβ and TBK1, or other potential binding partners upon *M. avium* infection. Two macrophage-like cell-lines; THP1 and U373- CD14 were initially chosen as candidates for a model cell-line, and both cell-lines were observed to phagocytose *M. avium* with different efficiency. We decided that the use of U373-CD14 cells as a model was more beneficial as they could be transiently transfected, hence they were chosen for further experiments. Since the U373-CD14 cell-line did not respond with a high up-regulation of inflammatory cytokines in response to infection, we chose to transiently transfect them with endogenously absent receptors TLR2 and TLR8. The TLR2 receptor is established to have an important role in *M. avium* recognition, while the role of TLR8 is still unclear [14,23]. Transfection was preformed to assess if the cytokine responses towards infection with *M. avium* were enhanced. We observed an increase in inflammatory responses, especially for TLR2 transfected cells, and further proceed by creating two cell-lines with lentiviral transduction of TLR2 or TLR8. The stable cell-line U373-CD14 TLR2 responded with a high up-regulation of inflammatory cytokines TNF-α and IL-8, and significant up-regulation of IFNβ, highlighting the importance of the receptor towards *M. avium* infections. The cell-line could be used in the future as a model to study the remaining aims of the project. The U373-CD14 TLR8 cell-line did not respond with cytokine up-regulation to *M. avium* infection, but it might be considered a longer infection time than 4 hours to study the role of the receptor for infection.

5.1 U373-CD14 as a model cell-line to detail Keap1's regulation of the NF-κB and IRF-pathways upon *M. avium* infection

The U373 cells are from an astrocytoma in the human brain. The astrocytes are a subtype of glial cells, and a macrophage-like cell-line that originate from neural stem cells and share common precursors with oligodendrocytes and neurons. They work to protect the central nervous system and mediate phagocytic clearance of apoptotic cells. In addition they possess several receptors involved in the innate immunity like Toll- like receptors, scavenger receptors, mannose receptors and components of the complement system. Activation of an astrocyte stimulates phagocytosis, secretion of cytokines and ROS [78]. The production of ROS would be beneficial as Keap1 is a ROS- sensor, and are recruited to *M. avium* containing phagosomes in a ROS- dependent manner [65].

5. Discussion

The U373-CD14 cell-line was available in the laboratory, and previously used for studies of inflammation pathways in the innate immune system in our group [35]. In addition previous studies in our laboratory provides evidence that the cells are transfectable, which is favorable when studying protein interactions and regulations. The initial cell-line U373 is stably transfected with the surface receptor CD14, as CD14 is an adaptor for TLR4, needed for efficiently activation of the NF-κB pathway [79].

After cells were verified to phagocytose *M. avium* efficiently in high MOI's, we studied its cytokine response and no efficient up-regulation of TNF-α, IL-8, IFNβ or IP10 was discovered towards the mycobacteria. We chose to measure the specific cytokines as they are released after activation of the NF-κB and IRF- pathways, sought to detail further. Only a low significant response was detected for TNF-α and IL-8 with fold-induction values below 4 compared to un-stimulated cells, and we presumed that it did not represent biological relevant responses. Kurt- Jones et al previously showed that the cell-line is equipped with TLR1, 3, 4, 5 (weak expression), 6, 7 and 9 expressed in contrast to PBMC's that are thought to have TLR1-9 [77]. As the cells did not respond to infection, we hypothesized it this could be due to the endogenously absent TLR2 and TLR8. This hypothesis was further confirmed by the up-regulation of inflammatory cytokines upon infection in cells transiently transfected with TLR2 Cherry. Stable cell-lines U373-CD14 TLR2 and TLR8 were established with lentiviruses, and it was confirmed that the TLR2 containing cell-line responded in terms of up-regulation of inflammatory cytokines 4 hours post *M. avium* infection. The observed enhanced response for *M. avium* infection because of modulations of the cell-line proved that they could be used as a model system for further detailing of the NF-κB and IRF- pathways.

The benefits of the U373-CD14 cell-line are the ability to transient transfect them and transduce them. If the cell-line with TLR2 were as easy to transduce with shRNA towards endogenous Keap1 for knockdown, a stable cell-line expressing TLR2 and knockdown of Keap1 would be the complete tool for further studies. A stable "background" would have been generated, and transient transfection of different complete and partial constructs of Keap1 could be performed to elucidate interaction partners with and without infection. Interaction studies and alterations in the cytokine profile could be assessed when overexpressing or knocking down proteins participating in the signaling pathways of NF-κB and IRF's.

One drawback of the model system would be that cell-lines are derived from metastatic tumors, and could possess properties and altered responses that do not naturally occur for the primary macrophage cells. This may pose challenges when extrapolating results to biological contexts. In addition cell-lines with integrated transgenes would insert the genes at random places in the cell, which could disrupt the function of important endogenous genes and provide "off-target" effects. A heterogeneous population of cells would be generated, with genes of interest inserted various places in the genome. Over time the cell-line could experience drift due to selection of sub-clones, which has to be evaluated when obtaining experimental data [7].

5.1.1 Other potential model systems

Commonly used model systems are cell-lines, primary cells, and animal models. Primary cells like human macrophages from PBMC's would be beneficial to use for the studies of Keap1, as it is a natural host for *M. avium* infection, and because of its previous use in our laboratory. Findings in these cells would be easier to extrapolate to the actual environment in the body at the site of infection, only excluding cell-to-cell communication. Primary cells are more challenging to work with in terms of replicating experiments, because they are tricky to transfect and donor variations occur. With a cell-line, the difference from biological replicates would only be different passage numbers of cells that could slightly change the cells over time due to accumulating spontaneous mutations. Low passage numbers of cells were used for all experiments to avoid genetic drifting in the cell- line.

The THP1 cells would be another option of a model cell-line to study the aims. As these cells previously have been used for publication studies of The genus mycobacterium's different subspecies, an upregulation of inflammatory cytokines are already established towards infection [75, 76]. The drawback with these cells is the challenges with transient transfection. To alter levels of protein expressions, lentiviruses could be used for each new experiment with cloned gateway vectors with genes of interest or shRNA towards endogenous genes. We could have established a stable cell-line for the THP1 cells with Keap1 knockdown, but the Keap1 constructs would in addition have been transduced with lentiviruses each time a new interaction study was preformed. Transduction is a more time-consuming and unsafe method than transient transfection, due to potential biohazards. In respect to this, the U373-CD14 cells would be more beneficial as they are transiently transfectable and easier to conduct interaction experiments in after establishing a stable cell-line.

HEK 293 cell-line was previously tried out by an earlier master student in our laboratory, Christina Dybdrodt Bjørnvall, as a model to accomplish the same aims as for this project. The cells gave a decreased inflammatory response towards Keap1 knockdown, opposite of shown in human macrophages by our group [65] and were discarded as a potential model. In addition, these cells are not macrophage- cells, and would not necessarily respond to *M. avium* in a biological relevant matter.

The use of mouse models as tools to detail further the pathogenesis of *M. avium* infections are suggested [14]. A live model with a knockout of Keap1 from myeloid cells would provide the advantage of a biological system as a whole, with included cell-to-cell communications in inflammation. The drawback with mouse models for this study purpose would be that the motif of IKKβ that is required for Keap1 binding is not present in rodent cells, and only conserved in advanced mammals [62]. Such drawbacks would make it hard to study interaction domains for the aims of the project, and careful considerations must be made before choosing appropriate models as diverse functions could be differently conserved between species.

5.2 U373-CD14- TLR2/8 cell-lines for detailing aims and future studies

Stable cell-lines expressing either TLR2 or TLR8 were established from the U373-CD14 cells with lentiviral transduction. After ligand stimulation and infection with *M. avium*, the U373-CD14-TLR2 cells was observed to respond to infection with significant up-regulation of inflammatory cytokines TNF-α, IL-8, and IFNβ, while IP10 levels was not significantly altered. The cytokine production was observed as stabile for three biological replicates, and the established cell-line could be used for studying remaining aims. The up-regulation of TNF-α and IL-8 had a fold-induction of around 20 compared to un-stimulated reference sample, which is sufficient to be able to observe alterations in the profile induced by over-expressions or knockdowns of proteins involved in the interesting pathways. The findings highlight the importance of the TLR2 receptor for an efficient inflammation response towards *M. avium* infections. In the cell-line with all TLR receptors except TLR2 and TLR8 the induction of cytokines were not significant after 4 hours but when TLR2 was introduced, the response was highly up- regulated after 4 hours infection. It is tempting to speculate based on the results if the TLR2 receptor is most important for *M. avium* recognition and initiation of inflammatory signaling in the cell-line as previously stated for macrophages [14].

