Investigation of changes in immunostimulatory properties of clinical *Mycobacterium avium* strains over time of infection

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Abstract

Mycobacterium avium complex (MAC) is a group of environmental bacteria that are able to become intracellular pathogens that cause disseminated diseases in immunocompromised hosts such as HIV-infected individuals. The following study aims for a better characterization of the in-patient evolution of strains and how immunostimulatory properties change over time for these opportunistic mycobacteria.

MAC isolated from patients at St. Olav's hospital were analyzed for their genotypic, phenotypic and immunostimulatory properties. A pulsed-field gel electrophoresis (PFGE) was established to determine genetic relatedness of the isolated strains and to compare the identity of the strains. By using the PFGE we could validate that the strains are able to mutate in the host as well as that patients got infected with different strains over time of infection. If the different strains co-existed in the host or if the patients got repeatedly infected needs to be further validated.

The different mycobacterial isolates displayed variable phenotypic properties concerning their growth and intracellular survival. The strongly varying growth characteristics between the isolates were not clearly relatable to observed genetic differences in the PFGE.

The immunostimulatory properties of the mycobacterial strains were validated by ELISA measurement of the pro-inflammatory cytokines TNF-α and IL-6. It was shown that the macrophage response to the mycobacterial strains can vary over time of infection even for strains that genetically looked identical in the PFGE. While for some strains a strong decrease in the cytokine levels was found, for other strains the expression level increased. Furthermore it was shown that a high cytokine response induced by the bacterial strains correlated with a lowered intracellular survival 24h past infection indicating that an induction of a strong immune response could be detrimental for the bacterium. The findings made in this study could be valuable for further investigations concerning the identification of new therapeutic approaches.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ADC	Albumin dextrose catalase
AP-1	Activated protein-1
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CCR	C-C motif receptor
COPD	Chronic obstructive pulmonary disease
CSF	Colony-stimulating factor
CR	Complement receptor
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome autoantigen 1
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPL	Glycopeptidolipid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
IL	Interleukin
IL-6R	Interleukin-6 receptor
INF	Interferon
JAK	Janus kinase
LAM	Lipoarabinomannan
LM	Lipomannan
LOS	Lipooligosaccharides

LPS	Lipopolysaccharide
MAA	*Mycobacterium avium avium*
MAC	*Mycobacterium avium* complex
MAH	*Mycobacterium avium hominissuis*
MAP	*Mycobacterium avium paratuberculosis*
MAPK	Mitogen-activated protein kinase
MAS	*Mycobacterium avium silvaticum*
M	Marker
mAGP	Mycolyl-arabinogalactan-peptidoglycan
Man-LAM	Mannosylated lipoarabinomannan
MC	Media control
MCP-1	Monocyte chemotactic protein-1
MIP	Macrophage inflammatory proteins
Mo-MuLV	Moloney murine leukemia virus
MR	Mannose receptor
MTB	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary response protein 88
NC	Negative control
NF	Nuclear transcription factor
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
OADC	Oleic albumin dextrose catalase
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PFGE	Pulsed-field gel electrophoresis
PIM	Phosphatidylinositol mannosides
p.inf.	Past infection
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RIP2	Receptor-interacting protein 2
RO	Reverse osmosis
RT	Room temperature

SA-PE	Strepatividin-phycoerythrin
SA-HRP	Strepatividin-horseradish-peroxidase
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TBE	Tris/Borate/EDTA
TE	Tris-EDTA
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF6	Tumor necrosis factor receptor-associated factor 6
wt/vol	Weight/ volume

International commonly used mathematical, chemical as well as physical abbreviations are not separately mentioned.

1. Introduction

1.1 The immune system

Immunity can be broadly categorized into innate immunity and adaptive immunity. The innate immune system works as a first line defense and it aims to eliminate microbial pathogens. Nevertheless, the innate immune system may cause harm to the host if excessive inflammation is induced[1]. The early innate immune response is mainly driven by the activation of fully germline-encoded pattern recognition receptors (PRRs). These receptors have the ability to recognize evolutionary conserved molecular patterns, called pathogen-associated molecular patterns (PAMPs), found in a wide range of pathogens[2]. PAMPs stimulate intracellular signaling as well as gene expression and hence activation of inflammatory and antimicrobial activities[3]. Due to the recognition of evolutionary conserved molecular patterns the innate immune response is rapidly evolving and mainly driven by the activation of macrophages and dendritic cells (DCs). In addition, it is also needed for the development of an adaptive immune response and establishment of an immunological memory.

Due to the ability of pathogens to mutate in order to avoid host detection, a second defense line called adaptive immunity can be found in vertebrates. While the innate immune response has limitations in the recognition of common pathogenic molecular patterns, the adaptive immune system relies on highly specific receptors selected through somatic recombination of a large array of gene segments. It is therefore possible to generate highly specific and flexible immune responses, which can persist in the host a whole lifetime. Due to this immunological memory a rapid immune response after re-exposure can be induced by the host organism. The cells of the adaptive immune system are T- and B-cells. These develop in the thymus and the bone marrow respectively, from common lymphoid progenitors. At later stages the cells migrate to secondary lymphoid organs like the lymph nodes and the spleen, which are capable to capture circulating antigens from lymph and blood. Upon infection, the adaptive immune response is initiated by activation of adaptive immune cells arising from these organs, directly followed by lymphocyte migration to the site of infection[4].

1.2 Mycobacteria

Mycobacteria are aerobic and except of the species *M. marinum* nonmotile. They are acid-fast, slightly curved rod-shaped bacteria without an outer cell membrane. The bacteria are 0.2µm to 0.6µm wide and 1µm to 10µm long. Especially the exclusively pathogenic strains are slow-growing such as *M. leprae* that can have doubling times of up to 20 days.

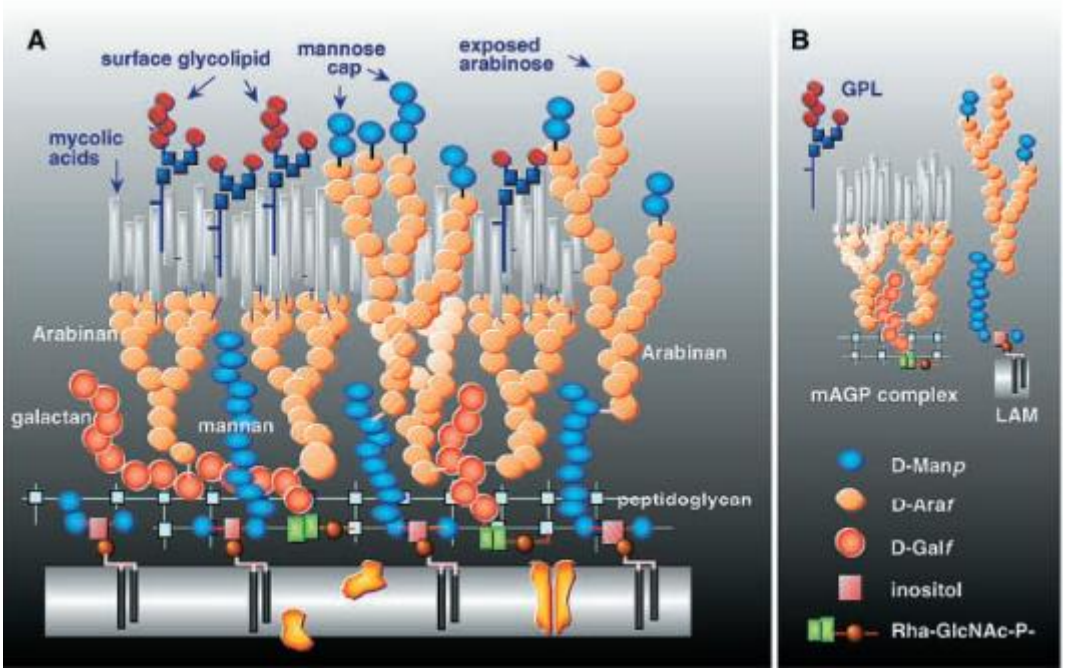


Figure 1: Schematic representation of the mycobacterial cell wall
(A). The cell wall of *M. tuberculosis* is largely devoid of surface glycolipids, whereas other nontuberculosis mycobacteria express one of the three classes of sero-specific glycolipids, namely the glycopeptidolipid (GPL), lipooligosaccharides (LOS), and phenolic glycolipids (PGL). The three major glyco-constituents of the *M. avium* cell wall are further illustrated in (B). mAGP, mycolyl-arabinogalactan-peptidoglycan complex; LAM, lipoarabinomannan, Lipomannan (LM) is related to LAM but lacks the arabinan, whereas the phosphatidylinositol anchor of LAM/LM also exists as phosphatidylinositol mannosides (PIM), e.g. PIM2 which carries two Man residues on the inositol ring. Figure taken from Chatterjee and Khoo, 2001[5].

All mycobacteria share a characteristic cell wall structure made of peptidoglycans, poly-saccharides, glycolipids and lipids (Figure 1A). The mycobacterial cell wall contains lipo-arabinomannan (LAM), that shares many physicochemical properties with lipopolysaccharide (LPS), a bacterial endotoxin found in many other bacterial species, but not in mycobacteria. The cell wall is thicker than of most other bacteria. Furthermore, the cell wall is hydrophobic, waxy and composed of a hydrophobic mycolic acid layer as well as a peptidoglycan layer. These two layers are connected by a polysaccharide called arabinogalactan. The peptido-glycan N-acetylmuramic acid, found in many other bacteria, is replaced by N-

glycolylmuramic acid. The outer layer is composed of extracellular polysaccharides and glycolipids, many of which are species specific. The *Mycobacterium avium* complex (MAC) for example contains type-specific glycopeptidolipids (GPLs)[6] (Figure 1B).

The GC-rich (65.6%) 4.4-Mbp genome of *M. tuberculosis* (MTB) encodes about 4,000 predicted proteins[7]. Mycobacterial species are known to have high mutation frequencies. For *M. avium* for example it is known that there are more than 50 different alleles of the *hsp65* gene already identified. Therefore, this gene is also often used to characterize and identify different *M. avium* isolates[8].

1.2.1 *Mycobacterium tuberculosis*

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a major health problem with an estimate of 8.7 million new cases and 1.4 million deaths in 2011[9]. Even if one third of the world's population is latently infected with MTB, only 10% of these individuals will develop an active disease[10]. Hence, a strong influence of the host genetics in the regulation of disease progression seems likely. The first possible genetic loci that could influence disease susceptibility are already identified[11]. The loci rs4331426, located in a gene-poor region on chromosome 18q11.2, was associated with the disease[11].

The currently used vaccine *M. bovis* Bacillus Calmette–Guérin (BCG) was first used in humans in 1921, but since then no major discoveries on new vaccines have been made. The current BCG vaccine protects against severe forms of childhood TB, but is not properly working against adult pulmonary TB, the most common form of the disease[12]. The treatment of TB also poses some major challenges. Due to the fact that MTB has developed effective immune evasion strategies, the treatment regimens are long lasting and a couple of different antibiotics are involved. The standard antibiotics for treatment of TB are isoniazid, rifampicin, pyrazinamide, and ethambutol for two months followed by only isoniazid and rifampicin for another four months. Patients are considered to be cured at six months, although there is still a relapse rate of 2 to 3%. For latent tuberculosis, the standard treatment is six to nine months of only isoniazid. Because of poor compliance of patients and premature termination of drug therapy the emergence of drug resistance is becoming a challenging problem. About one in five patients is infected with a multidrug-resistant TB strain and strains

of virtually untreatable extensively drug-resistance are on the rise[9]. Also the first cases of total drug-resistance were described[13].

One of the biggest challenges in a successful treatment is the granuloma formation. This consists of a complex interplay between activated αβ-T-cell-receptor-expressing cells and macrophages that have engulfed mycobacteria. Granuloma formation occurs to keep the mycobacteria in a closed environment and prevent further spread in the infected host. The signals that activate macrophages for granuloma formation are complex and still not fully understood. An important role for the cytokines TNF-α and INF-γ in granuloma formation was proven by several studies[14,15].

1.2.2 *Mycobacterium avium* complex

M. avium and *M. intracellulare* are grouped under the term *M. avium* complex (MAC) with a wide range of different serovars, strains, subspecies and morphologic forms that, in addition, are differing in virulence. *M. avium* complex is an opportunistic pathogen capable of causing disease in both animals and humans[5,16].

The bacterium is usually found in the environment like in water or soil. Healthy individuals exposed to MAC will rarely develop an infection. It is well documented that patients with predisposing risk factors like elderly women that are non-smokers but have skeletal deformities[17], patients with chronic obstructive pulmonary disease (COPD), cystic fibrosis or human immunodeficiency virus (HIV) infection, are more prone to develop a severe MAC infection[18,19]. The clinical diagnosis of MAC caused lung disease is usually done by radiography[20].

Mycobacterium avium is further subdivided into four subspecies: *M. avium avium* (MAA), *M. avium hominissuis* (MAH), *M. avium paratuberculosis* (MAP) and *M. avium silvaticum* (MAS)[21,22]. While MAA and MAS are mostly avian pathogens causing a tuberculosis-like disease in birds or infections in mammals but not humans[23], MAP and MAH are well known mammal and human pathogens[24]. MAP can cause Johne's disease[25], a chronic enteritis in ruminants. In consequence of similarities in the clinical characteristics and further microbiological evidence, MAP is also discussed to play a role in human Crohne's disease[26], an inflammatory bowel disease. Furthermore, MAH is a dangerous intracellular human

pathogen especially in the absence of normal T cell immunity found in advanced, untreated HIV infected patients. MAH is therefore considered as a major death factor in this patient group[27,28]. Patients with a slightly depressed T-cell immune response, e.g. elderly people and children have a higher risk to develop *M. avium* induced chronic lung diseases[29]. *M. avium* is one of the most important opportunistic pathogens associated with acquired immunodeficiency syndrome (AIDS) as it can be found in up to 70% of patients in advanced stages of AIDS[5].

The treatment of *M. avium* infections is controversial. Not all patients who have mycobacteria cultured from sputum or bronchoalveolar lavage are considered to have the disease. Hence, the decision on treatment should include an observation period of 3 to 12 months to evaluate a disease progression[20]. As the patients respond best to antibiotic treatment the first time it is administered, it is very important that patients initially receive a multi-drug treatment regimen[20,30]. The common applied antibiotic treatment involves a macrolide like clarithromycin or azithromycin, ethambutol and rifampicin. The treatment is very long lasting as the aim is 12 months of negative sputum cultures before stopping therapy[20]. To avoid drug-resistances a good compliance of the patient is crucial.

1.3 Immune recognition of mycobacteria

The susceptibility to mycobacterial infections especially tuberculosis varies due to an inborn variability. This was accidentally proven about one century ago, when in 1930 in Lübeck (Germany) new-born infants got vaccinated with living MTB instead of the BCG vaccine. Only some of the children fall severely ill, while others were completely unaffected[31]. This finding indicates that an effective immune response against MTB is displayed by some individuals and therefore also influences the outcome of the infection. Due to the fact that the adaptive immune system of new-born infants is not fully developed the importance of the innate immune system in anti-mycobacterial host defense was shown as well.

The usual uptake route of mycobacteria is via the inhalation of infected aerosols into the lungs of the host. Therefore, the first encounter of mycobacteria is with alveolar resident macrophages that take up the bacteria from the mucus layer of the lungs.

1.3.1 Pattern recognition receptors

The immune recognition of mycobacteria occurs mainly via the recognition of PAMPs that are recognized by PRRs[32]. The most common PRRs in mycobacterial recognition are toll-like receptors (TLRs), nucleotide-binding oligomerization domain receptors, in short NOD-like receptors (NLRs) and C-type lectins like dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and dectin-1 (Figure 2).