The low but significant increased response for IFNβ towards *M. avium* infection after 4 hours for the U373-CD14 TLR2 cells was up-regulated only after expression of TLR2 and not for the initial cell-line. We speculated based on these observations if the TLR2 receptor initiated these responses. Later studies have shown that bacterial TLR2 ligands can induce Type 1 Interferon responses through endolysosomal compartments after ligand stimulation and internalization. This signaling could be mediated through adaptor protein TRAM and IRF3 via TLR2/6 [80, 37], or via MyD88 and IRF1/7 dependent pathways [38]. It might be that the response would have been further up-regulated if the infection time was increased. We also observed a slight increase of IP10 towards *M. avium,* but not significant because of a high standard deviation. The TLR2 ligands LM, FSL-1, and PAM3CSK4 mediated the same cytokine response trend with highly up-regulated TNF-α and IL-8, and a low but significant response for IFNβ for FSL-1 and PAM3CSK4 stimuli. No significant up-regulation of IP10 was observed for any of the TLR2- ligands. It might be that a longer time- exposure to *M. avium* would increase the cytokine production of IFNβ and IP10, and that TLR2 fails to induce an IP10 response through the low IFNβ production after 4 hours. LPS stimulation managed to induce IP10 through TLR4 after 4 hours, and also had a higher up-regulation of IFNβ. TLR4 mediates the IFNβ activation thorugh IRF3 that is responsible for the early wave of the cytokine expression, and it is tempting to speculate if TLR2 in these cells mainly activates IFNβ through IRF7, as earlier reported [38], and fails to induce IP10 within 4 hours as IRF7 induces a later IFNβ response. In addition, the high background levels of IP10 for transduced, un-stimulated cells could be responsible for the absent IP10 responses as they could vary from time to time between samples. It is further tempting to conclude that TLR2 is the only potential inflammation mediator for sensing *M. avium* for these cells, as the cytokine profile was similar to the TLR2 ligand cytokine responses. In addition the IFNβ response was so low it did not seem likely that TLR4 played an important role in the recognition and up-regulation of inflammation. It could also be that TLR4 is actually activated, but that the

suggested TLR4- ligand PIM from *M. avium* is not as an efficient activator of TLR4, as LPS. The presence and levels of the different IRF's in the specific cell-line remains to be elucidated, and must be considered when drawing conclusions about the signaling pathways including the Toll-like receptors.

The role of TLR8 in recognition and initiation of response towards *M. avium* is not known, and is still remains unclear after this study. The stable cell-line expressing TLR8 did not show a significant response to the mycobacteria for any of the cytokines 4 hours post-infection. We hypothesized that a longer infection time was necessary for activation of TLR8, supported by studies of mycobacteria processing in the endolysosome-like compartments and signaling from our laboratory (unpublished, Alexandre Gidon). We observed that the U373-CD14-TLR8 cells responded significantly to the CL075 ligand after 4 hours, but the ligand do not require processing and can signal as it is after uptake. The up-regulation of all screened cytokine to CL075 stimulation, gave evidence of a functional receptor and signaling machinery required for mediating the response thorugh the IRF pathway. The up-regulation of IFNβ however was expected to be higher than a fold-induction of 1,7. IFNβ induction was observed to be higher for TLR4 stimulation with LPS for 4 hours, and as previously reported endocytosis of TLR4 initiates TRIF-IRF3 activation, essential for early IFNβ production [41]. The TLR8 receptor can only activate the MyD88 signal adaptor and IRF7 which works as a later IFNβ inducer than IRF3 [42], which could indicate the need for a longer stimulation time to observe a higher IFNβ production upon CL075 stimulation. Another explanation could be deficiencies in the pathway, leading to failure of IFNβ production like IRF7 absence.

IP10 however, was strongly induced for both LPS and CL075 stimulation, and are known to be initiated as a second response by stimulation of IFNβ [60]. As cells were only stimulated for 4 hours, it might not be sufficient time to activate IP10 expression thorugh the "late response" of IFN- α/β. TLR4 activation could initiate direct IP10 activation thorugh IRF3 [49], and could be the reason for the early present IP10 response for LPS activation. The reason for the early up-regulation of IP10 without a high IFN-β response was not certain, but as we did not known how much IFN- β that is needed to up-regulate IP10 significantly, it could be that the low up-regulation was sufficient. IP10 is a commonly produced cytokine in brain astrocytes in response to virus infections [81], and it could be that the cells have alternative mechanisms of activating IP10. The cytokine response for the U373-CD14 cells towards stimulation with TLR2 ligands was not significant. The stimulation was used as a control to quality check the absence of the TLR2 receptor in the cell-line.

5.3 Response towards infection with *M. avium* for the U373-CD14 cell-line

The U373-CD14 cell-line was not as efficient in clearing *M. avium* from the surroundings when exposed to low MOI's of the bacteria as THP1 cells. Only some cells were able to phagocytose the bacteria, and few bacteria per cell on an average were counted. When exposed to a higher MOI of *M. avium* (100), more cells were infected on an average, which is advantageous for interaction studies detailing protein interactions resulting from the infection. Since interactions and inflammatory cytokine production would be the read-out from later experiments, as many cells as possible infected would be beneficial. The phagocytosis of *M. avium* was more efficient for 50 MOI and 8 hours, but as cytokines released as an early immune response are studied, it was advantageous to keep the time-lapse of the infection low. It is also important with a high number of infected cells, as the endosomal TLRs would have to be exposed to mycobacterial nucleic acids to efficiently up-regulate the production of type 1 Interferon's.

When infecting cells to look for production of inflammatory cytokines with q-PCR, two different MOI's of 20 and 50 were used for a time-lapse study from 30 minutes to 8 hours. Even though 100 MOI was determined as most efficient and would be used for further studies, 20 and 50 MOI could still be enough to initiate an inflammatory response, especially for such a long infection time. LPS is one of the most established TLR4 ligands, and was used as a positive control towards TLR4 and CD14 [23,1] to ensure that the cells were responsive and experiments valid. We preformed experiments to assess at what time-point the highest up-regulation of inflammatory cytokines were present, and if the cells would be able to respond to *M. avium* at all. The cytokines TNF-α, IL-8, IFNβ, and IP10 were chosen as inflammatory targets as they are produced after initiation of the NF-κB and IRF pathways which was wanted to study further for Keap1's regulation and interaction's upon infection. In addition, these cytokines (except IL-8) were shown to be up-regulated when Keap1 was knocked down in monocyte-derived macrophages (MDM's) in our group [65]. As we sought to replicate these findings in the cell-line, an up-regulation of these cytokines towards an infection with *M. avium* was required, as it was expected to observe an increase of the cytokines as response to Keap1 knockdown.

The response to LPS induced production of TNF-α and IFNβ with highest levels after 2 and 4 hours, indicating that they were induced as an early innate response towards the stimulation. The levels of IL-8 and IP10 were increasing with longer exposure to the mycobacteria, highest induction after 8 hours stimulation. IP10 is induced by type 1 and 2 Interferon's and in cooperation with TNF- α [59], and could represent a second inflammatory response. The response towards LPS proved that the cells are capable of inducing production of all the chosen cytokines.

The response towards *M. avium* with MOI 20 and MOI 50 displayed no significant response towards the bacteria or a trend of significant down- regulation of IFNβ and IP10, and up-regulation of TNF-α and IL-8. The absence of high inflammatory responses could be due to the two absent TLR receptors TLR2 and TLR8, and less activation of the NF-κB and IRF

pathways as highlighted by the increase in inflammation when TLR2 was present in infection. The TLR1 and 6 receptor is also dependent on TLR2, for hetero-dimerization and initiation of downstream signaling. It could be speculated if it exists potential ligands for TLR1 and TLR6 activation alone without dimerizing with TLR2, since the cell possesses these receptors alone endogenously. The slight increase in signal for TNF-α and IL-8 upon infection by U373-CD14 cells could be induced by some signaling thorugh endogenous TLR4 and/or 9 that can lead to NF-κB signaling activation [1]. The TLR 4, 8 and 9 receptors are previously linked to Mtb infections or susceptibility, and their role in *M. avium* infection could be evaluated by this study. Since the initial cell-line was equipped with TLR4 and TLR9 endogenously, and the cells were not able to respond, the role of the receptors could be less important compared to TLR2 in infection, in least for this cell-line. In addition to TLRs the cells could have several other receptors able to respond to infection like NLR's and CLR's, inflammasomes etc., which could be responsible for the low NF-κB activation.

The down-regulation of IFNβ and IP10 could be caused by removal of variable basal levels for un-stimulated reference sample, and are not necessarily biologically relevant. Detecting changes in the responses due to modulation of protein levels in the cells with knockdown or over-expressions would be problematic with such low responses, and it was concluded that it would not be beneficial to use the U373-CD14 cells as they are for a model cell-line.