TLRs are a family of PRRs consisting of 12 members in mammals and are expressed either on the surface of the cell membrane or on the membrane of endocytic vesicles of antigen presenting cells (APCs) especially macrophages and DCs. Toll was first identified in *Drosophila* where it was shown to be a key player in antifungal immunity[33]. A homologous family of toll receptors was later found in mammals. TLR family members are expressed differentially among immune cells and respond to different stimuli[34]. Generally, signals initiated by the interaction of TLRs with mycobacterial ligands induce an activation of inflammatory and antimicrobial innate immune responses. Interaction of mycobacteria with TLRs leads to phagocyte activation, but not directly to an immediate ingestion of the mycobacteria. TLRs with established roles in recognition of mycobacteria are TLR2, TLR4, TLR8 and TLR9[35–37]. TLR2 forms heterodimers with either TLR1 or TLR 6 and these heterodimers can recognize bacterial cell wall glycolipids[38]. TLR4 is activated by the heat shock protein 60/65 secreted by a variety of mycobacterial species[39]. Furthermore LPS, mannan and glycoinositolphospholipids are potent TLR4-inducers as well[40]. TLR9 recognizes unmethylated CpG motifs in bacterial DNA[36]. TLR8 recognizes single-stranded RNA from viruses but Davila *et al.* demonstrated upregulation of TLR8 in macrophages after infection with mycobacteria[37]. The underlying mechanism of this recognition is still unclear. Interaction of mycobacterial ligands with TLRs result in the recruitment of specific adaptor molecules such as myeloid differentiation primary response protein 88 (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6). Via a signal transduction cascade nuclear transcription factor (NF)-κB translocates finally in the nucleus and leads to the activation of the innate host defense. As a consequence, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) are secreted[41,42]. In addition, several protein kinases like mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3 kinase and activated protein-1 (AP-1) are also activated by the TLR signaling cascade[43].

NLRs are a receptor family consisting of more than twenty members with a conserved structure. The recognition of PAMPs is thought to occur by the C-terminal part consisting of a series of leucin-rich repeats. Via the N-terminal caspase activation and recruitment domain (CARD), the receptor-interacting protein 2 (RIP2) is recruited and finally NF-κB becomes activated leading to pro-inflammatory cytokine expression[44,45].

C-type lectins recognize polysaccharide structures of pathogens. For the mycobacterial detection the mannose receptor (MR) plays the most important role. This receptor is highly expressed on the surface of alveolar macrophages[46] that are usually the first line defense against mycobacteria taken up by aerosols. Mannosylated lipoarabinomannan (Man-LAM) of the mycobacterial cell wall is usually the recognized ligand for the MR. In addition, the binding of mycobacteria to the MR induces phagocytosis, but only limited phagosome-lysosome fusion[47]. Another C-type lectin is DC-SIGN that is mainly involved in dendritic cell-mycobacteria interactions and the receptor is therefore also mainly found on DCs. DC-SIGN promotes an anti-inflammatory immune response by maturation of infected DCs and induction of IL-10 expression[48]. Dectin-1 belongs to the C-type lectins as well and is mainly found on macrophages, DCs, neutrophils and a subset of T-cells. Dectin-1 was shown to complement TLR signaling for the induction of a pro-inflammatory response[49]. It recognizes β-glucans present in fungal pathogens, but an important role in mycobacterial recognition is also known. The mycobacterial PAMP that is recognized by dectin-1 is still not known. Some mycobacterial species express α-glucan on the cell surface that functions as a ligand for dectin-1[50].

Activation through TLRs, NLRs and dectin-1 initiates essentially a pro-inflammatory response while the C-type lectins MR and DC-SIGN mainly have a modulatory function (Figure 2)[51].

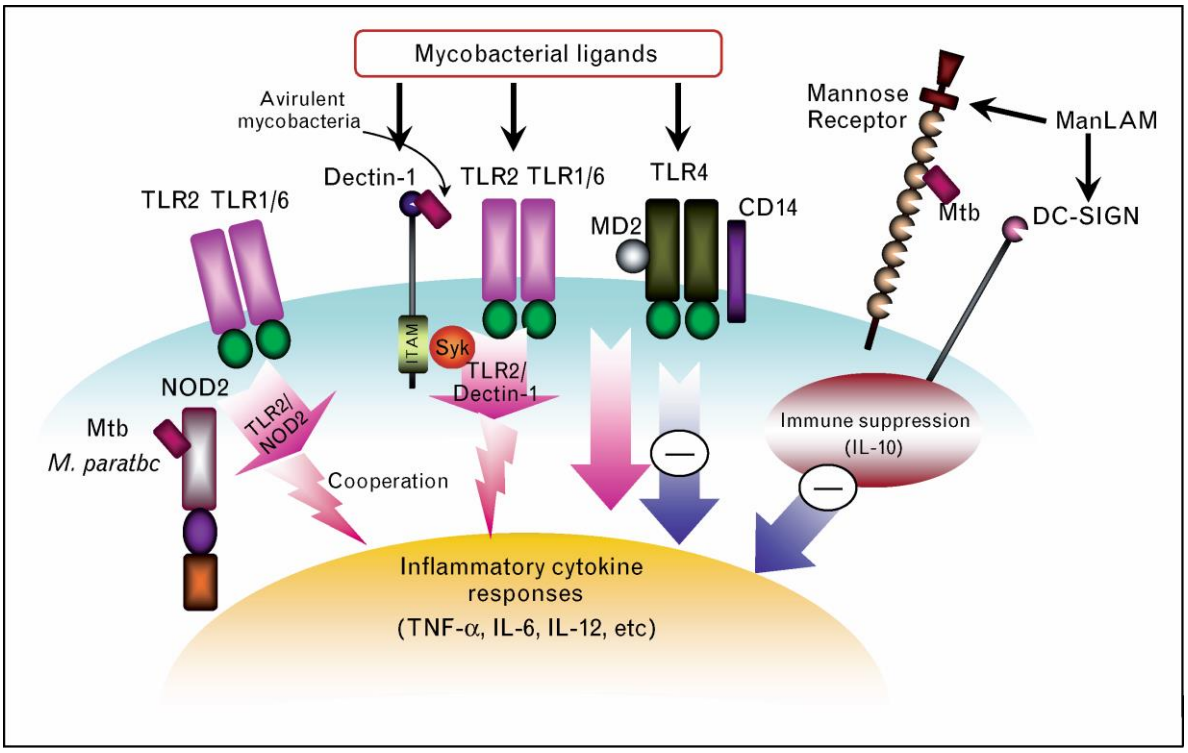


Figure 2: Pattern recognition receptors in the recognition of mycobacteria.
Engagement of macrophage innate receptors by mycobacteria or mycobacterial components initiates a series of intracellular responses, which culminate in the induction of inflammatory cytokine responses. Most TLR-dependent signals initiated by mycobacteria are positive, leading to activation of the inflammatory and antimicrobial innate immune responses. However, several are negative signals that may mediate feedback inhibition to limit macrophage activation and prevent excessive inflammation. Mycobacterial binding to the C-type lectins mannose receptors or DC-SIGN on macrophages principally induces a potent inhibitory effect on cytokine production, although that effect may be modulated by the degree of acylation of the mycobacterial lipoglycans. Dectin-1 also participates in regulation of Mtb-induced inflammatory signals in cooperation with TLR2. The cytosolic PRRs are more recently defined immunosensors. NOD2 is important for host cell cytokine responses to Mtb. It synergizes with TLR2 in mediating cytokine responses, and also serves as a nonredundant recognition receptor for Mtb. TLRs, Toll-like receptors; NOD, nucleotide-binding oligomerization domain; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin; IL, interleukin; Mtb, *Mycobacterium tuberculosis*; M. paratbc, *Mycobacterium paratuberculosis*; ManLAM, mannose-capped lipoarabinomannan; TNF, tumor necrosis factor. Figure taken from Jo[51].

1.3.2 Phagocytosis

Phagocytosis of mycobacteria by macrophages is receptor mediated. During this receptor interaction the macrophage is able to distinguish whether the phagocytized particle is cell debris or a harmful pathogen. Due to the ligation of the receptors by the invading pathogen or cell debris respectively, further action on the cell surface of the macrophage is determined. The combination of receptors that become activated determines if there will be an inflammatory response or not. The range of actions can vary from simple phagocytosis and

degradation of cell debris to the uptake of a pathogen and further activation of antimicrobial mechanisms inducing an immune response[52].

The entrance into the macrophage occurs by internalization after interaction with various surface receptors. The most recognized receptors are complement receptors (CR) 1, 3 and 4 that are needed for the uptake of opsonized bacteria. In addition, fibronectin[53] as well as type A scavenger receptors are well characterized. In conjunction with antibodies an uptake via Fc-γ receptors is possible[54]. MR is known to be involved in phagocytosis by human alveolar macrophages but not by murine macrophages[55,56]. Mycobacteria are interacting with many receptors to be phagocytized and induce mechanisms to defeat intracellular pathogens. Some receptors like CR allow a silent entry into the macrophages without causing a major immune response, while other receptors like Fc-γ force a strong immune response[12].

1.4 Cytokines and chemokines in mycobacterial recognition

1.4.1 Pro- and anti-inflammatory cytokines

Cytokines are proteins that can serve a lot of functions in the mammalian host. The release of cytokines as soluble messengers is fundamental for cell-cell communication and regulation within the immune system. Cytokines can act as growth factors that influence growth and differentiation of cells by induction or control of target cells or as mediators in the immune response. Cytokines can be divided into five main groups called interferons (INFs), interleukins (ILs), colony-stimulating factors (CSFs), tumor necrosis factors (TNFs) and chemokines. Macrophage-derived cytokines play a key role in the control of mycobacterial infections as they are released to activate and recruit other cells during inflammation or work as direct killing agents[57,58]. Cytokines are capable to link cells of the immune system to cells in the surrounding tissues and they can display reparative or destructive signals to other cells in response to stimuli like injury or tumor growth[59,60]. The most cytokines work in autocrine, effect on the same cell, or paracrine, effect on neighboring cells, fashions[61]. The early steps of the cytokine secretory pathways are common to all eukaryotic cells. The newly synthesized protein precursors translated into the endoplasmic reticulum are folded and quality checked. The first glycosylation occurs before the protein precursors are loaded into carrier vesicles for transport to the Golgi apparatus, where post-translational processing and glycosylation

continues[62–64]. After the modifications in the Golgi apparatus the secretory pathways begin to diverge and differ in the routes and carriers used (Figure 3).

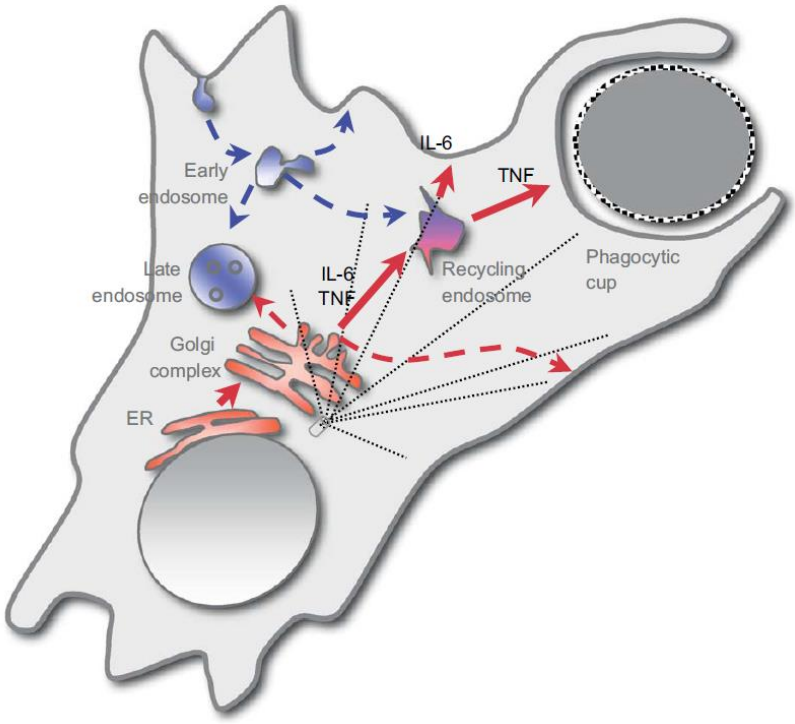


Figure 3: Cytokine secretion and trafficking pathways in macrophages.
The constitutive secretory route tracked by the inflammatory cytokines IL-6 and TNF in macrophages is shown (solid red lines). This pathway diverges at the recycling endosome for separate delivery of cytokines to the cell surface. Dotted lines indicate other major exocytic (red) and endocytic (blue) routes in cells connecting organelles, some of which intersect at the recycling endosome with cytokine trafficking. Figure taken from Stow et al.[62].

Following infection a number of cytokines is released by macrophages. Theses cytokines participate in the host response. TNF-α, IL-6, IL-12, IL-18, transforming growth factor-β (TGF-β) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are well characterized cytokines having a role in mycobacterial infection of the host[65]. These cytokines are needed for recruitment and subsequent control of mycobacterial infections.

1.4.2 TNF-α

TNF-α is a well characterized pro-inflammatory cytokine with multiple roles in mycobacterial infection as TNF-α deficient mice are known to be more susceptible to MTB infection and also display deficiencies in granuloma formation[66]. Macrophages synthesize and secrete TNF-α as an early response cytokine. TNF-α is involved in the induction of apoptosis of alveolar macrophages infected with MTB[67]. Furthermore, it is crucial for macrophage activation as well as granuloma formation. TNF-α triggers antimicrobial mechanisms against *M. avium* by increasing bactericidal proteins and enhancement of superoxide anion production[68]. Likewise, studies have shown that TNF-α induces changes in the contents of the mycobacterial phagosome leading to an impairment of the bacterial replication[69]. TNF-α in combination with INF-γ induces anti-mycobacterial activity in murine macrophages by the induction of reactive oxygen species[70,71]. In addition, TNF-α recruits monocytes and circulating antigen specific T-cells to the site of infection and influence the expression of chemokine receptors such as C-C motif receptor 2 (CCR2) as receptor for monocyte chemotactic protein-1 (MCP-1), CCR5 as receptor for RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and macrophage inflammatory proteins-1α/β (MIP-1α/β)[72–74]. Nevertheless, TNF-α can also have negative effects during mycobacterial diseases as it can cause severe tissue damage[75]. It is released in response to mycobacterial LAM, LPS, other bacterial products and IL-1[76].

TNF-α is predominantly produced by macrophages, but many other cells like dendritic cells or mast cells are able to produce it as well. The cytokine is mainly expressed as a transmembrane protein, arranged in stable homotrimers[77]. TNF-α is ultimately delivered to the cell surface, where it is rapidly cleaved by TNF converting enzyme to release its ectodomain as a soluble cytokine[78]. TNF-α production in macrophages infected with *M. avium* is dependent on MyD88 and TLR2 but not TLR4, MR, or CR3[56]. LAM and muramyl dipeptide but not arabinomannan from the mycobacterial cell wall are able to induce TNF-α and IL-6 secretion in macrophages.[79]

TNF-α can amplify its own production indirectly by enhancing gene expression of the pathogen in newly infected macrophages[80] and creates therefore a positive feedback loop for TNF-α production. In the same way the anti-inflammatory cytokines like TGF-β are capable to inhibit TNF-α secretion to ensure a negative feedback loop and to inhibit excessive inflammation[80].

1.4.3 IL-6

IL-6 is a pleiotropic mainly pro-inflammatory cytokine initiated in early inflammatory responses. It is produced for some time alongside with TNF-α. An increased production is found in many human chronic inflammatory diseases. IL-6 deficient mice developing rapidly lethal tuberculosis after infection with MTB due to altered cytokine secretion[81]. Established functions of IL-6 in mycobacterial infections are the induction of cytotoxic T-cells[82], inhibition of TNF-α receptor expression[83] and a block of antigen presentation by infected macrophages[84]. Furthermore it is essential for the generation of a protective Th1-immune response after vaccination with a tuberculosis subunit vaccine[85] and IL-6 suppresses the macrophage response to INF-γ[86]. Hence, IL-6 acts predominantly negatively in mycobacterial infections as the suppression of pro-inflammatory cytokines like TNF-α can lower the ability of intracellular killing and the development of granulomas. Mycobacteria upregulate the hosts IL-6 expression for the inhibition of type I interferon signaling[87]. IL-6 depletion exacerbates *M. avium* infection[82]. Nevertheless, in studies in mice using MTB it was shown that IL-6 is required for protective immune responses after infection with high doses of MTB administered intravenously[81], but it is rather dispensable for control of mycobacterial growth after low dose aerosolic infection[88]. Therefore, IL-6 appears to contribute to protective immune responses during mycobacterial infections, but it is not fundamental in containment of mycobacterial infections.

IL-6 acts via a receptor complex consisting of a specific IL-6 receptor (IL-6R) and the signal-transduction protein gp130[89]. The classical IL-6 signaling pathway is initiated by binding of IL-6 to membrane-bound IL-6R. Via a signal transduction cascade including the JAK (Janus kinase)-STAT (Signal Transducer and Activator of Transcription) pathway or via MAPK IL-6 responsive elements become finally activated and further gene expression is induced[89] (Figure 4).

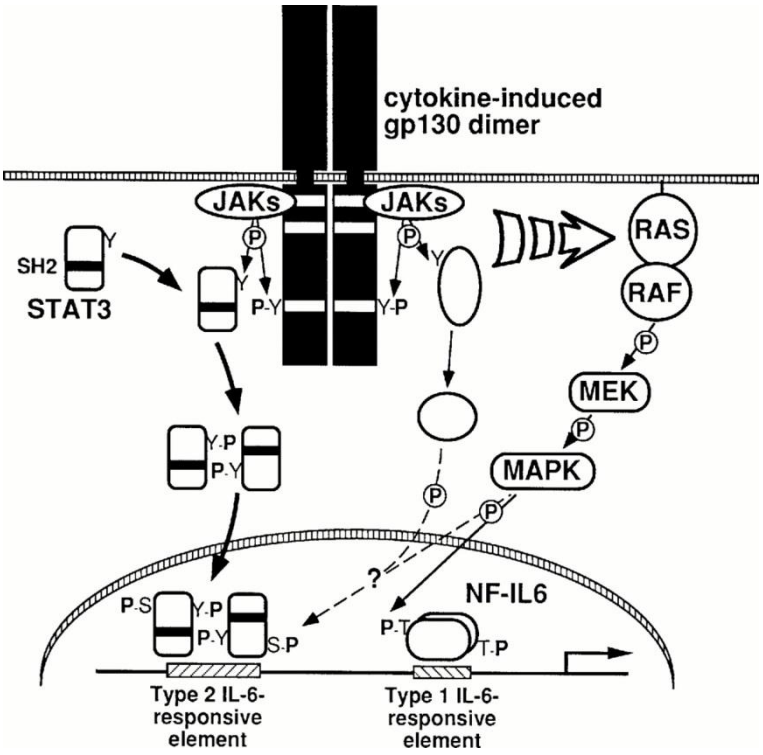


Figure 4: Signal transduction following gp130-dimerization.
Following dimerization of gp130 by cytokines, cytoplasmic protein tyrosine kinases in the JAK family (JAK1, JAK2, and TYK2; "JAKs" in this figure) that are already associated with the membrane-proximal portion of gp130 at box 1 become activated. Tyrosine residues in the cytoplasmic region of gp130 are then phosphorylated. A cytoplasmic latent transcription factor, STAT3, is recruited to a phosphotyrosine-containing sequence in box 3 and other C-terminal part of gp130 via its SH2 domain and is tyrosine-phosphorylated by the juxtaposed JAKs. Tyrosine-phosphorylated STAT3 proteins then form homodimers via intermolecular SH2-phosphotyrosine interactions and are translocated to the nucleus. Serine-phosphorylation on STAT3 protein by a thus-far-unidentified kinase is important for its full activation. Another type of transcription factor, NF-IL6, is activated after gp130-stimulation through threonine phosphorylation by MAPK. Blank ovals represent an unidentified serine/threonine kinase pathway component. Figure taken from Taga and Kishimoto[89].

IL-6 secretion is mediated through mycobacterial interaction with PRRs. Mycobacterial LAM is a potent inducer of the IL-6 secretion pathway as it is known to induce NF-IL6 and NF-κB that are enhancing the IL-6 gene expression and therefore the IL-6 secretion[90]. Receptors that can be induced for IL-6 secretion are dectin-1 and TLR4. TLR2 seems to be less important in the IL-6 secretion since TLR2 agonists were shown to poorly induce IL-6 secretion[47,91]. TNF-α in comparison shows a higher dependency on TLR2 induction[91]. However, TLR4 is not involved in TNF-α secretion due to *M. avium* infection[56].

1.5 Manipulation of the host immune system by *Mycobacterium avium*

1.5.1 Entering the host

M. avium is capable to manipulate its host's immune response to the infection. The usual uptake routes are either via the respiratory tract causing lung infections slowly disseminating to other organs, or via the intestinal epithelium causing rapidly disseminating infections[92,93]. Upon entering the macrophage by phagocytosis *M. avium* resides and replicates within the phagosomes which are organelles in the macrophage for the phagocytic destruction of taken up particles. Mycobacterial phagosomes are only capable to fuse with early endosomes, but fail to fuse with late endosomes and lysosomes[94,95]. Due to the lack of the proton ATPase the phagosomes also fail to acidify[96]. The missing phagolysosome maturation leads to a missing antibacterial environment in the phagosome and mycobacterial intracellular survival. The mechanism for this block of phagosome maturation is still not completely understood, but several studies indicate a failure in the recruitment of effector molecules important in phagosomal biogenesis[97]. 3-phosphoinositides and Rab5 effector EEA1 (early endosome autoantigen 1) are essential molecules for phagosomal maturation. Mycobacterial phagosomes exclude this regulators of vesicular trafficking within the endosomal pathway and therefore also inhibit phagosomal maturation[97].

1.5.2 Manipulation of the host response

M. avium is triggering anti-inflammatory cytokines and inhibiting essential pro-inflammatory cytokines and immune functions. Therefore, bacterial growth and reproduction inside host macrophages is possible. This is achieved by different mechanisms. The bacteria are either able to inhibit macrophage activation, or resist bactericidal mechanisms, or use a combination of both ways[98]. The lifestyle of *M. avium* in the host is similar to other pathogenic mycobacteria like *M. tuberculosis*.

The reduced induction of pro-inflammatory cytokines like TNF-α is a key mechanism in the avoidance of a strong immune response by *M. avium*[99,100]. The bacteria also interfere with responses to INF-γ and resist antimicrobial agents that damage the bacterial cell envelope as well as counters toxic reactive oxygen and nitrogen intermediates[101,102]. The stress response

factor SigH is known as an important factor in the suppression of macrophage activation and pro-inflammatory responses[103].

M. avium interferes with a lot of intracellular signaling pathways and is able to suppress them (Figure 5). Pro-inflammatory cytokines, such as TNF-α, IFNs and IL-6, induce an innate immune response when invading mycobacteria are detected. Especially the host signaling pathways such as MAPK and JAK-STAT result in the production of pro-inflammatory cytokines. Pathogenic mycobacteria have therefore evolved mechanisms to suppress these signal transduction cascades and thereby, manipulate the cytokine-induced immune response.

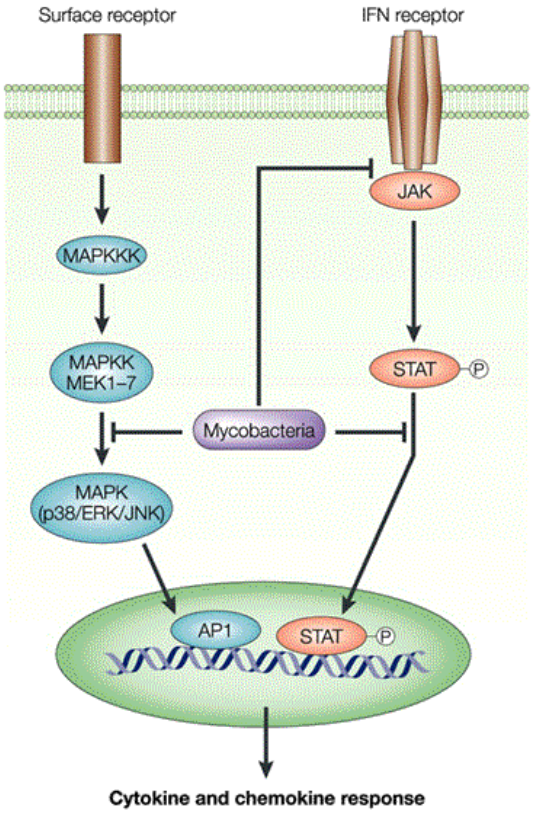


Figure 5: Disruption of macrophage signaling pathways by mycobacteria.
Mitogen-activated protein kinase (MAPK) signaling pathways are activated by stimuli such as pathogen entry, cytokines and growth factors, which lead to a cascade of kinase activity that ultimately results in the activation of MAPKs — for example, p38, extracellular signal-related kinases (ERKs) and Jun N-terminal kinases (JNKs). Activated MAPKs phosphorylate substrates such as transcription factors — for example, activator protein 1 (AP1) and nuclear factor (NF)-κB — which leads to the production of inflammatory mediators like tumor-necrosis factor-alpha (TNF-alpha) and interleukin (IL)-1. Pathogenic mycobacteria suppress this host response by inhibiting the activation of p38 and ERK1/2. The binding of INF-gamma to its receptor leads to the recruitment of Janus kinases (JAKs), which bind to the intracellular domain of the receptor, leading to its tyrosine phosphorylation and subsequent association with the signal transducer and activator of transcription (STAT) protein. Phosphorylated STAT is then translocated to the nucleus, where it activates the transcription of interferon (IFN)-gamma target genes, leading to a potent anti-bacterial response. Pathogenic *Mycobacterium avium* interferes with the JAK/STAT signaling pathways by downregulating the expression of the IFN-gamma receptor, whereas *M. tuberculosis* affects the DNA-binding activity of STAT1, which leads to reduced transcription of IFN-gamma-responsive genes. MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MEK, MAPK/ERK kinase. Figure taken from Koul et al.[104]

With all these mechanisms *M. avium* has evolved effective immune evasion strategies and it is able to elude from several immune killing strategies like block of phagosomal maturation, apoptosis or autophagy.

2. Aim of study

The aim of this study was to characterize phenotypic, genetic and immunostimulatory properties of clinical *Mycobacterium avium* complex strains isolated from patients at the St. Olav's hospital, Trondheim, Norway. Furthermore, a pulsed-field gel electrophoresis to epidemiologically characterize the bacterial isolates was established. Specifically, we wanted to test these hypotheses:

1. Patients are infected with the same mycobacterial strain during the entire infection, or get infected with new strains.

2. *M. avium* strains from different patients differ in their growth properties, but strains from the same patient grow fairly similar.

3. The intra-macrophage survival characterizes the ability of the strains to evade the detection by the host immune system and should increase over time of infection.

4. During long-term infection the mycobacterial strains differ in their immunostimulatory properties and the pro-inflammatory cytokine secretion is expected to decrease over time of infection due to better immune evasion strategies established by mycobacteria in long term infections.

3. Materials and Methods

3.1 Chemicals and Reagents

Dulbecco's Phosphate buffered saline (PBS)	Sigma Aldrich
Tween 80	Sigma Aldrich
β –mercaptoethanol	Sigma Aldrich
Albumin, from bovine serum (BSA)	Sigma Aldrich
RPMI 1640	Sigma Aldrich
EDTA	Sigma Aldrich
HEPES	Roth
Glycerol 85%	Merck
Fetal calf serum (FCS)	Gibco
SnaBI	New England Biolabs
NEBuffer 4	New England Biolabs
BSA 100x	New England Biolabs

3.2 Buffers and media

Table 1: Buffers and media used in the experiments

Media/ buffer	Composition
Difco™ Middlebrook 7H9 Broth per 900ml	0.5g Ammonium Sulfate, 0.5g L-Glutamic Acid, 0.1g Sodium Citrate, 1mg Pyridoxine, 0.5mg Biotin, 2.5g Disodium Phosphate, 1g Monopotassium Phosphate, 0.04g Ferric Ammonium Citrate, 0.05g Magnesium Sulfate, 0.5mgCalcium Chloride, 1mg Zinc Sulfate, 1mg Copper Sulfate, 2ml Glycerol
BBL™ Middlebrook ADC Enrichment per liter	8.5g Sodium Chloride, 50 g Bovine Albumin (Fraction V), 20g Dextrose, 0.03g Catalase
BBL™ Prepared Plated Media for the Isolation of Mycobacteria Middlebrook 7H10 per liter	0.05g Magnesium Sulfate, 0.04g Ferric Ammonium, 0.4g Sodium Citrate, 0.5g Ammonium Sulfate, 0.5g Monosodium Glutamate, 1.5g Disodium Phosphate, 1.5g Monopotassium Phosphate, 13.5g Agar, 1mg Pyridoxine, 1mg Zinc, 1mg Copper Sulfate, 0,5mg Biotin, 0.5mg Calcium Chloride, 0.25mg Malachite Green, 100ml OADC Enrichment, 5ml Glycerol
OADC Enrichment per liter	8.5g Sodium Chloride, 50g ,Bovine Albumin (Fraction V), 20g Dextrose, 0.03g Catalase, 0.6ml Oleic Acid

Spheroplasting buffer	50mM EDTA, 29mM sodium hydrogen phosphate, 8.4mM citric acid, 0.1%(w/v) Tween 80
TE buffer	10 mM Tris and 1 mM EDTA; pH 7.5
ES buffer	0.5 M EDTA, 1% (w/v) lauryl sarcosine; pH 8.0
TBE buffer	44.5mM Tris, 44.5mM boric acid, 1mM EDTA

3.3 Kits

The kits used in the study and their respective manufacturers are listed with in Table 2.

Table 2: Kits used with respective manufacturer

Kit	Manufacturer
Mouse TNF-α and IL-6 ELISA DouSet	DuoSet® ELISA development kits R&D Systems
Bio-Plex Pro™ 10-plex and 4-plex	Bio Rad
RNeasy Mini Kit	QIAGEN
RNA to cDNA kit	Invitrogen
TaqMan® Gene Expression Assays	Applied Biosystems
TaqMan® Fast Advanced Master Mix	Quanta BioSciences

3.4 Pulsed-field gel electrophoresis (PFGE) for *Mycobacterium avium*

3.4.1 Preparation of bacterial plugs

The protocol was taken from Stevenson et al[105] and modified. DNA for PFGE analysis was prepared from single colonies of mycobacterial samples grown on 7H10 agar plates with oleic albumin dextrose catalase (OADC) enrichment. The colonies were transferred in 7H9 broth with albumin dextrose catalase (ADC) enrichment and 0.02% Tween 80. The strains were grown at 37°C to an optical density (OD) at 600nm of approximately 0.5 which correlates to a McFarland standard of 2[106] used in the original protocol[105]. This usually took between 12 and 14 days and the colonies were shaken once every 24h.

The whole bacterial solution was centrifuged at 2,500g for 25min at room temperature (RT) and afterwards washed with 5ml spheroplasting buffer. After another centrifugation under the conditions mentioned before, bacteria were resuspended in 1ml spheroplasting buffer.

After pre-warming the bacterial suspension and 1.5% (wt/vol) low-melting-point agarose to 55°C, equal volumes (500µl) of bacterial suspension and 1.5% (wt/vol) low-melting-point agarose were mixed and approximately 90µl of the suspension were poured into plug molds. The plugs were left to solidify for 20min.

3.4.2 Lysis of bacteria and DNA digestion

The plugs were incubated in 3ml Tris-EDTA (TE) buffer containing 20mg/ml lysozyme for at least 18h at 37°C and afterwards in 5ml ES buffer and 20mg/ml proteinase K for 3 days at 55°C. Afterwards, the plugs were washed four times with 3ml TE buffer for at least one hour each time. The plugs were stored in the fridge at 4°C until further use.

An approx. 1-2mm thick gel slice was cut from the plug for restriction endonuclease treatment. The DNA digestion was done with restriction enzyme SnaBI. The digestion mix contained 20U of the restriction enzyme and the supplied restriction buffer NEBuffer4 and bovine serum albumin (BSA). DNA digestion was done for 3h at 37°C with shaking (300rpm) on a heating block. After DNA digestion the gel slices were loaded onto a 1% (wt/vol) agarose gel and electrophoresed in 0.5x Tris/Borate/EDTA (TBE) buffer.

3.4.3 Gel electrophoresis

Electrophoresis was conducted using a Bio-Rad CHEF Mapper XA and the running conditions were as follows. For SnaBI restriction fragments the running time was 22h with an initial switch time of 1s and a final switch time of 23s, ramped linearly. Also a gradient of 6V cm^{-1} was applied and the included angle was 120°. The electrophoresis was carried out at 14°C. A lambda midrange marker was always loaded onto the gel as a molecular size standard.

The staining of the gel was finally done with GelRed™ for 30min followed by a destaining in reverse osmosis (RO)-water for at least 1h with a change of RO-water after 30min.

3.5 Cell culture of RAW 264.7 macrophages

The RAW 264.7 cell line was established about 30 years ago from a tumor in a BAB/14 mouse induced by the Abelson murine leukemia virus[107]. BAB/14 is a BALB/c IgH congenic strain that was inoculated with Abelson murine leukemia virus. This virus belongs to the class of retroviruses and it contains the v-abl tyrosine kinase oncogene. It is commonly used together with replication-competent Moloney murine leukemia virus (Mo-MuLV) that serves as a helper virus.

The RAW 264.7 macrophages were cultured in RPMI 1640 with addition of L-glutamine, 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 10% fetal calf serum (FCS). The culture conditions used were 37°C and 5% CO_2 in the atmosphere.