5.4 Transient transfection of U373-CD14 and inflammatory response to *M. avium* with TLR2 and TLR8.

The U373-CD14 cells were transient transfected with various plasmids of different concentrations and incubation times to observe efficient parameters for further experiments. We observed that transient transfection with Gene Juice did not provide higher efficiency than 30-40 % transfected cells on an average counted visually with imaging, and various protein expression for different plasmids detected with Western blotting. We concluded that the number of cells would most likely be sufficient to detect protein interactions later in a stable cell-line, and the transfection could also be further optimized. By the variable cytokine responses detected for stimulated transfected cells with TLR2 and TLR8, we decided that it was not sufficient to induce a stable response towards *M. avium*. If cells would have been transient transfected with several different plasmids for over-expression and knockdown shRNA, it would have been difficult to know which cells integrated which plasmids, and there would be a heterogeneous mixture of expressing cells. This would not be convenient in terms of studying interactions and inflammatory cytokine responses upon infection as only one variable parameter should be present, and the response towards the bacteria should be consistent. The transfection experiments gave evidence of a response to *M. avium*, especially for the cells transfected with TLR2, which was enough confirmation to proceed with establishing stable cell-lines.

We observed that some of the inflammatory responses towards infection with *M. avium* varied between biological different experiments and had a high standard deviation, which

could be caused by different efficiency of transfection from time to time. In addition, it could be caused by differences in the background response levels of the cytokines for the reference sample to the transfection procedure. Different factors could influence the degree of transfection like reagent used, incubation time, degree of confluence for cells, passage number, and DNA quality and quantity [82].

Cells transfected with TLR2 wildtype did not initiate a strong response towards either TLR2-ligands or *M. avium*, compared to TLR2 Cherry that gave an increased response towards all TLR2-ligands especially for IFNβ and IP10, and all screened cytokines for *M. avium* infection with a fold-induction of 10-30 compared to reference sample. The low cytokine induction could be due to the impaired transfection efficiency of the plasmid with TLR2 wild-type, as seen for TLR2 expression with Western blotting. If the receptors were present and functional, a higher activation of NF-kB, and increased TNF-α and IL-8 responses would be expected for stimulation with TLR2-ligands. Even though some responses were calculated as significantly up-regulated, the low values obtained for some of the parameters was not thought to be biologically relevant. Based on the results, we hypothesized that the TLR2 Cherry plasmid gave expression of functional TLR2 receptors that were able to respond to *M. avium*. Also, the TLR2 Cherry did not respond to the TLR8-ligand, which further increased the evidence. The TLR8-YFP transfected cells did not initiate increased cytokine responses towards the TLR8-ligand, and we presumed if this could be due to the YFP fluorescent tag interfering with protein signaling, folding, or its transportation sequence for correct localization in the cell. The TLR8 wild-type protein was also presumed to be functional in transfected cells as it responded with an upregulation towards all screened cytokines (RQ 5-15) to the TLR8-ligand stimulation. We did not observe an efficient response to *M. avium* infection, but as the function of TLR8 is not elucidated for *M. avium* infection we could not determine if its native function was preserved.

The cells co-transfected with both TLR2 Cherry and TLR8 was expected to have an increased response towards the mycobacteria, as both the plasmids were presumed to be functional based on the results. The co-transfection with both 0,2 ug and 0,4 ug of each did not give significant responses to either ligands or infection, lower than for the TLR2 Cherry receptor alone. We did not expect a decreased response when transfecting the receptors together, and especially not for 0,4 ug concentration, same as used for transfecting the receptors separate. It is tempting to speculate if the responses are down-regulated or absent due to inhibition of receptor activity when co-transfecting the two receptors. Also the low degree of transfection could give a heterogeneous mixture of cells with none, one, or two plasmids present, or perhaps inhibit the transfection efficiency.

The observed variation of transfection efficiency for GFP-Keap1 and GFP in same plasmid backbone could be due to the size of the gene insert, as it has previously been observed for other transfection methods that molecular weight of transgene can affect the efficiency of transfection [83]. We also observed two phenotypes of transfected cells with fluorescence; either totally colored cells with a high expression distributed in the cytosol, or cells with spots of fluorescence probably representing cluster of protein expression. Cells transfected with Cherry and GFP were observed to express the proteins in the whole cell, compared to Cherry-

TLR2 and GFP- Keap1 that were expressed only limited places observed as fluorescent dots. We suggested if this could be due to regulated expression of mammalian proteins like TLR2 and Keap1 due to transporter sequences at the genes, coding for expression at limited regions in the cells. The fluorescent proteins possibly do not have limited expression as they are not naturally found in humans, and are expressed continuously in the whole cell.

To optimize transfection later for pull-down and interaction studies, other transfection methods could have been examined like other chemical or physical treatments, and the Gene Juice method could have been optimized further with respect to concentrations of plasmids and Gene Juice reagent and incubation time.

The auto-fluorescence observed from the U373-CD14 cells after excited with the 405 nm laser was unfortunate as some of the studied proteins were linked to GFP flurophores. Even though the fluorescence was not as prominent as for the GFP proteins, it was still hard to distinguish some of the signals and could have affected the results. The auto-fluorescence could be excited from the cell growth medium e.g. from phenol red aromatic rings and/ or from the cell compartments, often observed for macrophages [84]. The detection highlights the importance of including negative controls in future experiments to avoid bleed-through, and to be able to correct for auto-florescent background from cells.

5.5 Background cytokine responses to transfection and transduction

We chose virus transduction for making stable cell-lines as transfection reagents are shown to destabilize the cell membrane and be toxic to cells. It has been previously shown that viruses like HIV-1 in lentiviruses can avoid a host-cell IFN response towards transduction by evolved mechanisms, while commonly used transfection methods usually provoke an increased IFN response in the host cells [85]. Lentiviral vectors have also shown to be less inflammatory than adenovirus vectors [86]. With lentiviral transduction, the foreign DNA is stably integrated into the genome, and the cells would not constantly be exposed to foreign un-modified nucleic acids after the integration. Up-regulation of IFN- responses towards transfection or transduction itself would be unfortunate, as the responses would be studied after *M. avium* infection and alterations in the pathways thought to be caused by the infection alone. As the transduced cell-line was left for 1 week after transduction before used in experiments, it was thought that the potential inflammation caused by the virus transduction was decreased.

The inflammatory response towards LPS in the stable cell-lines was lower compared to un-treated U373-CD14 cells. LPS induced significant responses of all cytokines for both cell-lines, however slightly lower for the TLR2 cell-line, especially for TNF-α and IP10. It was presumed that the cell-lines would have approximately the same delta Ct values and "background" responses for reference samples, as they were transduced with viruses containing lentivectors with the same plasmid backbone. We observed that this was not the case and that the delta Ct values varied. The TLR8 cell-line had a lower delta Ct value for the IP10 cytokine compared to the TLR2 cell-line, which could explain the lower LPS response by subtraction of "background" inflammation. The background inflammatory responses could

be variable from biological independent experiments, and lead to inconsistent results. In addition the subtraction of reference sample background values from the results would assume that all samples have the same background responses, and could lead to potentially variance of this is not the case. The LPS responses were generally lower for the transduced cells compared to untreated cells, and the difference in the corresponding delta Ct values could explain the difference. However, the calculated RQ values from delta Ct values for virus-transduced cells with untreated cells as a reference sample did not directly correlate to the response differences and could not be used to correct for the variations. The values were also obtained from different biological experiments, and could only be used as an indication of the background responses. As we thought transduced cells would not have high basal levels of inflammatory cytokines after 1 week of selection, and that it provided a better option than transfection, it was surprising that the basal levels of particularly IP10 was so high, and varied between the two stable cell-lines.

To remove potential background responses in the cells, transduced cells without stimulation was used as reference samples. Ideally, cells should have been transduced with empty lentiviral vectors without transgenes to make a cell-line that could be used as reference sample in the studies to remove potential background inflammation induced by the transduction process alone.

When we transfected cells with plasmids containing TLR2 or TLR8 and observed the responses towards *M. avium*, reference sample used was pcDNA.3.1- transfected and un-stimulated cells. This was assessed to remove the background basal inflammation signal that could be initiated thorugh the transfection method itself and introduction of foreign DNA to the cell cytosol. Previous publications have highlighted the plasmid DNA's capability of inducing Type 1 Interferon responses in host cells with transient transfection [85]. The plasmid pUNO1 hTLR8b did not possess the pcDNA3.1 backbone, and it was observed that pUNO1 hTLR8b transfected cells up-regulated the expression of IFNβ and IP10 towards TLR2-ligand stimulations, which was not expected. An increased response for IFNβ was observed for the transfection of the pUNO1 hTLR8b vector alone without stimulation, while no significant increase for IP10 was observed. When the pUNO1 hTLR8b responses towards TLR2 stimulations were normalized to these values, the IFNβ response was diminished, while the IP10 response remained significant. The IP10 responses most likely represent a second inflammation wave as previously explained, and it could be that the up-regulation of IP10 was variable from response to vector alone and to TLR2- ligand stimulations of the cells. Ideally, cells transfected with an empty pUNO1 vector would have been used as reference sample for cells transfected with the plasmid used for stimulation experiments, to remove the correct background response. We purposed that the vector pUNO1 induces a higher IFNβ response in the cells than the pcDNA3.1 vector, but this could not be verified, as the empty vector was not available. All previous responses were normalized to the responses towards the vectors alone to remove all possible background for the plasmids. The other signals from transfected cells with pcDNA3.1 vectors were also normalized to corresponding vector responses. Whether this is an appropriate way of presenting the results remains to be considered, as transfection of genes in pcDNA3.1 plasmids would already be supposedly controlled for with

the reference sample. Hence the un-normalized results were chosen to discuss further, with the pUNO1 vector response as an important factor.