The cells were split every 3 to 4 days according to the density of growth to prevent them of reaching confluence as this might change their characteristics. For the splitting the cells were detached with 5ml EDTA (Ethylenediaminetetraacetic acid) diluted in PBS incubated for 5min at 37°C. Afterwards, the cells were detached mechanically and spun down for 6min at 1500rpm. The cells were resuspended in 1ml culture media and 20µl were transferred to a new 75cm^2 culture flask containing 12ml fresh culture medium.

The cell line was usually kept for circa 15 passages before a new batch was taken up from the liquid nitrogen storage.

3.6 Culture of *M. avium* complex

The *M. avium* strains were cultured either on 7H10 agar plates with OADC enrichment or in liquid 7H9 with ADC enrichment. The bacteria were always incubated at 37°C in a bacterial incubator. The cultures in 7H9 agar were shaken once every 24h to avoid clumping. If necessary, the cultures were diluted in fresh 7H9 agar to prevent reaching the stationary phase. Tween 80 was added at a concentration of 0.02% to the 7H9 for the growth curve measurement and, if necessary for growth before infection experiments. If Tween 80 was added to the cultures for infection, an additional washing step was carried out to remove the Tween 80 before infection of macrophages.

3.7 Preparation of infection aliquots

3.7.1 Growth of bacterial cultures

The patient samples for the experiments were provided by the Department of Medical Microbiology at St. Olav's Hospital. The samples were collected over the last 10 years and stored at -80°C. All the samples were identified as *Mycobacterium avium* complex but the subspecies have not been further characterized.

The mycobacterial samples were first streaked out on 7H10 agar plates with OADC enrichment and grown at 37°C until single colonies could be picked. This usually took around seven days. Afterwards, a single colony was transferred into approx. 6ml of 7H9 agar with ODC enrichment and 0.02% Tween 80. The mycobacteria were incubated at 37°C and shaken once every 24h. The mycobacteria were grown to an OD_{600} of approx. 0.5. To avoid any influence of Tween 80 on the experiments 100µl of the bacterial suspensions were transferred into 6ml fresh 7H9 media without Tween 80 and grown as mentioned before for circa five days to reach an OD_{600} of 0.5 again.

3.7.2 Freezing of infection aliquots

Before freezing the bacteria were spun down at 3,000rpm for 10min and resuspended in 500µl PBS mixed with approx. 500µl of glass beads (0.5mm diameter) to disrupt potential bacterial clumps. 5ml of PBS was added and the suspension was centrifuged at 200g for 10min to spin down the glass beads. The supernatant was pipetted out to avoid glass beads in the suspension.

The aimed OD_{600} for freezing down was approx. 0.2 and the bacterial solutions were diluted with PBS as needed. An amount of glycerol equal to a final concentration of 5% was added and aliquots of 400µl were finally frozen down in cryo tubes at -80°C. An overview about the strains used in the experiments and how they were grown is shown in Table 3.

Table 3: Overview of the mycobacterial strains achieved from the patient isolates.
Numbers for the strains were assigned by patient ID (first number) and order of isolate collection (second number). MAC 11 and MAC 104 are reference strains established in the laboratory.

Patient	MAC - Strain Nr.	Date Received	Cultured without Tween 80	Cultured with Tween 80
1	1-1	22.03.2006	x	x
1	1-2	01.06.2007	x	x
2	2-1	29.12.2008	x	x
2	2-2	09.02.2009	x	x
3	3-1	26.01.2010	x	x
3	3-2	05.08.2010		x
3	3-3	26.10.2010		
4	4-1	11.03.2009		x
4	4-2	07.09.2009		x
4	4-3	01.07.2010		x
5	5-1	23.05.2003	x	
5	5-2	05.01.2005	x	
6	6-1	23.03.2007		
6	6-2	16.04.2008		
6	6-3	28.12.2008		
7	7-1	07.12.2005	x	
7	7-2	25.05.2007	x	
8	8-1	22.05.2008	x	
8	8-2	26.05.2008	x	
9	9-1	07.04.2005	x	
9	9-2	12.08.2005	x	
9	9-3	09.12.2005	x	
9	9-4	05.05.2006	x	
9	9-5	21.02.2007	x	
9	9-6	10.10.2007	x	
10	10-1	25.04.2005	x	
10	10-2	11.06.2007	x	
10	10-3	21.09.2007	x	
11	11-1	07.07.2009	x	
11	11-2	22.09.2010	x	
12	12-1	09.04.2006	x	
12	12-2	28.02.2007	x	
13	13-1	20.12.2006	x	
13	13-2	05.02.2007	x	
13	13-3	14.02.2008	x	
13	13-4	25.02.2009	x	
14	14-1	10.08.2007	x	
14	14-2	19.12.2008		
15	15-1	19.01.2006	x	
15	15-2	19.12.2006	x	
X	11	X	x	
X	104	X	x	

3.8 Infection of RAW 264.7 macrophages with *M. avium* strains

3.8.1 Seeding of RAW 264.7 macrophages

The RAW 264.7 macrophages were seeded in flat bottom 96 or 24 well culture plates depending on the kind of planned experiment. For harvesting of ELISA supernatants usually 96 well plates were used while the generation of cell lysates for qRT-PCR was done in 24 well plates due to the higher number of cells needed. Approximately 50,000 cells in 100µl per well in a 96 well plate respectively appoximately 200,000 cells in 400µl per well in a 24 well plate were used. The macrophages were seeded 24h prior to the infection to let them recover and adhere to the culture dish.

3.8.2 Bacterial infection of RAW 264.7 macrophages

The next day the bacterial strains were thawed and diluted in culture media as necessary for an infection ratio of 1:50. Afterwards, the bacteria were added in equal volumes to the prior seeded macrophages and incubated for 4h. After 4h the medium was exchanged to remove non internalized bacteria. The ELISA supernatants were harvested 8h and 24h past infection (p.inf.), while supernatants for qRT-PCR were harvested 4h, 6h, 8h and 24h p. inf..

3.9 Bacterial viability and survival plating

To measure the bacterial viability as well as the intracellular survival at different time points during the infection, a plating assay was carried out. For the viability test the bacteria from the dewed infection aliquot were directly used and diluted, while for the survival plating the macrophages were lysed for 30min with distilled water. The bacterial dilutions were plated on 7H10 agar plates in 5µl droplets and triplicates for each of the used dilutions. For the calculation of the results the dilution with countable single colonies were used and the mean of the triplets were the basis for the calculations.

3.10 Bacterial growth curve analysis

For the bacterial growth curve measurement the strains were first grown again on 7H10 agar plates and after seven days a single colony was transferred into 7H9 agar with addition of 0.02% Tween 80. The strains were grown to an optical density of 0.3-0.5 and then diluted to a theoretical OD_{600} of 0.001 to achieve similar starting conditions for all strains.

The used volume in the 100 well honeycomb plates was 200µl and every strain was run in triplicates. The growth analyzer was set up to 37°C and to constantly shake the plate. An OD_{600} measurement was scheduled every 8 hours and the strains were grown for 8 days. For the calculation of the graphs the mean and the standard deviation out of the triplicates were calculated.

3.11 Multiplex immunoassay

3.11.1 Principle

The multiplex immunoassay was carried out with the xMAP technology from Bio Rad. The big advantage compared to the traditional ELISA technique is that it is not limited by the ability to measure only one single antigen at a time. Unlike with traditional ELISA, xMAP capture antibodies are covalently attached to a bead surface. This is effectively allowing a greater surface area to react with the analytes. The assay procedure is similar to a sandwich ELISA.

3.11.2 Example protocol

The assay was carried out in accordance to the supplier's instructions. As first step the capture antibodies were diluted 1:13, 50µl were dispensed on the plate and washed two times with 100µl wash buffer. The next step was the addition of 50µl of each sample to the beads and incubation in the dark for 45min at RT with shaking at 300rpm. After 3 times washing the plate 25µl of the detection antibody was added and incubated for 25min as mentioned before. Another 3 times of washing was followed by the addition of 50µl strepatividin-phycoerythrin conjugate (SA-PE) and incubation for 10min. Finally, the plate was washed again 3 times and

the beads were resuspended in Bio-Plex assay buffer and shaken at 1,100rpm for 30sec before reading of the plate. The beads were passed through the array reader, which measured the fluorescence of the bound SA-PE.

3.12 Enzyme-linked immunosorbent assay (ELISA)

3.12.1 Principle

The ELISAs were always made according to the recommendation of the supplier. The procedure is a sandwich ELISA where it is possible to determine an antigen concentration by binding of the antigen on a known amount of capture antibody. The development of the ELISA is done by the help of a detection antibody on which streptavidin conjugated to horseradish-peroxidase (HRP) is bound. Therefore, a color reaction is initiated after addition of tetramethylbenzidine and H_2O_2. The intensity of the color can be quantitatively measured at 450nm. The samples analyzed for IL-6 were used undiluted while the samples for TNF-α were diluted 1:2 volume units.

3.12.2 Example protocol

The 96-well half volume plate was incubated with 50µl capture antibody dilution according to the supplier's recommendation and incubated at 4°C overnight. The plate was then washed once with 150µl per well with wash buffer consisting of 0.05% Tween 20 in PBS. 150µl blocking buffer containing 1% BSA and casein dissolved in PBS was added for one hour and the plate was washed again once with the wash buffer. After that 50µl of the samples and the standard was added and incubated for 2h. The plate was washed three times and 50µl detection antibody was added as recommended by the supplier. After a washing step (3 times) SA-HRP was added for 20 minutes and the plate was washed again 3 times. 50µl substrate solution containing equal amounts of tetramethylbenzidine and H_2O_2 was added and incubated for 20min. The last step was the addition of 50µl 2N H_2SO_4.

3.12.3 Data analysis

The plate was read at a wavelength of 450nm and a second measurement at 570nm was made and subtracted from the 450nm measurement to minimize the influencing effect of liquid reflections. The data was analyzed with the MPM microplate manager software and a 4PL standard curve fit function was used for the calculation of the sample concentrations from the standard curve.

3.13 Quantitative real-time-PCR (qRT-PCR)

3.13.1 Principle

Quantitative real-time PCR is a PCR method where samples got illuminated in a thermal cycler by a beam of light of a specified wavelength. The emitted fluorescence by the excited flourochrome is measured by detectors integrated in the thermal cycler. The TaqMan probe principle relies on the 5´–3´ exonuclease activity of a Taq polymerase, a thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus*, to cleave a dual-labeled probe during hybridization to the complementary target sequence. Afterwards, a fluorophore-based detection allows a quantitative measurement of the accumulation of the product during exponential stages of the PCR.

The PCR process generally consists of 25-40 cycles depending on the template DNA and is carried out in different stages with varying temperatures. The first step is the incubation at 95°C for the separation of the DNA double helix and the second step is at 50-60°C depending on the used primers to allow annealing of the primers on the single strand DNA template.

3.13.2 Example protocol

The infected macrophages (see 3.8.2 Bacterial infection of RAW264.7 macrophages) were lysed with commercial RLT buffer and stored at -80°C until further use. The DNA extraction was done using the Qiagen QIAcube and the RNeasy mini kit according to the supplier’s instructions. The extracted RNA was measured by NanoDrop to determine the concentration and purity of the extracted RNA. Subsequently, the RNA was transcribed to cDNA using the

RNA-to-cDNA kit from Invitrogen in a thermal cycler at 37°C for 60 minutes. The cDNA was diluted to a final concentration of 5ng/µl and an amount of 10ng was used for the qRT-PCR.

TaqMan® Fast Advanced Master Mix and TaqMan® Gene Expression Assays were mixed in low tube strips and 11 µl of the mix was pipetted to each well in a MicroAmp® Fast Optical 96-well reaction plate, before the addition of water and cDNA template was done. The plate was sealed and centrifuged at 1,500rpm for 2 minutes to eliminate air bubbles, and then loaded onto the thermal cycler. The samples were processed using the quantitative ΔΔCT program at the following settings: 45 ºC for 2 minutes, 95 ºC for 30 seconds and 40 cycles of 95 ºC for 1 second and 60 ºC for 20 seconds.

Table 4: TaqMan® Gene Expression Assays

Gene	Assay ID
TNF-α	Mm00443258_m1
IL-6	Mm00446190_m1
GAPDH endogenous control	Mm99999915_g1

4. Results

4.1 Tween 80 can be a growth influencing factor

To ensure a reproducible and comparable infection of macrophages, the strains for bacterial infection experiments were grown from single colonies and frozen in aliquots to be ready to use for infection experiments. The samples obtained from the Department of Microbiology were usually taken from sputum or bronchoalveloar lavage. The protocol for the generation of infection isolates and an overview of the bacterial strains can be found in Materials and Methods p. 23-24.

To evaluate the difference in the immune response towards two bacterial isolates it is important that the growth of the infectious inoculum is identical. Mycobacteria are often grown in 7H9 medium with Tween 80 as a detergent to avoid clumping. Ideally we would avoid using Tween 80 in the growth medium because of detrimental effects of the detergent on macrophages. During the generation of the infection isolates the first difference that could be noticed was that the ability to grow without detergent varied between the strains from the different patients and also from strains from the same patient (Table 5). Some strains were impossible to grow without the addition of Tween 80, since they started to clump after some days in 7H9 media without Tween 80 supplementation. When bacteria clump it is difficult to use them for infection experiments as the number of bacteria for an infection cannot be defined and reproducible results would therefore not be possible. To prevent a possible influence of Tween 80 in experiments with strains that would require Tween 80, the infections were mainly done with strains that were cultured without Tween 80 supplementation. Only in some of the ELISA experiments the strains grown with Tween 80 were included to give a complete overview. To minimize the effects of Tween 80 in these experiments the bacterial strains were washed more extensively to remove the Tween.

Table 5: Dependence of Tween 80 supplementation on the growth of bacterial isolates sorted by patient ID
If only the patient ID is mentioned, all bacterial isolates from the patient grow under the same conditions.

	Growth without Tween 80 supplementation	**Growth only with Tween 80 supplementation**
Patient ID	1,2,5,7,8,9,10,11,12,13,15 3 (only strain MAC 3-1) 14 (only strain MAC 14-1)	4,6 3 (only strains MAC 3-2 and MAC 3-3) 14 (only strain MAC 14-2)

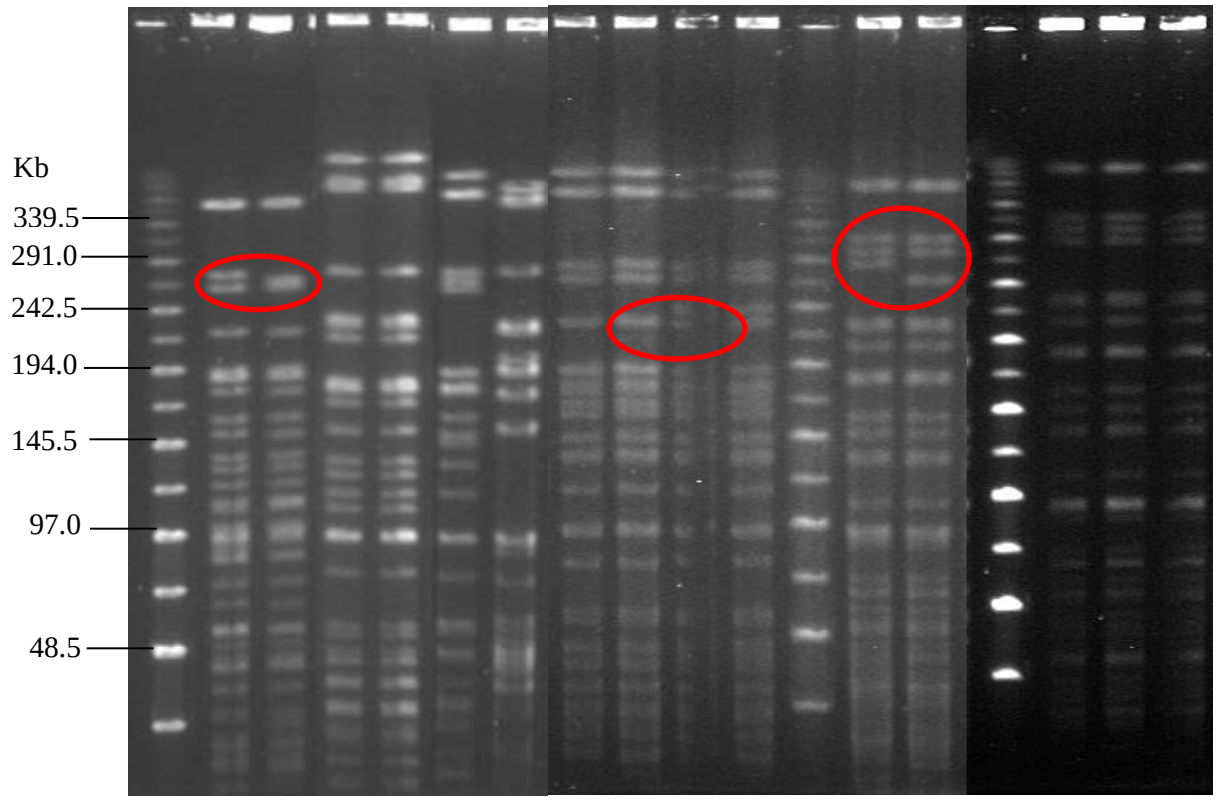
The growth characteristics, depending on Tween 80 supplementation, seemed usually similar within each patient, as for patients 4 and 6 it was generally not possible to grow them without addition of Tween 80. Only in Patient 3 and 14 the growth characteristics concerning usage of Tween 80 were varying even in between the same patient. Hence, a further analysis of these strains in the PFGE could show if the patients were infected with different strains or, alternatively, if infected by the same strain, if any major changes in the genome may have occurred over time of infection.

4.2 The strains can mutate in the host over time of infection

Our main hypothesis was that the isolates are different in between patients and that strains isolated from the same patient either are the same strain over time or patients get repeatedly infected with new strains. To characterize the genetic background of the bacterial isolates from patients, a PFGE was performed.

Generally, the PFGE enabled us to group the strains into three different categories. The most frequently occurring one was where exact the same pattern appeared in all isolates from a patient indicating that it is clonally related. In the second category single band differences between bacterial isolates collected from the same patient occurred, while in the third category completely different band patterns were observed for the analyzed strains. The different patterns found in the PFGE analysis are exemplified in Figure 6.

The strains MAC 8-1 and MAC 8-2 derived from patient 8 looked identical in their DNA pattern. Thus, no major changes in the DNA of the bacterial strain seemed to have happened in the time between the samples were taken. Nevertheless, minor changes that are not detectable by the use of the PFGE can still be possible and could be detected by further sequencing analysis. Strains MAC 5-1 and MAC 5-2 show a clear band difference at approx. 260kb. Single band differences are usually a clear sign that the infection is still caused by the same bacterial strain. However, the genetic mutation that occurred could strongly influence the characteristics of the strain. Patient 11 seemed to be infected with two different *M. avium* strains as the band pattern shows a substantial variation between the first and the second isolate. The time period between sampling was 14 months and such a great variation in such a short time frame is a clear sign that it cannot be the same mycobacterial strain. An overview of the PFGE profiles of all strains can be found in Appendix I.



MAC strain	M	5-1	5-2	8-1	8-2	11 -1	11 -2	13 -1	13 -2	13 -3	13 -4	M	14 -1	14-2	M	3-1	3-2	3-3
Patient		5		8		11		13					14			3		

Figure 6: Overview of different DNA patterns
Representation of different patterns found in the PFGE analysis with single band difference (labeled with red circle) for the isolates of patient 5, no difference for the isolates of patient 8 and two different strains for patient 11.The strains from patient 3 displayed no band difference between the isolates, while the strains of patient 14 showed a clear band difference as well as the isolates from patient 13 between the strains MAC 13-2 and MAC 13-3 (labeled with red circles); M – Marker.

The PFGE profiles of all isolates from patients 13, 14 and 3 are shown because of the growth characteristics that were observed during the cultivation of the bacterial strain. For the isolates of patient 3 no visible band differences could be found in the PFGE patterns. The different growth characteristics in connection to the usage of Tween 80 under growth were not correlated to any changes observed in PFGE patterns. For strains MAC 14-1 and MAC 14-2 from patient 14 a single band difference at approx. 270 kb was observed (Figure 6). These strains also showed differences in their ability to grow without the supplementation of Tween 80. The PFGE indicates that these two strains have undergone some major genetic changes that may have changed their growth properties. Further PFGE analysis carried out by Kathleen Lilleness showed that the PFGE profiles of patient 3 and 14 as well as 4 and 6 were identical (Appendix II). Therefore it was not surprising that these strains posed the same difficulties in the ability to grow without Tween 80 supplementation.

Nonetheless, in most of the patients no visible changes in the DNA patterns were observed. Due to the picking of single colonies from the samples this is just a one-time point analysis. For the patients that showed the same or similar patterns it is very likely that they kept the same strain over the time of infection. However, these patients can still be infected with other strains. To verify that some patients like patient 11 or 15 are infected two times with different mycobacterial strains the PFGE should be redone with several single colonies picked from the samples. It is also possible that both strains or even more can be found over the whole period of infection.

4.3 The growth characteristics of the strains can vary over the period of infection

In some strains differences in the PFGE were observed, whereas in most strains no visible changes occurred. To evaluate whether more phenotypic changes can be found, bacterial growth experiments were performed on the strains.

The strains were grown for 8 days so that the growth should have reached the stationary phase. The strains were then diluted to an OD_{600} that was similar to the blank medium to ensure equal starting conditions for all strains and obtain a visible lag-phase. The growth curve measurement was carried out with Tween 80 supplementation so that all available isolates could be analyzed. In the PFGE the isolates from patients 5, 11, 13, 14 and 15 showed visible changes in the DNA pattern. Therefore, it should be validated whether there are also measurable growth differences in these strains or not. They underwent major DNA changes over the period of infection or they were completely different strains respectively. Figure 7 shows the growth curves for the mycobacterial isolates from the patients mentioned above. The strains MAC 5-1 and MAC 5-2 grew fairly similar and no major differences could be seen (Figure 7A). That means, the growth properties did not seem to have changed measurably during the period of infection even if there were major genetic changes observed in the PFGE. As shown before patient 11 seemed to be infected by two different *M. avium* strains at the different time points of sample collection, strain MAC 11-1 at the first time point and strain MAC 11-2 at the second. The growth curves of the two strains are totally different as strain MAC 11-1 seemed to grow more rapid with a shorter but stronger exponential phase, whereas strain MAC 11-2 has a less strong exponential growth instead and was in addition

overall growing slower than strain MAC 11-1 (Figure 7B). The reasons for the stationary phase between day three and five in the growth curve for strain MAC 11-2 cannot be clarified so far. The samples of patient 15 were completely different in the PFGE profile and also in the growth characteristics (Figure 7E). Strain MAC 15-1 was growing faster and up to a much higher OD_{600} compared to strain MAC 15-2. Because of this, the hypothesis that both patients were infected with different mycobacterial strains over the period of infection is strongly supported.

A totally different result appeared for patient 14. While strain MAC 14-1 grew well and showed an expected growth curve, strain MAC 14-2 was not growing at all, even though the growth in a tube before dilution exhibited no difficulties (Figure 7D). All growth curves were run in triplicates in 7H9 media with addition of Tween 80 and none of the runs showed a measurable growth for strain MAC 14-2. It was also impossible for this strain to grow without supplementation of Tween 80, during the preparation of the infection aliquots. So far, it is only known that this strain showed a single band difference in the PFGE, but this mutation has not necessarily to be the reason for the observed growth differences.

The strains isolated from patient 13 displayed a wide range of growth characteristics (Figure 7C). The first collected isolate MAC 13-1 grew slow but very constant over time of measurement. For the last taken isolate, strain MAC 13-4, we saw that after a short exponential and stationary phase, a strong decrease in the measured OD_{600} occurred, which is most likely connected to dying bacteria or extensive clumping. The two isolates MAC 13-2 and MAC 13-3 collected in between MAC 13-1 and MAC 13-4 were displaying similar growth properties as MAC 13-4 especially in the exponential growth phase. The only difference known so far for these isolates is a single band difference in the PFGE first observed between strain MAC 13-2 and MAC 13-3. Further differences need to be validated later by characterization of immunostimulatory properties that can possibly be connected to these different growth characteristics.

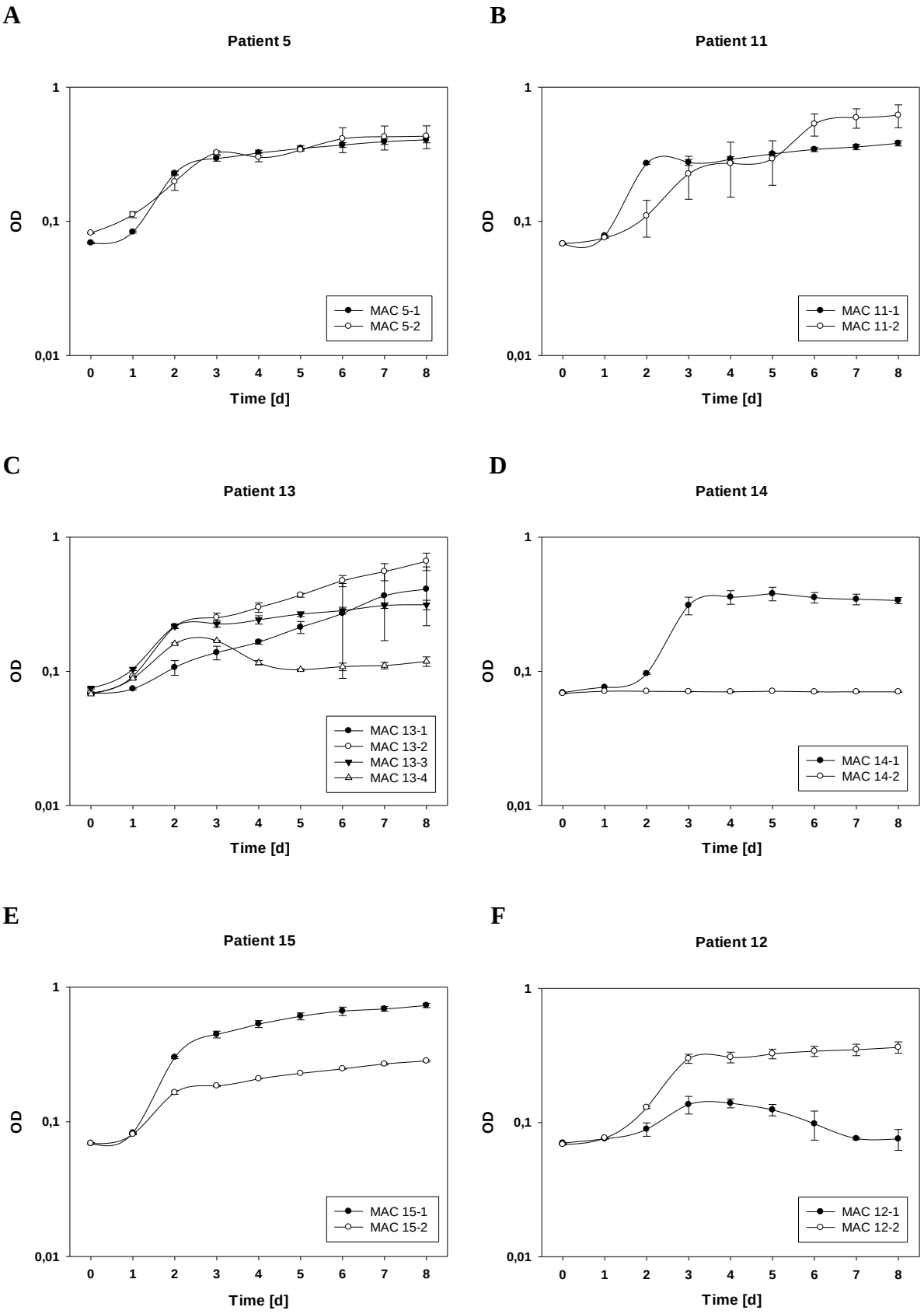


Figure 7: Bacterial growth curves for the different MAC strains
A-F) Growth curves of bacterial strains isolated from patients. The legend shows strains in chronological order of collection from the patients. The experiment was run in triplicates and the mean of the triplicates with the standard deviation is shown.

The strains MAC 12-1 and MAC 12-2 from patient 12 were also displaying very different growth characteristics (Figure 7F). While strain MAC 12-1 grew to a high OD_{600} of approx. 0.6 with a pronounced exponential growth phase, strain MAC 12-2 grew slow and even seemed to die after some days as the OD_{600} after 8 days reached the basic level of the blank media again. Hence, it seems likely that these strains underwent some genetic changes during period of infection.

For all the other tested strains the curves appeared quite similar (Appendix III). The growth curves for the patient isolates can be grouped in different categories. There were fast and similar growing strains like the isolates from patients 1, 5, 7 and 8. These strains were starting to grow exponentially already after approx. two days, while slow growing strains like the strains from patient 4 and 6 needed up to five days for an exponential growth. For the other patients it is difficult to define a clear pattern as the strains of the same patient were still strongly varying in their growth characteristics.

4.4 The intracellular survival of the bacteria in the macrophage is varying

The measurement of the growth rate is one approach to define differences between the different bacterial isolates. Another possibility to characterize the different strains is the determination of intracellular survival in the macrophage over the period of infection. Therefore, bacterial survival plating at different time points of infection was carried out.

The intracellular survival rate was overall strongly varying in between the patients (Figures 8, 9 and Appendix IV). Whereas strains like MAC 11-1 and MAC 11-2 achieved survival rates of up to 90% 24h p. inf., some other strains like MAC 10-1 did not have a measurable intracellular survival 24h past infection.

The strains from patient 5 were displaying similar growth rates independently of the observed PFGE band difference. The intracellular survival for strains MAC 5-1 and MAC 5-2 was slightly varying 8h p. inf. but becoming similar at 24h p. inf. (Figure 8A). It seems that strain MAC 5-1 got more effectively killed shortly after infection while this took more time for strain MAC 5-2. The strains from patient 11 varied in their intracellular survival, but both showed survival rates in the macrophage higher than expected (Figure 8B). These strains seem to have effective immune system evasion strategies so that the killing of the bacteria by

macrophages was overall on a low level. However, the variation between MAC 11-1 and MAC 11-2 is still relatively big which is most likely connected to the fact that genetically these strains are totally different from each other. Therefore, they also show different growth characteristics and immunostimulatory properties. The same applies to MAC 15-1 and MAC 15-2 from patient 15. While strain MAC 15-2 underwent a fast killing in the first hours after infection, strain MAC 15-1 seemed to be equally killed over the whole time of the survival measurement and the survival rate would maybe decrease even more at later stages that were not tested in this experiment (Figure 8C).