The decrease in LPS response as seen with the stable cell-lines with TLR2 and TLR8, was also observed for the transient transfected cells, and could be caused by the removal of basal "background response" as explained previously. The calculated RQ values of the reference pcDNA3.1 samples were especially prominent for IL-8 and IP10, and inflammatory response could be caused by the transfection procedure itself with exposure to foreign DNA. We were expecting a higher up-regulation of IFNβ as this response is typically seen when activating cytosolic receptors with foreign nucleic acids, and as it also would have explained the high IP10 response. Compared to the stable cell-lines made by lentiviruses, stimulation with LPS gave increased responses for pcDNA3.1-transfected cells except for IL-8 and IP10 for U373-CD14 TLR8 cells. The transiently transfected cells were incubated with the plasmids for 24 hours before the remaining plasmids were washed away, and this procedure gave the cells 48 hours to "rest" before infecting them with *M. avium*. It was thought that this time would be sufficient to decrease the basal background responses to the foreign nucleic acids in the plasmids, but this was not the case. The same was expected for the lentiviral transduced cells, as they were left to rest for a week after virus infection before preforming experiments, but we also observed background responses for these cell-lines. The up-regulated inflammation could potentially "prime" the cells and lead to anergy, lack of "normal" induction or negative regulation of cytokines because of earlier exposure to foreign nucleic acids by the plasmids. This again could lead to a less potent response towards the *M. avium* infection, however this needs to be assessed further with additional studies. Still, the observed cytokine induction for *M. avium* infection and TLR- ligands was significantly up-regulated for the stable cell-lines and sufficient for further detailing of the pathways.

The TLR receptors were only transiently transfected into the U373-CD14 cells, which means that the DNA was delivered into the cell nucleus but not randomly integrated into the cell genome, and will be diluted over time with cell division and through degradation [87]. This would also mean that cells would be exposed to the foreign DNA consistently when incubated, probably initiating a background inflammation response, making it harder to study inflammatory pathways in the cells unless the response is efficiently removed by a reference sample. Stable transfection could also have been provided for randomly integration into the host genome over time, and would have required a co-expressed marker gene like antibiotic resistance, to allow selection of the few cells integrating the DNA into the genome, expanding the population. The U373-CD14 cells were already stably transfected with CD14 and the neomycin resistance gene as a selectable marker, which limited the possibilities for another transient transfection marker gene. As lentiviral transduction provides a high efficiency of integrating cells fast, and lentiviral vectors with Puromycin resistance was available, it was chosen to proceed with this method to establish stable cell-lines.

Another possible reason for the different LPS responses was that the levels of transfected CD14 could vary from different passage numbers and between experiments, as the cells for experiments were seeded without selection antibiotics.

In general, we concluded that both transfection and transduction mediated a "background" inflammatory response for the cells. The transduced cells mainly up-regulated IP10 as a response to the procedure, but induction of the other cytokines was also observed in slighter degree (RQ 2-8) compared to un-treated cells. The transient transfected cells with pcDNA3.1 gave especially prominent values of IL-8 and IP10 as a background response, and some induction of the other cytokines (RQ 2-16) compared to un-transfected cells. The pUNO1 vector gave evidence of up-regulating IFN-β with an RQ of 25 compared to pcDNA3.1-transfected cells.

5.5 Gateway cloning of expression vectors

Gateway cloning was used as a tool to create expression vectors with genes of interest both for lentiviral transduction and for transfections, for cell-lines U373-CD14 and THP1. For lentiviral vectors with TLR2 and TLR8, we chose to not fuse the genes to a fluorescent gene because it could interfere with the native function of the protein. As previously seen for the YFP-TLR8 protein, it was not functional when transfected into and expressed in the cells. A fluorescent tag could interfere with the protein folding, signal- mediation downstream in the cell, or interaction partners that would be unfortunate for the purpose of studying signaling and response towards *M. avium*. The drawback with leaving out a fluorescent marker gene is that cellular localization and transportation would be more difficult to observe, as it would require fixation and immunofluorescence staining. In addition, when selecting for cells transfected or transduced with the genes, it would be hard to know when the majority of the cells would express the proteins of interest without further examination. To be able to distinguish efficiently transduced cells with the TLR2 and TLR8 pLenti CMV Puro Dest vectors, it could have been placed a fluorescent protein in the vector backbone. The fluorophore would not be fused to the protein of interest and possibly not interfere with protein function as it would be expressed as a separate protein. In addition the TLRs could have been fused to smaller tags than flurophores like myc, FLAG, and HA tags, as well-characterized antibodies towards these tags are available. Because of the small size of the tag, it would be unlikely that they interfere with signaling and folding, and the function of the proteins. Preferable this would have been assessed instead of using another cell-line with GFP-Keap1 as a control for transduction efficiency.

Because the cloned vectors of full-length Keap1 had both a GFP and a myc-tag fused to the protein, we wanted to further elucidate the functionality for Keap1. We observed that around 30 % of the proteins associated with *M. avium* 4 hours post infection, and we presumed that the GFP and myc-tag did not interfere with the proteins native function [65]. However, as the images only represented a random selection of the sample, we could not exclude the possibility of the associations being co-incidences and not representing the majority of the proteins. If preforming interaction studies or cell localization studies with the protein, we would have to keep in mind that the tags might interfere with other native protein functions. The proteins GFP and Keap1 were only observed to associate some places in some cells. This could be due to low efficiency of the Keap1 antibody, failing to recognize Keap1 at all present places in the cell. Since the Keap1 protein was fused to both myc- and GFP tags, this

could interfere with the antibody failing to recognize Keap1 efficiently. In addition the transfection efficiency was low and it was observed that some cells expressed the plasmids highly efficiently while others did not, creating a heterogeneous mixture of cells with two different phenotypes present. We have to consider when preforming interaction studies that the phenotype displaying a high over-expression could induce artificial interactions that do not occur natively [88]. For immunofluorescently stained endogenous Keap1 in cells, it was observed fewer proteins compared to transfected cells that could imply further evidence of the constructs containing Keap1. When creating expression clones of GFP-Keap1, expression clones of GFP were also created to use as a control for the GFP tag.

When entry clone plasmids were generated by gateway cloning, restriction enzyme digestion gave evidence of several positive clones of GFP-Keap1 even though only one turned out to be positive according to sequencing. It could be speculated if the strategy with predicted product sizes were incorrect by comparing to sizes created by restriction enzyme digestion. The positive clone for GFP-Keap1 displayed a slightly larger top band than the other clones that could indicate a difference, but according to the strategy the difference in size should be present in the lowest band size. The band size variance was about proportional to the GFP gene size that was presumed to be excluded for some of the recombination reactions, because of the attB1 site present between the genes, but because the ladder was not thoroughly separated it was hard to distinguish the different band sizes. We did not really know how the two present attB1 sites upstream and downstream of the GFP gene would affect the BP reaction, but based on the sequencing results it seemed like the recombination preferred the shortest possible fragment with only Keap1.

When the entry plasmids with genes of interest were sequenced, the alignment with known sequences gave from 97- 99% similarity. Some common sequencing errors were observed in the beginning and the end of the sequences [89]. In addition, one or two base-differences could be observed in the middle of the sequences for some of the constructs. These mutations were not investigated any further, but could have been potentially problematic if they represented mutations that gave rise to loss-of- function for the proteins.

In addition to quality check the entry plasmids with cloning, we screened the entry plasmids and the expression clones for genes of interest with Go Taq Green PCR. When bacteria culture was added directly in the reaction it was observed that no PCR product was mediated for several of the samples, thought to be caused by an “overload” for the primers. The bands present in the bottom of the lanes were thought to represent the primers, which were not efficiently extended by amplification. It was easier to detect bands when purified plasmids were added to the reaction mix.

6. Conclusions and further perspectives

In this project we established tools to detail the regulation of IKKβ and TBK1 by Keap1, upon infection with *M. avium*. Primary macrophages from PBMC's were used for previous studies in our laboratory of Keap1 knockdown, but these cells are challenging to work with in terms of transfections and protein interaction studies, and donor variations occur between biologically different experiments. Two macrophage-like cell-lines were established by modification of the U373-CD14 astrocytoma cells to stably express TLR2 or TLR8 by lentiviral transduction and gateway cloning of expression vectors. The initial cell-line mediated low or none up-regulation of inflammatory cytokines TNF-α and IL-8, while low or none down-regulation of IFNβ and IP10, as a response to infection. It was found that they lacked endogenous expression of TLR2 and TLR8, which was thought to affect the response towards the mycobacteria. However, they managed to clear *M. avium* by phagocytosis efficiently when exposed to high MOI's. With transient transfection of various plasmids and different incubation time, it was observed with imaging, Western blotting and stimulation experiments that the majority of cells were not efficiently transfected, and that two phenotypes of cells were observed transfected. Only some cells had a high over- expression of the proteins distributed in the whole cell, while others had spots with fluorescence thought to indicate proteins expressed in clusters. The transfections of different transgenes also lead to different amount of protein expression in the cells.