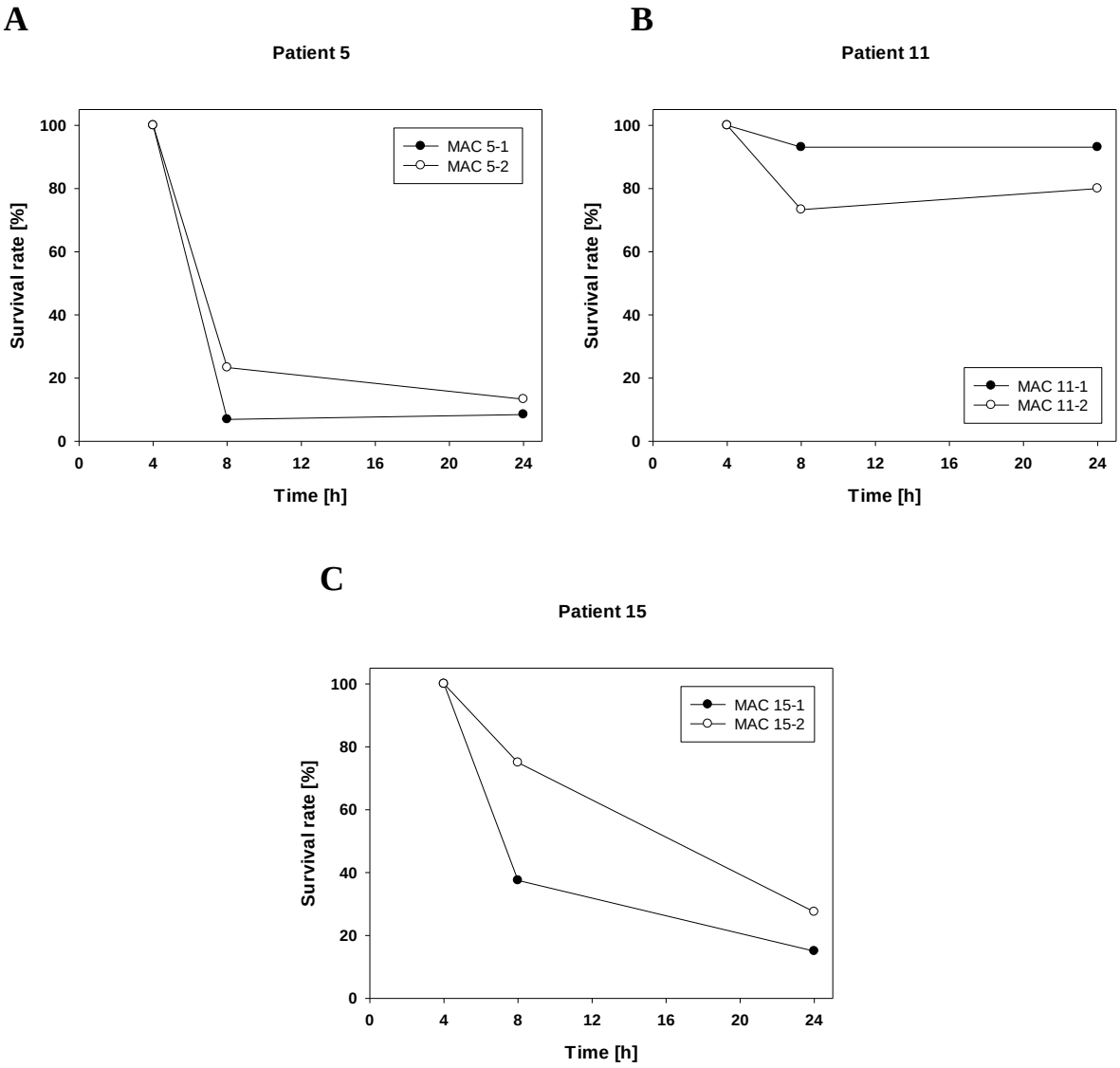


Figure 8: Intracellular survival of MAC strains after infection of macrophages
The infection ratio macrophage to mycobacteria was 1:50. Non-phagocytized bacteria were removed 4h p. inf. and this time point was also chosen for a relative survival rate of 100%. The values at 8h and 24h p. inf. were calculated from the decrease in bacterial numbers compared to 4h p. inf.. Data from one of two experiments is shown.

The strains from patient 13 were again displaying slightly unexpected results (Figure 9A). While the first isolated strain MAC 13-1 showed a high survival rate 8h p. inf., the rate decreased strongly 24h p. inf. The same applies to strain MAC 13-4 that was the last isolate collected. The two strains MAC 13-2 and MAC 13-3 which were collected in between the first and last sample exhibited very similar survival rates but these are way lower than observed for MAC 13-1 and MAC 13-4. The growth curve measurements showed that MAC 13-2 and MAC 13-3 grew similar whereas the other two strains behaved completely different. As the single band difference observed in the PFGE occurred between strain MAC 13-2 and MAC 13-3 a connection to the observed growth and survival differences are not plausible. A similar pattern was observed for patient 12. While strain MAC 12-1 was slow growing to a low OD_{600}, MAC 12-2 was growing fast. Consequently, the lower survival rate of MAC 12-1 compared to MAC 12-2 especially 8h p. inf. could be connected to observed differences these strains also displayed in the growth analysis.

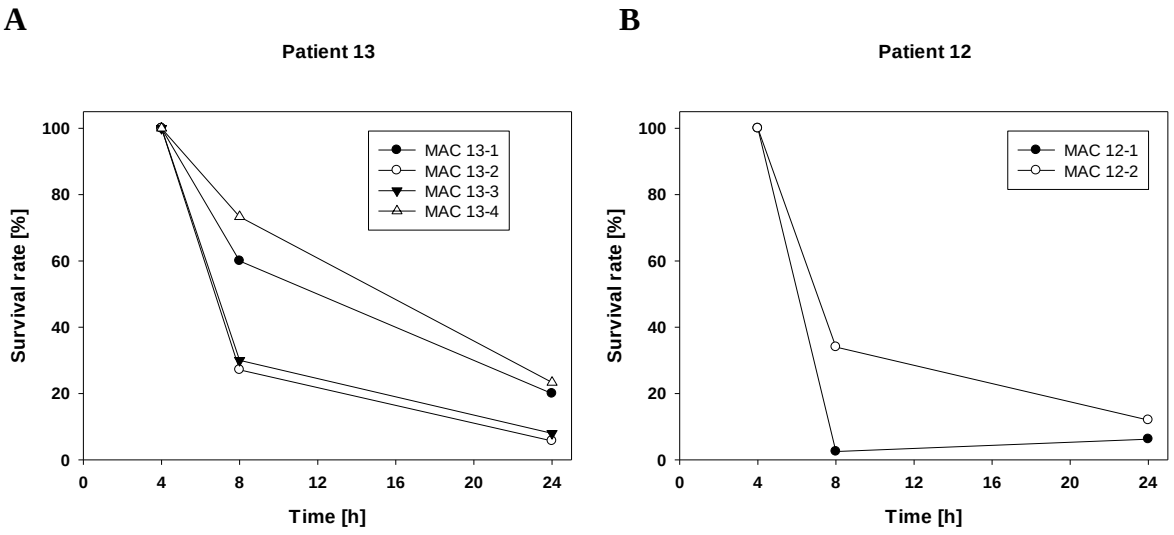


Figure 9: Intracellular survival of the different MAC strains
The infection ratio macrophage to mycobacteria was 1:50. Non-phagocytized bacteria were removed 4h p. inf. and this time point was also chosen for a relative survival rate of 100%. The values at 8h and 24h p. inf. were calculated from the decrease in bacterial numbers compared to 4h p. inf.. Data from one of two experiments is shown.

To sum up, a direct connection between the growth characteristics and the intracellular survival during the infection does not seem to exist. No clear pattern, like poorly growing strains are more easily killed or well growing strains survive better in the macrophage, could be observed. However, it seems that the last collected isolate often survives better in the macrophage. This supports the hypothesis that the mycobacteria develop more efficient evasion strategies over time of infection. Further analyses would be useful to validate these results.

4.5 TNF-α, IL-6 and chemokines are upregulated after infection with *M. avium* strains

The level of secretion of pro-inflammatory cytokines is a good parameter for the characterization of immunostimulatory properties. The main hypothesis was that in long term infections MAC strains would evolve in a way that reduce their immunostimulatory properties. To evaluate the immunostimulatory properties of the strains, a mouse macrophage cell line (RAW 264.7) was infected with the same amount of bacteria for every strain and the cytokine release was compared.

To determine which cytokines and chemokines are mainly produced after infection of RAW 264.7 macrophages with different MAC strains, a multiplex immunoassay for multiple cytokine measurement was carried out. Seven different MAC strains were chosen for initial analysis based on preliminary ELISA results that showed a robust TNF-α response (Data not shown). Due to the limited capacity of the multiplex immunoassay only one strain was analyzed for the three different time points 4h, 8h and 24h p.inf. whereas the remaining six strains were only analyzed 8h p. inf..

Multiplex analysis of samples from one strain over time showed that the response of macrophages to MAC infection was varying between the different cytokines and chemokines (Figure 10). Most cytokines, like IL-1α/β, IL-10 and IL-12, did not show a high enough secretion to be considered as having an influencing effect on the immune system at any tested time point. Especially for IL-12 and IL-1β the results were unexpected as these cytokines are known to be highly upregulated after *M. avium* infection in human macrophages[23,108] but at

least for IL-1β studies have shown that this cytokine is not upregulated in RAW 264.7 macrophages[109].

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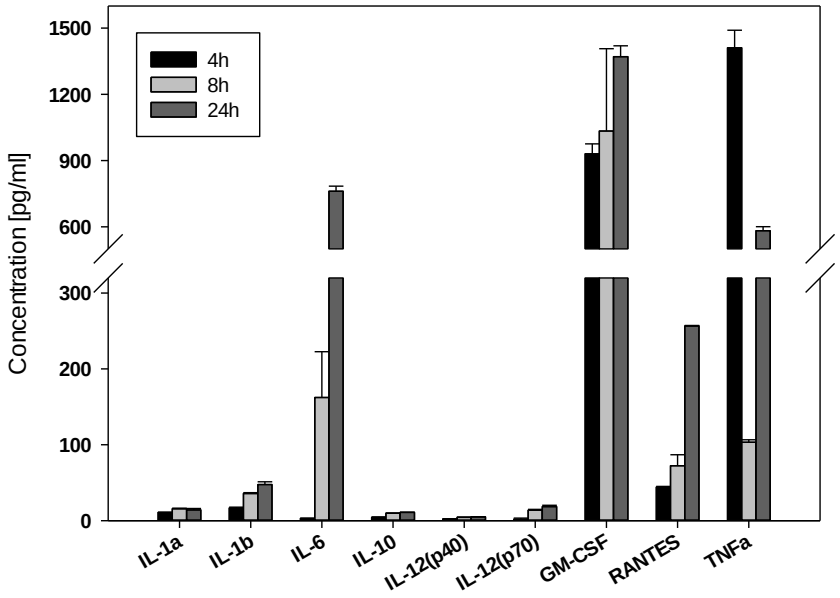

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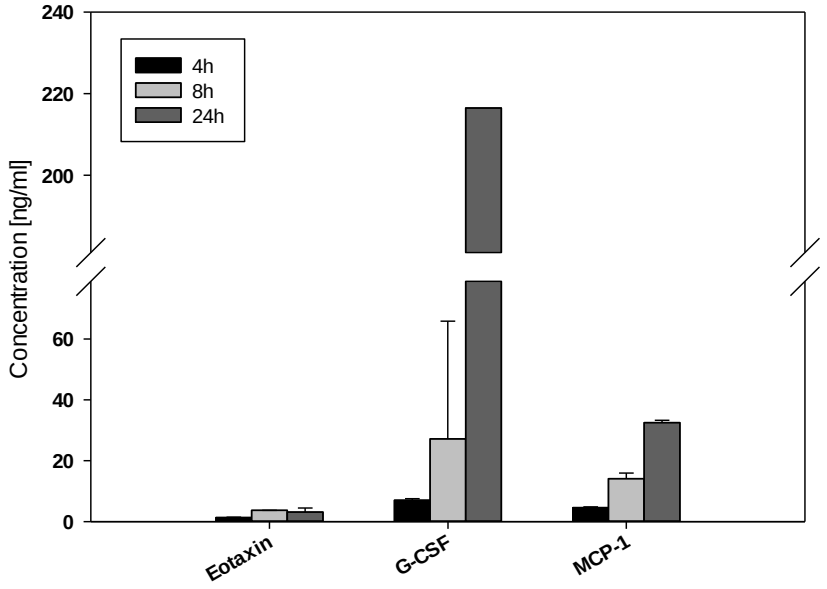

Figure 10: Cytokine/ chemokine concentration at 8h p.inf. after infection of macrophages with MAC
Supernatants were collected from RAW 264.7 macrophages infected with strain MAC 11-1 at different time points after initial infection. A) Cytokine and chemokine concentration in pg/ml; B) Chemokine concentration in ng/ml; The concentrations of the chemokines MIP-1α and MIP-1β were above the maximum detection limit of 25ng/ml and are not shown. The samples were run in duplicates and the standard deviation of these duplicates is shown.

All the chemokines like GM-CSF, G-CSF (granulocyte colony-stimulating factor), Eotaxin and MCP-1 are highly raised after infection with MAC at all tested time points. The chemokines MIP-1α and MIP-1β showed responses even above the maximum detection limit for the multiplex immunoassay of 25ng/ml and are not explicitly shown. IL-6 showed a low secretion at the 4h time point that steadily increased towards the 8h and 24h time point. On the contrary TNF-α showed a high response 4h p. inf. that were decreasing towards 8h p. inf., but raised again 24h p. infection.

We then went on to analyze the samples taken on the 8h time point using several strains. The cytokines with the greatest variation in the level of secretion connected to the different MAC strains were TNF-α and IL-6 (Figure 11). These cytokines where consequently selected to evaluate and quantify the immune response of RAW 264.7 macrophages to the different MAC strains. Figure 11 shows the varying responses for TNF-α (A) and IL-6 (B) between the tested strains and at different time points. Moreover, it can be seen that the strains like MAC 7-1 and MAC 7-2, which displayed a single band difference in the PFGE, still showed similar high levels in the cytokine response after MAC infection. The strains MAC 11-1 and MAC 11-2 as well as MAC 15-1 and MAC 15-2 are from the same patient but according to the PFGE totally different bacterial strains. Thus, the different cytokine expression of these strains was not unexpected. A further validation about a link of the PFGE results and cytokine expression levels needs to be verified.

Originally MCP-1 should be analyzed as well, but due to the high MCP-1 production a further analysis by ELISA was not possible because of a limited detection capacity of the ELISA kit. The same applies to other chemokines like G-CSF and GM-CSF, as well as Eotaxin. Most of the other cytokines had concentrations below the detection limit of an ELISA kit like IL-12(p70) or IL-1β, and the multiplex analysis did not show a huge variation between the different tested strains (Appendix V).

Due to these results a further analysis of the cytokines TNF-α and IL-6 was planned to determine the differences in the cytokine response of different MAC strains.

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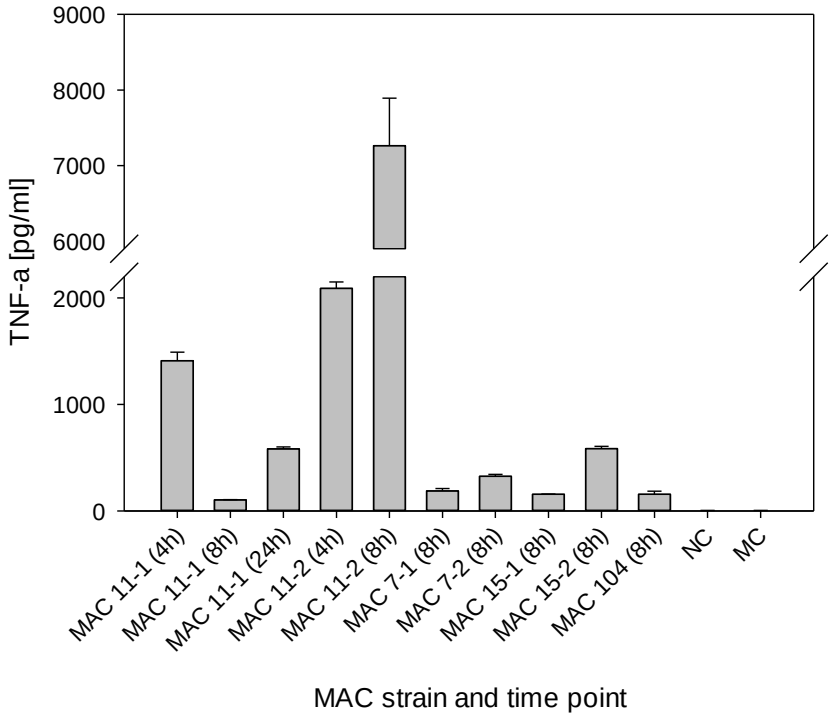

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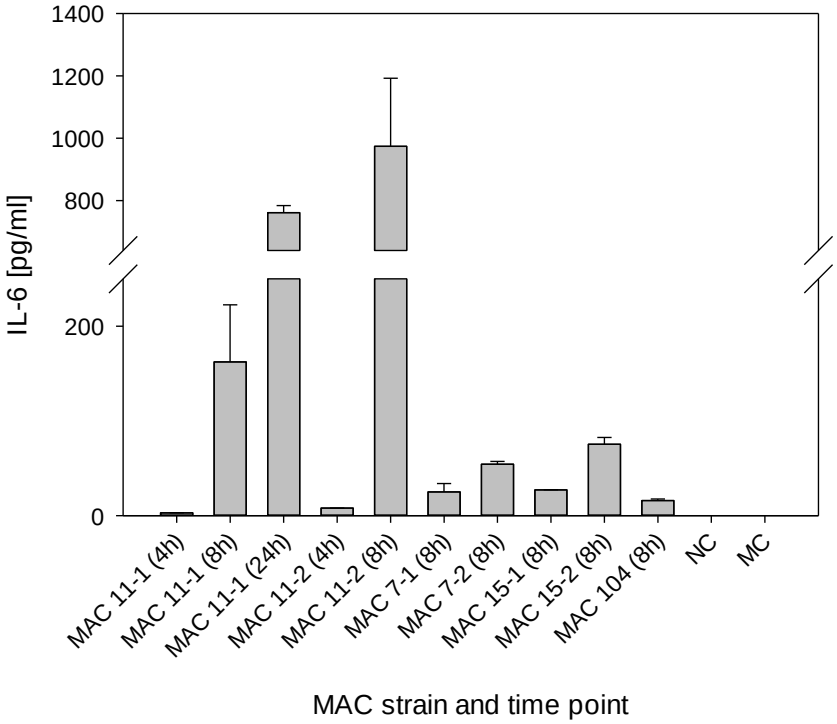

Figure 11: TNF-α and IL-6 secretion of the different MAC strains at different time points
Supernatants were collected from RAW 264.7 macrophages infected with different *M. avium* strains at several time points after initial infection. The infection ratio macrophage to mycobacteria was 1:50. A) TNF-α concentration in pg/ml; B) IL-6 concentration in pg/ml; NC – negative control; MC – media control. The samples were run in duplicates and the standard deviation of these duplicates is shown.

4.6 The infection ratio and the time point for collection of supernatants influences the intensity of the cytokine response

The first ELISAs showed quite inconsistent results in the cytokine response which was most likely due to a suboptimal infection ratio and also a not optimal chosen time point for the collection of the supernatants. Therefore, a further analysis of these parameters was carried out to ensure good functioning experimental conditions.

Preliminary ELISAs carried out with an infection ratio for macrophages to mycobacteria of 1:50 showed good results. To ensure that this ratio provides optimal results, a test with several infection ratios (1:5; 1:10, 1:50 and 1:100) was made. In addition, different time points (4h, 6h, 8h and 24h p. inf.) for collection of supernatants were tested. The time points were basically chosen according to established protocols in the workgroup.

For TNF-α measurable responses were obtained for nearly all chosen infection ratios and time points (Figure 12A). The decrease in the amount of secreted TNF-α after 6h compared to 4h p. inf. is mainly due to a necessary change of the medium after 4h for the removal of non-phagocytized bacteria. This is also the main reason that the 4h p. inf. supernatants were not chosen for further analysis due to a potential stimulatory effect of the high infection dose and from bacteria just floating in the culture medium. At 8h p. inf. the cytokine responses for the ratios 1:50 and 1:100 reached the same level as at 4h p. inf.. As the cytokine expression levels for the infection ratios 1:50 and 1:100 were equally, the infection ratio 1:50 with supernatants collected 8h p. inf. was considered as the optimal option. This was mainly to prevent an unnatural strong infection with unnatural high responses. It seems likely that at some point too high infection doses are not increasing the immune response anymore and that even higher infection ratios may be able to cause a reverse effect and even lower the cytokine levels again. However, also a 1:50 infection ratio still reflects a very high and probably unnatural high infection dose for naturally occurring infections which should be kept in mind when analyzing the data.

IL-6 did not show a measurable cytokine concentration at 4h p. inf. for any of the tested infection ratios (Figure 12B). This indicates that the IL-6 secretion occurs later than the TNF-α secretion. In addition it seems that generally a higher infection ratio is needed to gain a cytokine response. For the 1:5 as well as the 1:10 ratio no measurable IL-6 secretion was obtained. Due to the reasons mentioned before for TNF-α, the time point for collecting the

supernatants is 8h p. inf.. The infection ratio is 1:50 as the slight increase in the cytokine response at the 1:100 infection ratio would not compensate for the potential side effects of the unnatural high infection dose.

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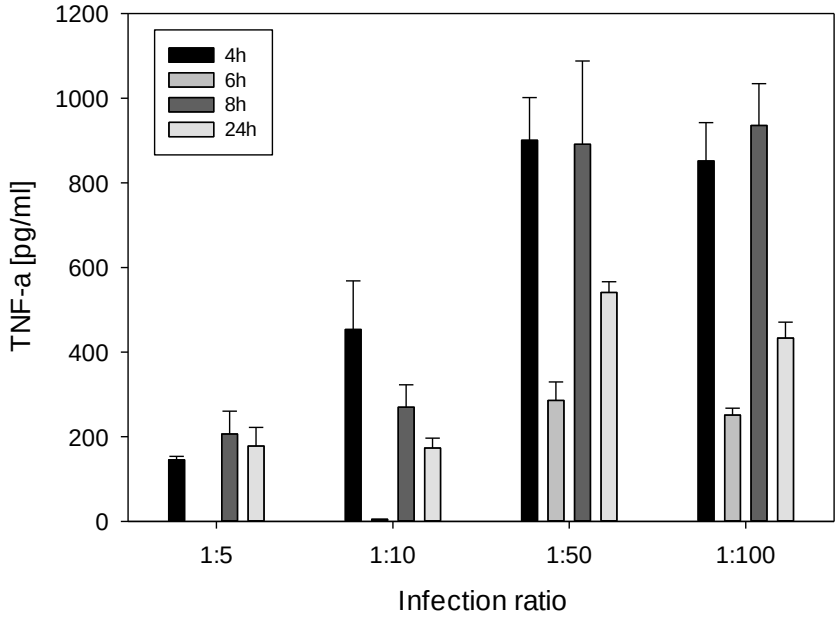

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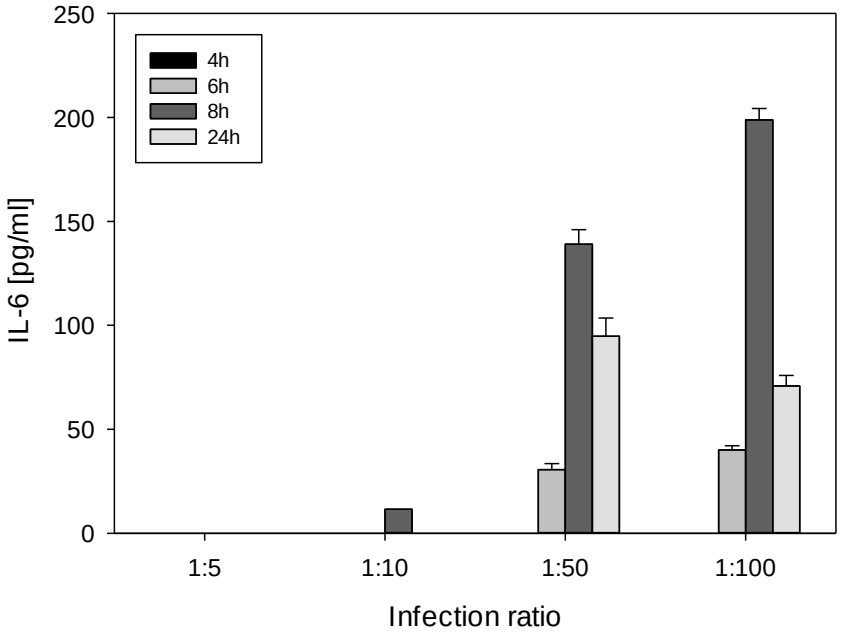

Figure 12: Determination of optimal infection ratio and time point for collection of supernatants
Supernatants collected from RAW 264.7 macrophages infected with strain MAC 104 at different infection ratios and time points after initial infection. A) TNF-α concentration in pg/ml; B) IL-6 concentration in pg/ml; The samples were run in triplicates and the standard deviation from these triplicates is shown.

All further ELISAs were carried out with supernatants harvested 8 hours after infection with a ratio of macrophage to mycobacteria of 1:50.

4.7 IL-6 and TNF-α secretion varies between the different *M. avium* strains

Based on the optimization of the ELISA conditions, we decided to measure IL-6 and TNF-α responses to identify *M. avium* strains that differ in the ability to activate macrophages. In particular we wanted to detect if *M. avium* strains isolated from the same patient over time would give varying responses. Furthermore, we wanted to connect the ELISA data to the results from the PFGE to determine if large genetic variations in the bacterial strains over time were reflected in a similar variation in the cytokine production.

As seen in Figure 13, the ELISA result for TNF-α showed substantial variation of responses between the strains from different patients. The hypothesis was a decrease in the cytokine response in the patients over the period of infection. This hypothesis seems to be valid only for some of the patients, especially when the first response was very high (above 1500pg/ml). Examples for strains that follow the trend of reduced response over the period of infection were the strains of patients 1, 5, 7 and 8 as well as to some extent of patient 13. The cytokine response for some of the strains from a single patient, such as patient 10, did not vary that strongly and the response over the period of infection was similar. If these results are compared with the PFGE some of the observed variations can be connected to these results. The huge variation in cytokine secretion between strain MAC 11-1 and MAC 11-2 was most likely a result of the fact that these are two completely different strains which therefore seem to display different immunostimulatory properties. However, similar patterns can also be seen for strains from patients without PFGE differences like the isolates of patients 7 and 8. The difference in the cytokine expression of strains MAC 5-1 and MAC 5-2 could possibly be connected to the observed band difference which may have changed the characteristics of the strain measurably. For the strains where a major change on the PFGE were observed, it is likely that more DNA changes have occurred without being identified by the PFGE because of the limited sensitivity of the method. For the two different isolates from patient 15, the cytokine response was different in the first and the second isolate collected. The strong difference in cytokine secretion for strains MAC 1-1 and MAC 1-2 could not be related to the

PFGE as the strains did not show any measurable genetic changes. The same strains had no difference in the phenotypic assays either and were both independent on Tween 80 for growth. A strong decrease in the ability to induce secretion of pro-inflammatory cytokines like TNF-α can be connected to the hypothesis that the cytokine response decreases over the period of infection due to the fact that the bacterial strains develop more effective mechanisms to prevent detection by the immune system. This increases their chances of a better intracellular survival and replication in the host.

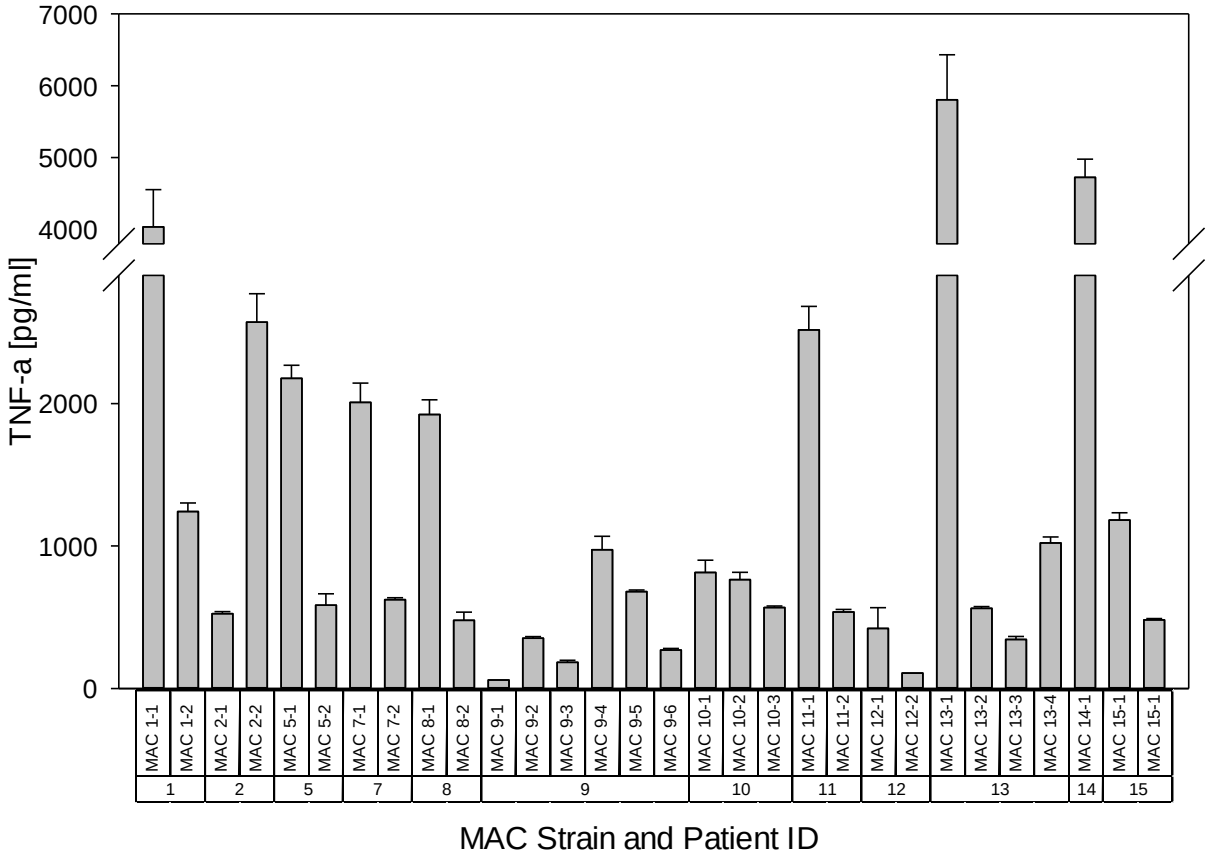

Figure 13: TNF-α concentrations for all MAC strains determined by ELISA
The ELISA was carried out with a ratio macrophage to mycobacteria of 1:50. Supernatants were collected 8h p. inf. The samples were run in triplicates and the standard deviation from these triplicates is shown. Data from one of two experiments is shown.

The cytokine production for IL-6 compared to TNF-α was generally on a lower level or not detectable (Figure 14). The pattern of response showed similarities to TNF-α, however some differences could clearly be seen. The strains which caused a strong TNF-α secretion were also able to induce a measurable IL-6 response. Thus, an influence of TNF-α on the IL-6 expression seems possible, especially since the IL-6 response arose measurably later than the

TNF-α response (compare Figure 12). Another reason could be the more specific receptor activation for IL-6 as it can be induced by TLR-4, but not TLR2, while TNF-α secretion is stimulated mainly by TLR2. Therefore, a measurable immune response for IL-6 may need more time. Nonetheless, the results from the supernatants harvested at 24h p. inf. showed even lower responses for most of the strains than the supernatants taken at 8h p. inf. for most of the strains (Appendix VI).

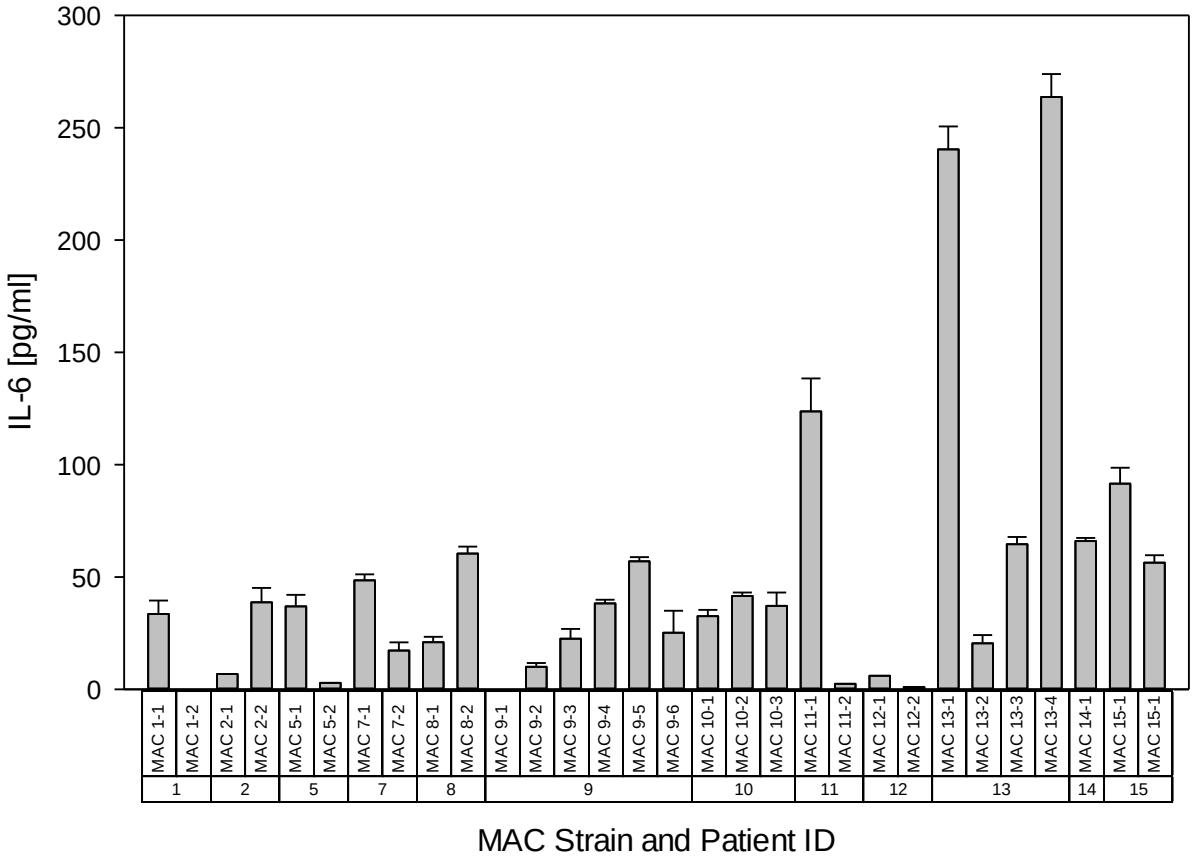

Figure 14: IL-6 concentrations for all MAC strains determined by ELISA
The ELISA was carried out with a ratio macrophage to mycobacteria of 1:50. Supernatants were collected 8h p. inf. The samples were run in triplicates and the standard deviation from these triplicates is shown. Data from one of two experiments is shown.