Cells transiently transfected with TLR2 and TLR8 gave evidence of an enhanced inflammatory response towards infection for 4 hours, especially for TLR2 transfected cells. The TLR2 stably expressing cell-line responded efficiently to *M. avium* after 4 hours infection with up-regulation of TNF-α and IL-8 involved in the NF-κB pathway, and a significant lower increase of IFNβ involved in IRF-pathways. It was assumed that the 4 hours infection was sufficient for activating cell membrane receptors, while the further activation of TLRs in the endosomal compartments would require a longer infection time to expose them to nucleic acids from *M. avium*. We assumed that the low up-regulation of IFNβ was triggered by the activation of NF-κB by TLR2 in cooperation with IRF's for transcription of type 1 Interferon's. The TLR8 stably expressing cell-line responded efficiently with up-regulation of all screened cytokines except IFNβ towards the TLR-8 ligand CL075, but did not respond significantly towards the infection. The observations reflect the hypothesis of 4 hours infection not being sufficient for the exposure of mycobacteria nucleic acids in endolysosomal compartments, and not sufficient for IFNβ response through activation of IRF7 which is a "late" route for type 1 Interferon production. It was observed a high "background" inflammation response towards transient transfection and transduction itself, as the delta Ct values of reference samples were low compared to untreated cells. This highlighted the importance of using correct reference samples to ensure that the "read-outs" are not affected.

To accomplish the objectives, we established cell-lines to further study the regulation of IKKβ and TBK1 by Keap1 upon infection with *M. avium*. The cell- line expressing TLR2 can be used to further elucidate and investigate other mechanisms of *M. avium* infection, as it is a macrophage- model cell-line and responds to the mycobacteria infection with production of

inflammatory cytokines. For the future plan regarding Keap1 regulations, a cytokine profile would be created for the cells with 100 MOI as it was established as the best efficient MOI for sufficient phagocytosis. Next, gateway cloning and lentiviral transduction could be used as tools to knock down endogenous Keap1, and establish a cytokine profile in the new stable cell-line, which would be expected to up-regulate cytokines in the NF- κB and IRF-pathways as previously shown for MDM's. The knockdown of Keap1 should target the 3'UTR (un-translated regions) that influences gene expression of the Keap1 mRNA, so that transfected Keap1 constructs are not targeted by the shRNA implemented into the cell genome. Gateway cloned vectors with tagged Keap1 and recombined truncated versions of Keap1 could be transiently transfected, and pull-down experiments performed with immunoprecipitation after *M. avium* infection, to detect interaction partners of Keap1 and binding domains involved. Hopefully, this would lead to a novel understanding of how IKKβ and TBK1 and their respective pathways can be regulated upon infection, and how this affects the survival of the mycobacteria. As another tool to study the remaining aims of the project, some vectors with Keap1 and GFP tags were created with gateway cloning.

Future plans to further establish the TLR2 and TLR8- expressing cell-lines would be to conduct a time study experiment with *M. avium* infection and known TLR-ligands to closer inspect when and if the TLR8 receptor gets activated by *M. avium* nucleic acids, and up-regulates type 1 Interferon's as an inflammatory response. This could gain further insight in molecular mechanisms involving the dormant state and immune recognition of the bacteria inside the endolysosomal-like compartments. The levels of TLR2 and TLR8 could also be further studied with Western blotting to assess the amounts of proteins expressed in the cells, and confocal microcopy used to assess the cellular localization. At last, a cell-line stably transduced with both TLR2 and TLR8 could be a efficient tool to study further how the signaling thorugh the receptors are cross- linked and regulated by each other, as previously suggested by recent publications. As q-PCR mainly was used as a tool to investigate the up-regulation of inflammatory cytokine levels at an mRNA level, other methods like ELISA could be used to detect actually protein levels of released cytokines in the future.

Since modified U373-CD14 cells proved to be functional for responding to the bacteria, the THP1 cells could for now be discarded, as they are known to be harder to work with. If it turns out that the modified cells do not regulate inflammation as expected when knocking down Keap1 in the cells, the THP1 cells could be reconsidered as a model cell-line to work with for further investigating the remaining aims. In the future, primary human macrophages could be used to replicate important findings to be able to extrapolate the biological processes further to increase the biological relevance of the results.

This study has evaluated the importance of TLR2 specifically in *M. avium* recognition and inflammation, and provided important tools for future work with investigating molecular mechanisms on cell levels as a response to infection.

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7. References

8. Appendix

8.1 Appendix I

PureYield Miniprep protocol, Promega:

Before lysing cells and purifying DNA, prepare the Column Wash Solution by adding ethanol. Cap tightly after addition. See Technical Bulletin #TB374 for detailed instructions.

DNA Purification by Centrifugation

Prepare Lysate:

1. Add 600µl of bacterial culture to a 1.5ml micro centrifuge tube.
   Note: For higher yields and purity use the alternative protocol below to harvest and process up to 3ml of bacterial culture.
2. Add 100µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a micro centrifuge for 3 minutes.
5. Transfer the supernatant (~900µl) to a PureYieldTM Minicolumn without disturbing the cell debris pellet.
6. Place the minicolumn into a Collection Tube, and centrifuge at maximum speed in a micro centrifuge for 15 seconds.
7. Discard the flow- through, and place the minicolumn into the same Collection Tube.

Wash:

8. Add 200µl of Endotoxin Removal Wash (ERB) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Add 400µl of Column Wash Solution (CWC) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.

Elute:

10. Transfer the minicolumn to a clean 1.5ml micro centrifuge tube, then add 30µl of Elution Buffer or nuclease-free water directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
11. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the micro centrifuge tube, and store eluted plasmid DNA at –20°C.

Alternative Protocol for Larger Culture Volumes

1. Centrifuge 1.5ml of bacterial culture for 30 seconds at maximum speed in a micro centrifuge. Discard the supernatant.
2. Add an additional 1.5ml of bacterial culture to the same tube and repeat Step 1.
3. Add 600µl of TE buffer or water to the cell pellet, and resuspend completely.
4. Proceed to Step 2 of the standard protocol above.

8.2 Appendix II

Reagents and recipes for Western blotting:

1 x Lysisbuffer:

Table 26: Lysis buffer (2x) recipe

Reagent	Amount (ml) in 100 ml	Purchased from
Glyserol 87 %	23	Merck Millipore
Natriumfluorid 0,5 M	20	Sigma Aldrich
Tris/Hcl pH 8,0 1M	10	Sigma Aldrich
EDTA pH 8,0 0,2M	1	Sigma Aldrich
EGTA 0,2M	1	Calbiochem
NaCl 5M	15,4	Merck millipore
Trition X-100 10%	20	Sigma Aldrich
Na_3VO_4 0,2M	1	Sigma Aldrich
Sodium Deoxychelat 10%	10	Sigma Aldrich
MiiliQ water	Up to 100	

Mix reagents to get 2x lysis buffer.

Benzonase and proteinase inhibitor cocktail solution:

- Benzonase 0,25U/ml 1,3 ul (in 5 ml)
- Proteinase inhibitor cocktail 1 tbl (in 5 ml)
- MilliQ water up to 5 ml.

Mix reagents and dilute 1:1 of 2x lysis buffer and benzonase and proteinase inhibitor cocktail solution to get a final 1x lysis buffer. Stored in -20 C freezer.

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TBS-T:

Table 27: Tris buffered saline- with Tween (TBS-T) recipe

Reagent	Amount (ml) in 1000 ml	Purchased from
Tris (pH: 7,5) 1M	9,9	Sigma Aldrich
Tween-20	1	Sigma Aldrich
NaCl 5M	19,8	Merck millipore
Deionized water	Up to 1000	-

8.3 Appendix III

Reagents for Immunoprecipitation:

HA- lysis buffer:

Reagent	Amount in 5000 ul	Concentration in stock	Concentration in buffer	Purchased from
NP-40	50	100 %	1 %	Sigma Aldrich
Triton-X 100	12,5	100 %	0,25 %	Sigma Aldrich
Tris-HCl pH 8	250	1 M	0,05 M	Sigma Aldrich
NaCl	750	1 M	0,15 M	Merck millipore
EDTA pH 8	10	0,5 M	0,001 M	Sigma Aldrich
PI/ Complete	200	25 X	1 X	Sigma Aldrich
PIC2	100	100 X	2 X	P5726, Sigma Aldrich
PIC3	100	100 X	2 X	P0044, Sigma Aldrich
MQ water	3527,5			-

Conjugation buffer: 20 mM Sodium phosphate from Merck Millipore + 0,15 M NaCl (pH 7-9).

- 0,1 M Sodium phosphate: 20 ml in 100 ml dH_2O
- 1 M NaCl: 15 ml in 100 ml dH_2O

10 ml 0,1 M Sodium phosphate + 7,5 ml 1 M NaCl + 32,5ml dH_2O

Quenching buffer: 1 M Tris HCl (pH 7,5), adjust pH with HCl.

8.4 Appendix IV

Gene sequences used for Gateway cloning:

TLR2 gene:

ATGCCACATACTTTGTGGATGGTGTGGGTCTTGGGGGTCATCATCAGCCTCTCCAAGGAAGAATCCTCCA
ATCAGGCTTCTCTGTCTTGTGACCGCAATGGTATCTGCAAGGGCAGCTCAGGATCTTTAAACTCCATTCC
CTCAGGGCTCACAGAAGCTGTAAAAAGCCTTGACCTGTCCAACAACAGGATCACCTACATTAGCAACAG
TGACCTACAGAGGTGTGTGAACCTCCAGGCTCTGGTGCTGACATCCAATGGAATTAACACAATAGAGGA
AGATTCTTTTTCTTCCCTGGGCAGTCTTGAACATTTAGACTTATCCTATAATTACTTATCTAATTTATCGTC
TTCCTGGTTCAAGCCCCTTTCTTCTTTAACATTCTTAAACTTACTGGGAAATCCTTACAAAACCCTAGGGG
AAACATCTCTTTTTTCTCATCTCACAAAATTGCAAATCCTGAGAGTGGGAAATATGGACACCTTCACTAA
GATTCAAAGAAAAGATTTTGCTGGACTTACCTTCCTTGAGGAACTTGAGATTGATGCTTCAGATCTACAG
AGCTATGAGCCAAAAAGTTTGAAGTCAATTCAGAATGTAAGTCATCTGATCCTTCATATGAAGCAGCAT
ATTTTACTGCTGGAGATTTTTGTAGATGTTACAAGTTCCGTGGAATGTTTGGAACTGCGAGATACTGATT
TGGACACTTTCCATTTTTCAGAACTATCCACTGGTGAAACAAATTCATTGATTAAAAAGTTTACATTTAG
AAATGTGAAAATCACCGATGAAAGTTTGTTTCAGGTTATGAAACTTTTGAATCAGATTTCTGGATTGTTA
GAATTAGAGTTTGATGACTGTACCCTTAATGGAGTTGGTAATTTTAGAGCATCTGATAATGACAGAGTTA
TAGATCCAGGTAAAGTGGAAACGTTAACAATCCGGAGGCTGCATATTCCAAGGTTTTACTTATTTTATGA
TCTGAGCACTTTATATTCACTTACAGAAAGAGTTAAAAGAATCACAGTAGAAAACAGTAAAGTTTTTCTG
GTTCCTTGTTTACTTTCACAACATTTAAAATCATTAGAATACTTGGATCTCAGTGAAAATTTGATGGTTGA
AGAATACTTGAAAAATTCAGCCTGTGAGGATGCCTGGCCCTCTCTACAAACTTTAATTTTAAGGCAAAAT
CATTTGGCATCATTGGAAAAAACCGGAGAGACTTTGCTCACTCTGAAAAACTTGACTAACATTGATATCA
GTAAGAATAGTTTTCATTCTATGCCTGAAACTTGTCAGTGGCCAGAAAAGATGAAATATTTGAACTTATC
CAGCACACGAATACACAGTGTAACAGGCTGCATTCCCAAGACACTGGAAATTTTAGATGTTAGCAACAA
CAATCTCAATTTATTTTCTTTGAATTTGCCGCAACTCAAAGAACTTTATATTTCCAGAAATAAGTTGATGA
CTCTACCAGATGCCTCCCTCTTACCCATGTTACTAGTATTGAAAATCAGTAGGAATGCAATAACTACGTT
TTCTAAGGAGCAACTTGACTCATTTCACACACTGAAGACTTTGGAAGCTGGTGGCAATAACTTCATTTGC
TCCTGTGAATTCCTCTCCTTCACTCAGGAGCAGCAAGCACTGGCCAAAGTCTTGATTGATTGGCCAGCAA
ATTACCTGTGTGACTCTCCATCCCATGTGCGTGGCCAGCAGGTTCAGGATGTCCGCCTCTCGGTGTCGGA
ATGTCACAGGACAGCACTGGTGTCTGGCATGTGCTGTGCTCTGTTCCTGCTGATCCTGCTCACGGGGGTC
CTGTGCCACCGTTTCCATGGCCTGTGGTATATGAAAATGATGTGGGCCTGGCTCCAGGCCAAAAGGAAG
CCCAGGAAAGCTCCCAGCAGGAACATCTGCTATGATGCATTTGTTTCTTACAGTGAGCGGGATGCCTACT
GGGTGGAGAACCTTATGGTCCAGGAGCTGGAGAACTTCAATCCCCCCTTCAAGTTGTGTCTTCATAAGCG
GGACTTCATTCCTGGCAAGTGGATCATTGACAATATCATTGACTCCATTGAAAAGAGCCACAAAACTGTC
TTTGTGCTTTCTGAAAACTTTGTGAAGAGTGAGTGGTGCAAGTATGAACTGGACTTCTCCCATTTCCGTCT
TTTTGATGAGAACAATGATGCTGCCATTCTCATTCTTCTGGAGCCCATTGAGAAAAAAGCCATTCCCCAG
CGCTTCTGCAAGCTGCGGAAGATAATGAACACCAAGACCTACCTGGAGTGGCCCATGGACGAGGCTCAG
CGGGAAGGATTTTGGGTAAATCTGAGAGCTGCGATAAAGTCCTAG

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TLR8 gene:

ATGGAAAACATGTTCCTTCAGTCGTCAATGCTGACCTGCATTTTCCTGCTAATATCTGGTTCCTGTG
AGTTATGCGCCGAAGAAAATTTTTCTAGAAGCTATCCTTGTGATGAGAAAAAGCAAAATGACTCAG
TTATTGCAGAGTGCAGCAATCGTCGACTACAGGAAGTTCCCCAAACGGTGGGCAAATATGTGACA
GAACTAGACCTGTCTGATAATTTCATCACACACATAACGAATGAATCATTTCAAGGGCTGCAAAAT
CTCACTAAAATAAATCTAAACCACAACCCCAATGTACAGCACCAGAACGGAAATCCCGGTATACA
ATCAAATGGCTTGAATATCACAGACGGGGCATTCCTCAACCTAAAAAACCTAAGGGAGTTACTGCT
TGAAGACAACCAGTTACCCCAAATACCCTCTGGTTTGCCAGAGTCTTTGACAGAACTTAGTCTAAT
TCAAAACAATATATACAACATAACTAAAGAGGGCATTTCAAGACTTATAAACTTGAAAAATCTCTA
TTTGGCCTGGAACTGCTATTTTAACAAAGTTTGCGAGAAAACTAACATAGAAGATGGAGTATTTGA
AACGCTGACAAATTTGGAGTTGCTATCACTATCTTTCAATTCTCTTTCACATGTGCCACCCAAACTG
CCAAGCTCCCTACGCAAACTTTTTCTGAGCAACACCCAGATCAAATACATTAGTGAAGAAGATTTC
AAGGGATTGATAAATTTAACATTACTAGATTTAAGCGGGAACTGTCCGAGGTGCTTCAATGCCCCA
TTTCCATGCGTGCCTTGTGATGGTGGTGCTTCAATTAATATAGATCGTTTTGCTTTTCAAAACTTGA
CCCAACTTCGATACCTAAACCTCTCTAGCACTTCCCTCAGGAAGATTAATGCTGCCTGGTTTAAAAA
TATGCCTCATCTGAAGGTGCTGGATCTTGAATTCAACTATTTAGTGGGAGAAATAGCCTCTGGGGC
ATTTTTAACGATGCTGCCCCGCTTAGAAATACTTGACTTGTCTTTTAACTATATAAAGGGGAGTTAT
CCACAGCATATTAATATTTCCAGAAACTTCTCTAAACTTTTGTCTCTACGGGCATTGCATTTAAGAG
GTTATGTGTTCCAGGAACTCAGAGAAGATGATTTCCAGCCCCTGATGCAGCTTCCAAACTTATCGA
CTATCAACTTGGGTATTAATTTTATTAAGCAAATCGATTTCAAACTTTTCCAAAATTTCTCCAATCT
GGAAATTATTTACTTGTCAGAAAACAGAATATCACCGTTGGTAAAAGATACCCGGCAGAGTTATGC
AAATAGTTCCTCTTTTCAACGTCATATCCGGAAACGACGCTCAACAGATTTTGAGTTTGACCCACAT
TCGAACTTTTATCATTTCACCCGTCCTTTAATAAAGCCACAATGTGCTGCTTATGGAAAAGCCTTAG
ATTTAAGCCTCAACAGTATTTTCTTCATTGGGCCAAACCAATTTGAAAATCTTCCTGACATTGCCTG
TTTAAATCTGTCTGCAAATAGCAATGCTCAAGTGTTAAGTGGAACTGAATTTTCAGCCATTCCTCAT
GTCAAATATTTGGATTTGACAAACAATAGACTAGACTTTGATAATGCTAGTGCTCTTACTGAATTGT
CCGACTTGGAAGTTCTAGATCTCAGCTATAATTCACACTATTTCAGAATAGCAGGCGTAACACATC
ATCTAGAATTTATTCAAAATTTCACAAATCTAAAAGTTTTAAACTTGAGCCACAACAACATTTATAC
TTTAACAGATAAGTATAACCTGGAAAGCAAGTCCCTGGTAGAATTAGTTTTCAGTGGCAATCGCCT
TGACATTTTGTGGAATGATGATGACAACAGGTATATCTCCATTTTCAAAGGTCTCAAGAATCTGAC
ACGTCTGGATTTATCCCTTAATAGGCTCAAGCACATCCCAAATGAAGCATTCCTTAATTTGCCAGCG
AGTCTCACTGAACTACATATAAATGATAATATGTTAAAGTTTTTTAACTGGACATTACTCCAGCAGT
TTCCTCGTCTCGAGTTGCTTGACTTACGTGGAAACAAACTACTCTTTTTAACTGATAGCCTATCTGA
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CTTTCTGAAGTCAGTAGTCTGAAGCACCTCGATTTAAGTTCCAATCTGCTAAAAACAATAAACAAA
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TGCACCTGTGACATTGGAGATTTCCGAAGATGGATGGATGAACATCTGAATGTCAAAATTCCCAGA
CTGGTAGATGTCATTTGTGCCAGTCCTGGGGATCAAAGAGGGAAGAGTATTGTGAGTCTGGAGCTA
ACAACTTGTGTTTCAGATGTCACTGCAGTGATATTATTTTTCTTCACGTTCTTTATCACCACCATGGT
TATGTTGGCTGCCCTGGCTCACCATTTGTTTTACTGGGATGTTTGGTTTATATATAATGTGTGTTTAG
CTAAGGTAAAAGGCTACAGGTCTCTTTCCACATCCCAAACTTTCTATGATGCTTACATTTCTTATGA
CACCAAAGATGCCTCTGTTACTGACTGGGTGATAAATGAGCTGCGCTACCACCTTGAAGAGAGCCG
AGACAAAAACGTTCTCCTTTGTCTAGAGGAGAGGGATTGGGACCCGGGATTGGCCATCATCGACA
ACCTCATGCAGAGCATCAACCAAAGCAAGAAAACAGTATTTGTTTTAACCAAAAAATATGCAAAA
AGCTGGAACTTTAAAACAGCTTTTTACTTGGCTTTGCAGAGGCTAATGGATGAGAACATGGATGTG
ATTATATTTATCCTGCTGGAGCCAGTGTTACAGCATTCTCAGTATTTGAGGCTACGGCAGCGGATCT
GTAAGAGCTCCATCCTCCAGTGGCCTGACAACCCGAAGGCAGAAGGCTTGTTTTGGCAAACTCTGA
GAAATGTGGTCTTGACTGAAAATGATTCACGGTATAACAATATGTATGTCGATTCCATTAAGCAAT
ACTAA

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GFP gene:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTA
AACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAA
GTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTGGGGCGTG
CAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGC
TACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTC
GAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCT
GGGGCACAAGCTGGAGTACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACG
GCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC
AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCG
CCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGA
TCACTCTCGGCATGGACGAGCTGTACAAG

GFP-Keap1 gene (GFP in blue, Keap1 in red):

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTA
AACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAA
GTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTG
CAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGC
TACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTC
GAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCT
GGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG
GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC
AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCG
CCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGA
TCACTCTCGGCATGGACGAGCTGTACAAGTCCGGACTCAGATCCGCTGGCCCAGGAACAAGTTTGTACA
AAAAAGCAGGCTCTTTAAAGGAACCGCAGCCAGATCCCAGGCCTAGCGGGGCTGGGGCCTGCTGCCGAT
TCCTGCCCCTGCAGTCACAGTGCCCTGAGGGGGCAGGGGACGCGGTGATGTACGCCTCCACTGAGTGCA
AGGCGGAGGTGACGCCCTCCCAGCATGGCAACCGCACCTTCAGCTACACCCTGGAGGATCATACCAAGC
AGGCCTTTGGCATCATGAACGAGCTGCGGCTCAGCCAGCAGCTGTGTGACGTCACACTGCAGGTCAAGT
ACCAGGATGCACCGGCCGCCCAGTTCATGGCCCACAAGGTGGTGCTGGCCTCATCCAGCCCTGTCTTCAA
GGCCATGTTCACCAACGGGCTGCGGGAGCAGGGCATGGAGGTGGTGTCCATTGAGGGTATCCACCCCAA
GGTCATGGAGCGCCTCATTGAATTCGCCTACACGGCCTCCATCTCCATGGGCGAGAAGTGTGTCCTCCAC
GTCATGAACGGTGCTGTCATGTACCAGATCGACAGCGTTGTCCGTGCCTGCAGTGACTTCCTGGTGCAGC
AGCTGGACCCCAGCAATGCCATCGGCATCGCCAACTTCGCTGAGCAGATTGGCTGTGTGGAGTTGCACC
AGCGTGCCCGGGAGTACATCTACATGCATTTTGGGGAGGTGGCCAAGCAAGAGGAGTTCTTCAACCTGT
CCCACTGCCAACTGGTGACCCTCATCAGCCGGGACGACCTGAACGTGCGCTGCGAGTCCGAGGTCTTCC
ACGCCTGCATCAACTGGGTCAAGTACGACTGCGAACAGCGACGGTTCTACGTCCAGGCGCTGCTGCGGG
CCGTGCGCTGCCACTCGTTGACGCCGAACTTCCTGCAGATGCAGCTGCAGAAGTGCGAGATCCTGCAGT
CCGACTCCCGCTGCAAGGACTACCTGGTCAAGATCTTCGAGGAGCTCACCCTGCACAAGCCCACGCAGG
TGATGCCCTGCCGGGCGCCCAAGGTGGGCCGCCTGATCTACACCGCGGGCGGCTACTTCCGACAGTCGC
TCAGCTACCTGGAGGCTTACAACCCCAGTGACGGCACCTGGCTCCGGTTGGCGGACCTGCAGGTGCCGC
GGAGCGGCCTGGCCGGCTGCGTGGTGGGCGGGCTGTTGTACGCCGTGGGCGGCAGGAACAACTCGCCCG
ACGGCAACACCGACTCCAGCGCCCTGGACTGTTACAACCCCATGACCAATCAGTGGTCGCCCTGCGCCC

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CCATGAGCGTGCCCCGTAACCGCATCGGGGTGGGGGTCATCGATGGCCACATCTATGCCGTCGGCGGCT
CCCACGGCTGCATCCACCACAACAGTGTGGAGAGGTATGAGCCAGAGCGGGATGAGTGGCACTTGGTGG
CCCCAATGCTGACACGAAGGATCGGGGTGGGCGTGGCTGTCCTCAATCGTCTCCTTTATGCCGTGGGGG
GCTTTGACGGGACAAACCGCCTTAATTCAGCTGAGTGTTACTACCCAGAGAGGAACGAGTGGCGAATGA
TCACAGCAATGAACACCATCCGAAGCGGGGCAGGCGTCTGCGTCCTGCACAACTGTATCTATGCTGCTG
GGGGCTATGATGGTCAGGACCAGCTGAACAGCGTGGAGCGCTACGATGTGGAAACAGAGACGTGGACT
TTCGTAGCCCCCATGAAGCACCGGCGAAGTGCCCTGGGGATCACTGTCCACCAGGGGAGAATCTACGTC
CTTGGAGGCTATGATGGTCACACGTTCCTGGACAGTGTGGAGTGTTACGACCCAGATACAGACACCTGG
AGCGAGGTGACCCGAATGACATCGGGCCGGAGTGGGGTGGGCGTGGCTGTCACCATGGAGCCCTGCCG
GAAGCAGATTGACCAGCAGAACTGTACCTGTTGA

8.5 Appendix V

Stimulated U373-CD14 cells, presented after stimulation time:

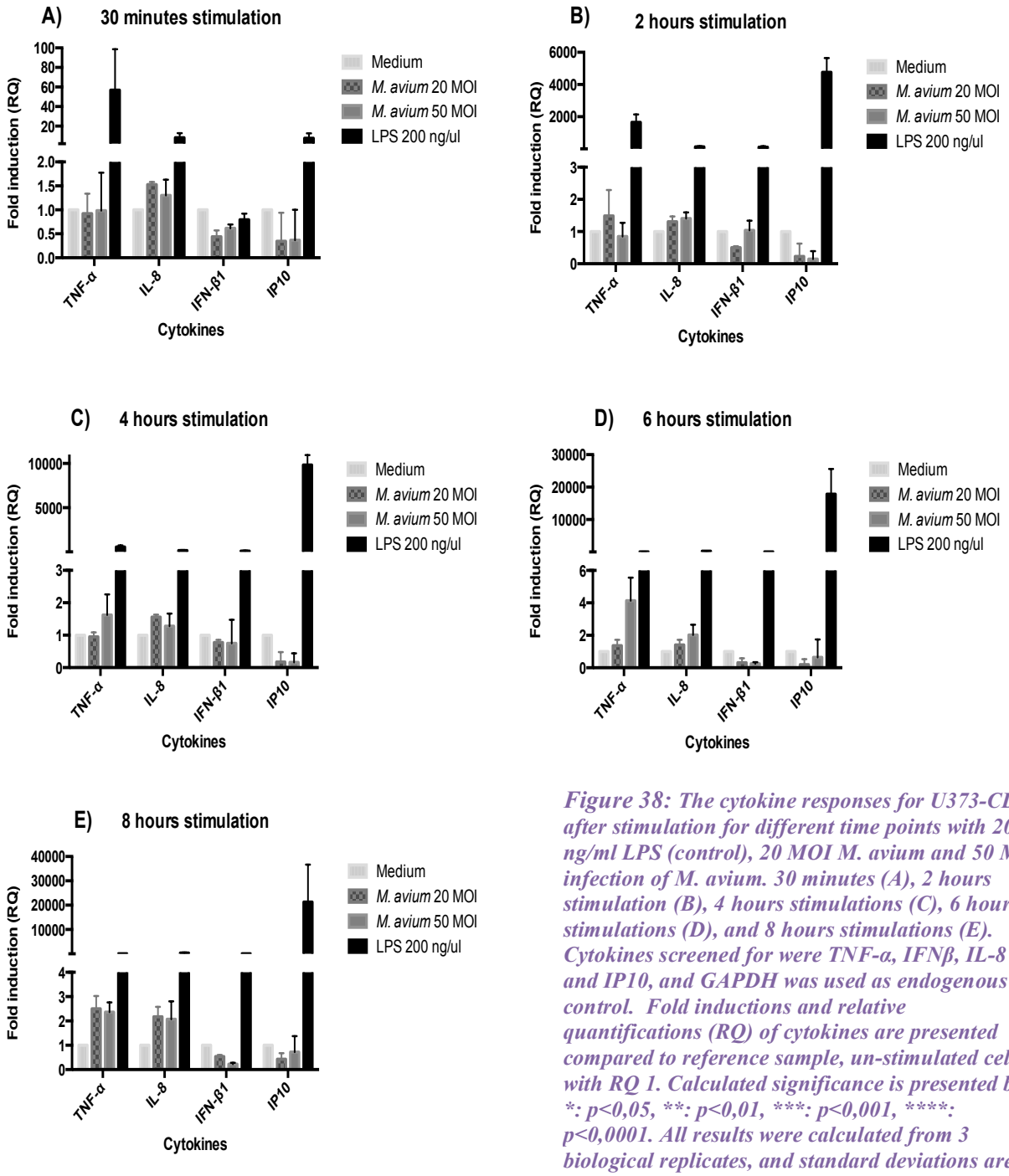

*Figure 38: The cytokine responses for U373-CD14 after stimulation for different time points with 200 ng/ml LPS (control), 20 MOI M. avium and 50 MOI infection of M. avium. 30 minutes (A), 2 hours stimulation (B), 4 hours stimulations (C), 6 hours stimulations (D), and 8 hours stimulations (E). Cytokines screened for were TNF-α, IFNβ, IL-8 and IP10, and GAPDH was used as endogenous control. Fold inductions and relative quantifications (RQ) of cytokines are presented compared to reference sample, un-stimulated cells with RQ 1. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. All results were calculated from 3 biological replicates, and standard deviations are presented in the figures.*

8.6 Appendix VI

Normalized responses for transfected U373-CD14 cells:

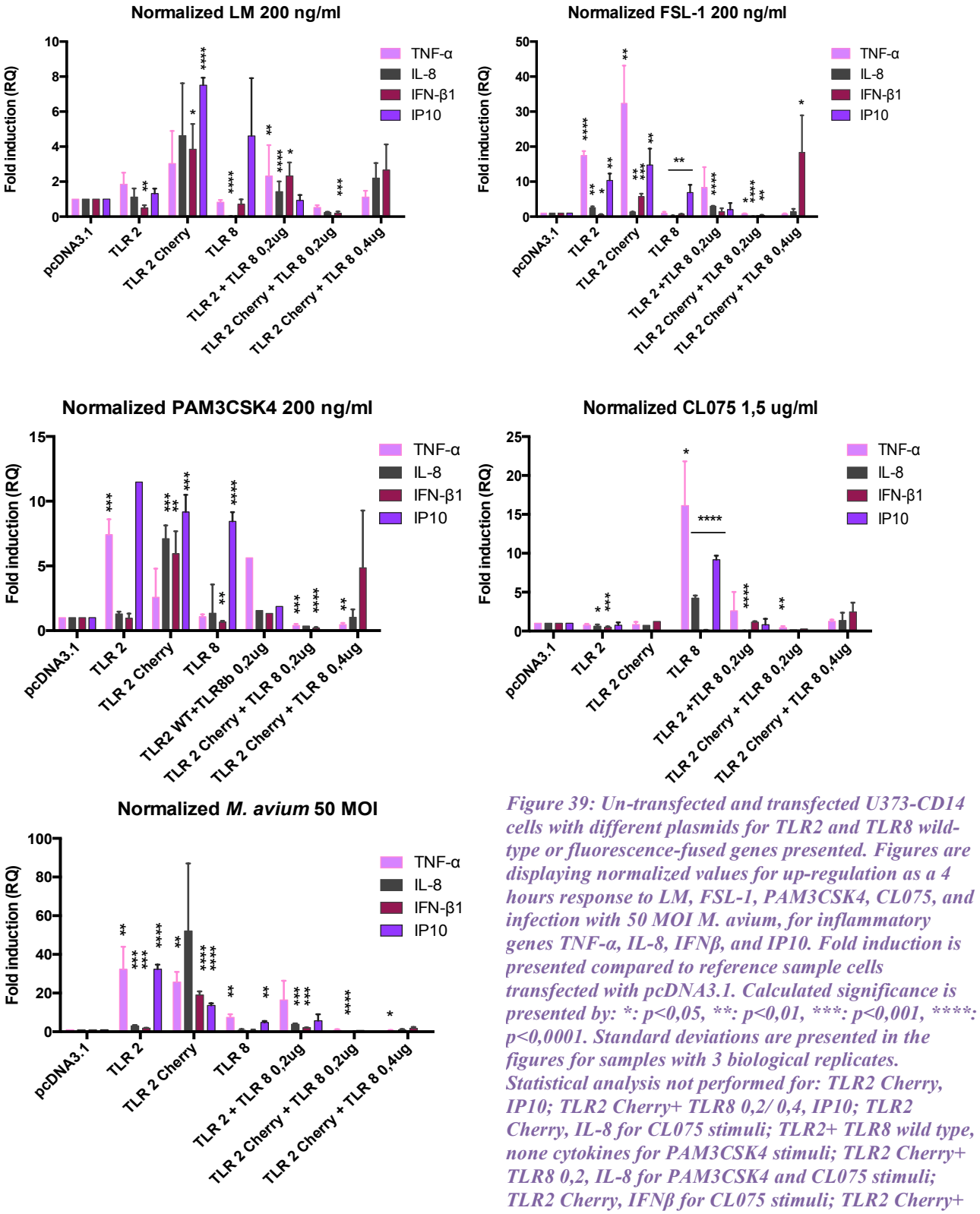

*Figure 39: Un-transfected and transfected U373-CD14 cells with different plasmids for TLR2 and TLR8 wild-type or fluorescence-fused genes presented. Figures are displaying normalized values for up-regulation as a 4 hours response to LM, FSL-1, PAM3CSK4, CL075, and infection with 50 MOI M. avium, for inflammatory genes TNF-α, IL-8, IFNβ, and IP10. Fold induction is presented compared to reference sample cells transfected with pcDNA3.1. Calculated significance is presented by: *: p<0,05, **: p<0,01, ***: p<0,001, ****: p<0,0001. Standard deviations are presented in the figures for samples with 3 biological replicates. Statistical analysis not performed for: TLR2 Cherry, IP10; TLR2 Cherry+ TLR8 0,2/ 0,4, IP10; TLR2 Cherry, IL-8 for CL075 stimuli; TLR2+ TLR8 wild type, none cytokines for PAM3CSK4 stimuli; TLR2 Cherry+ TLR8 0,2, IL-8 for PAM3CSK4 and CL075 stimuli; TLR2 Cherry, IFNβ for CL075 stimuli; TLR2 Cherry+ TLR8 0,2, IFNβ for CL075 stimuli because of too few biological replicates.*