Some results are especially interesting. The decrease in cytokine expression could be seen for the strains from patients 1, 5 and 7 again. The isolates from patient 8 showed a rise in cytokine secretion of IL-6 at a later stage of infection, while for TNF-α a strong decrease in cytokine expression was observed. The IL-6 secretion levels from the strains of patient 13 showed high expression levels for strain MAC 13-1 and MAC 13-4, while the two isolates

collected in between displayed relatively low levels of IL-6 secretion. The strains of patient 13 have undergone genetic changes clearly seen by a single band difference in the PFGE pattern. The mutation was first detected between strains MAC 13-2 and MAC 13-3 so it was unlikely that the mutations were the main reason for the varying cytokine expression.

Both cytokines seem to show the ability to characterize the immunostimulatory properties of the *M. avium* strains. To further determine how the immune response is influenced due to the cytokine responses, the patient history and treatment regime could provide useful data.

4.8 *M. avium* and *M. intracellulare* display similar immunostimulatory and phenotypic properties

In addition to the PFGE results, a qRT-PCR melting curve analysis was carried out by Kathleen Lilleness to determine the species of the MAC isolates (Figure 15). In this analysis all isolates were identified as members of MAC, but the strains from patients 7, 10 and 12 were additionally identified as *M. intracellulare*, whereas all other strains appeared to be *M. avium*.

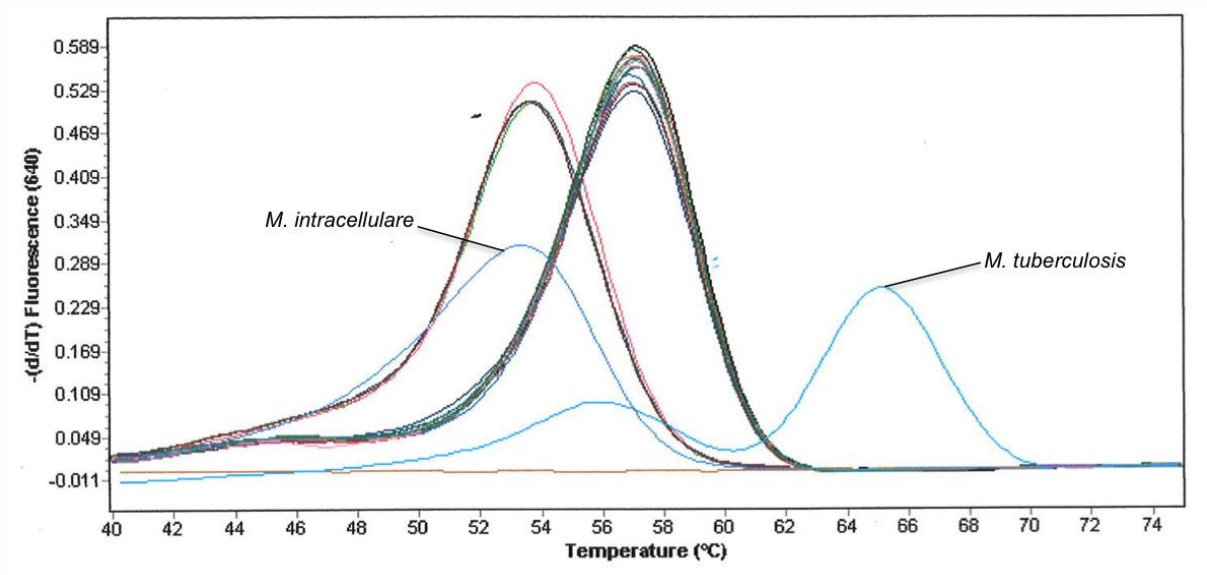

Figure 15: MAC species identification by qRT-PCR melting curve analysis
The graphs show differential melting curves from qRT-PCR for clinical MAC isolates as well as positive and negative controls for *M. intracellulare*, *M. avium* and *M. tuberculosis*. The experiment was carried out by Kathleen Lilleness.

With this sub-classification it could be checked if the changes in immune response were related to the species of MAC. So far no connection can be identified between the cytokine response to infection and the species of MAC. The observed cytokine responses for the *M. intracellulare* strains are not obviously different to results observed from the *M. avium* strains. The same applies for the growth analysis as well as the intracellular survival. Therefore the species of MAC seemed to be less important even if this needs to be further verified with more bacterial isolates especially for *M. intracellulare*.

4.9 ELISA measurement shows some advantages for quantitative analysis compared to qRT-PCR

Although ELISA showed differences in stimulatory effects of MAC strains which infected macrophages, some results were still surprising. To evaluate if changes could be reflected in a second method, a qRT-PCR was carried out. In addition, it should be tested if qRT-PCR would be better for identifying changes and discriminating between the strains.

Based on the results of the PFGE, the strains MAC 5-1 and MAC 5-2, MAC 8-1 and MAC 8-2 as well as MAC 11-1 and MAC 11-2 were chosen for a more detailed analysis. The selected strains represent all different DNA patterns that were seen in the patients during the period of infection. The strains from patient 5 showed a single band difference while the isolates of patient 11 seemed to be two completely different strains. The strains from patient 8 did not acquire visible changes during the period of infection.

As the RNA upregulation should arise earlier than the cytokine secretion the time point at 6h p. inf. was included in addition to the time points at 4h, 8h and 24h p. inf., where the supernatants for ELISA were sampled.

Figure 16A shows the results of the qRT-PCR for TNF-α. The highest relative gene expression was seen at 4h past infection. The expression level at 6h p. inf. decreased strongly for all of the strains. The expression level at 6h and 8h p. inf. is similar, whereas after 24h nearly no gene expression was measurable anymore. These findings confirmed the ELISA results for testing the optimal time point for collection of supernatants (compare Figure 12). Figure 16B shows the ELISA results for TNF-α with the supernatants harvested together with the collection of the cell lysates used for the qRT-PCR. As known from former experiments

the 8h supernatants give good cytokine responses and the trend of the cytokine levels is similar to the results of the q-RT-PCR, especially the high expression levels of MAC 5-1 and MAC 11-2 that still showed a high gene expression at 8h past infection.

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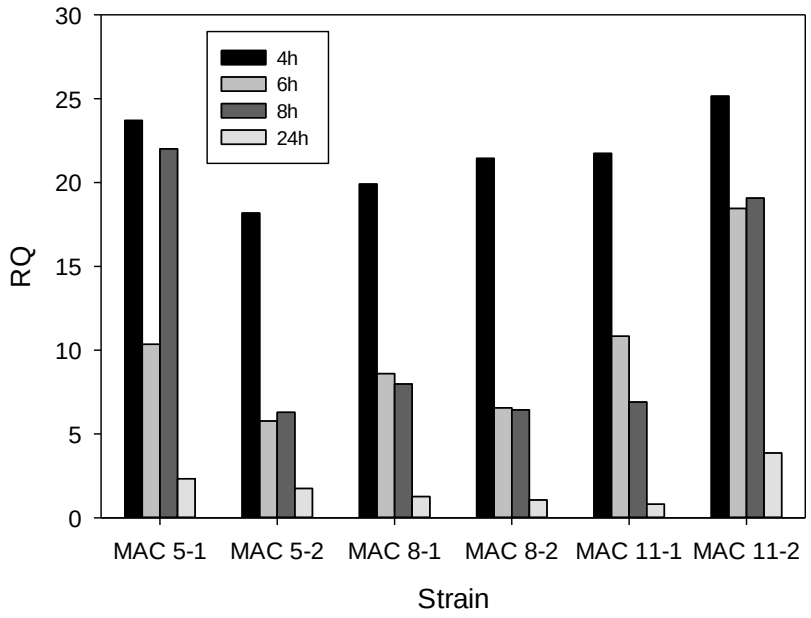

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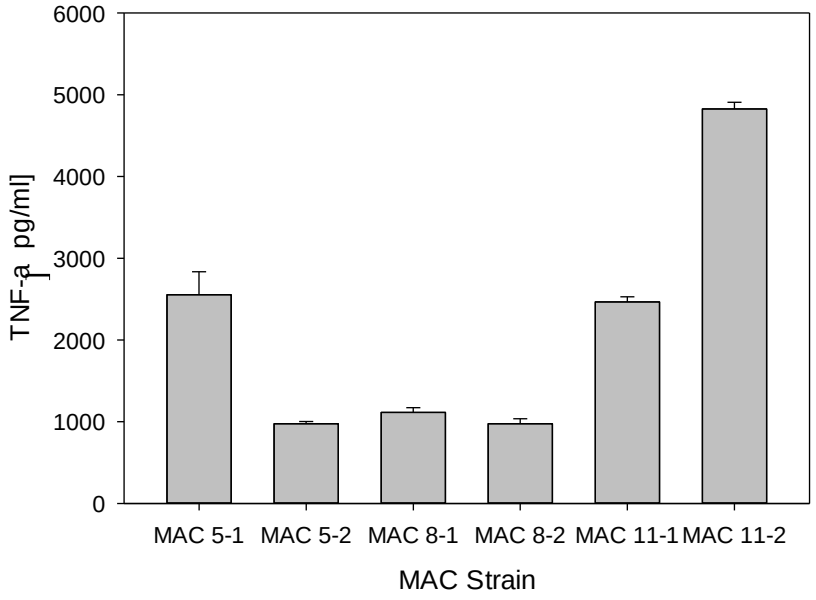
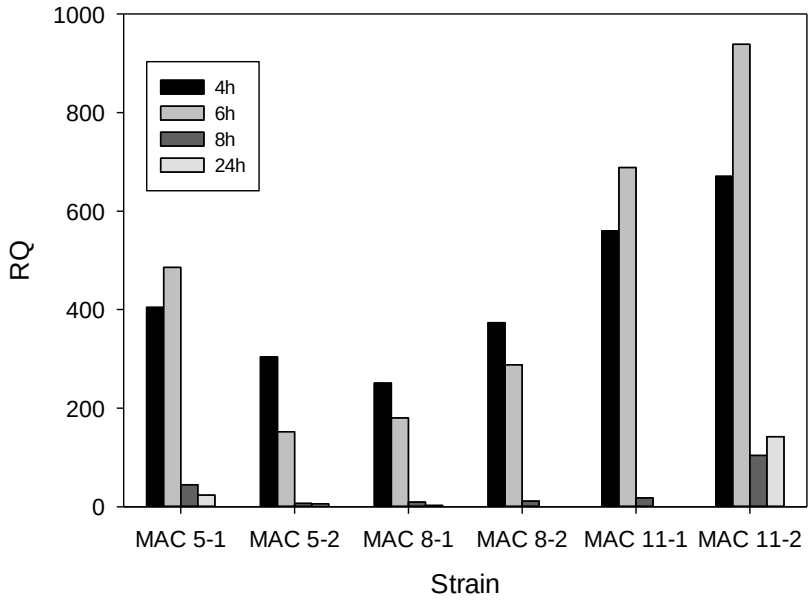


Figure 16: Comparison of ELISA and qRT-PCR results for TNF-α
A) qRT-PCR for TNF-α with cell lysates from infected RAW 264.7 macrophages and an infection ratio macrophage to mycobacteria of 1:50. B) ELISA for TNF-α with supernatants harvested 8h p. inf. from infected RAW 264.7 macrophages and an infection ratio macrophage to mycobacteria of 1:50. The ELISA samples were run in triplicates and the standard deviation of the triplicates is shown. Data from one of two experiments is shown.

For IL-6 (Figure 17) the results appeared similar to the results for TNF-α (Figure16). The gene expression was strongly upregulated at 4h and 6h p. inf., whereas the expression already strongly decreased at 8h p. inf.. The strains MAC 5-1, MAC 11-1 and MAC 11-2 displayed a high gene expression (Figure 17A) as it was already seen for TNF-α (compare Figure 14A). For all tested bacteria a measurable gene expression was measured. Thus, it would be expected that there is also a cytokine expression. Indeed, the ELISA measurement just showed a measurable cytokine level for the strains MAC 5-1, MAC 11-1 and MAC 11-2 (Figure 17B) which were also confirmed to display a high gene expression. For the other strains no measurable cytokine secretion could be verified. Hence, it seems that there are some compensating effects in the signaling system of macrophages so that only a very high gene expression led to cytokine secretion and therefore, further immunostimulatory actions. The reason for this need to be further determined, but could be just due to the sensitivity of the ELISA.

Both methods showed the ability to characterize the intensity of cytokine responses to infection of macrophages with different mycobacterial strains. Nevertheless, ELISA seems to be the preferred method for further use, as it is possible to quantitatively measure the amount of secreted cytokines and not only a relative gene expression.

A



B

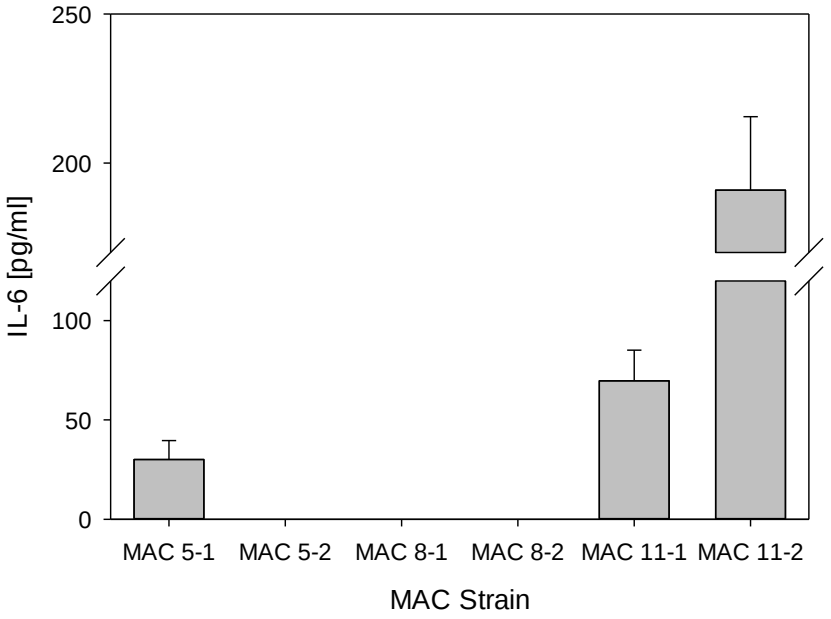


Figure 17: Comparison of ELISA and qRT-PCR for IL-6
A) qRT-PCR for TNF-α with cell lysates from infected RAW 264.7 macrophages and an infection ratio macrophage to mycobacteria of 1:50. B) ELISA for TNF-α with supernatants harvested 8h p. inf. from infected RAW 264.7 macrophages and an infection ratio macrophage to mycobacteria of 1:50. The ELISA samples were run in triplicates and the standard deviation of the triplicates is shown. Data from one of two experiments is shown.

4.10 High cytokine secretion leads to more effective bacterial killing

The main hypothesis of a decrease in cytokine secretion over the period of infection could be verified only to some extent and seem to be connected to the intensity of the cytokine expression induced by the bacterial isolate. It was also expected that a lower cytokine secretion led to a better intracellular survival as the bacteria may be able to circumvent detection by the macrophage. Especially for the strains with strongly varying cytokine expression levels like the isolates from patient 1, 2 or 5 the survival rates were not strongly varying (compare Figure 13 and Figures 8-9). Anyways, a connection could be possible as the strain with the higher cytokine secretion was usually more easily eradicated during the time of measurement. The only difference to this pattern was again seen for the isolates from patient 13 where the high cytokine inducing strains MAC 13-1 and MAC 13-4 also displayed good intracellular survival. The reason for these varying characteristics cannot be further determined by the current results.

A detailed overview over the phenotypic and genotypic changes that were investigated and validated in this work can be found in Table 6.

Table 6: Overview of differences in phenotype, genotype and cytokine response
The MAC strains are in chronological order of collection for each patient. TNF-α response – low: less than 500pg/ml, medium: 500-1500pg/ml, high: more than 1500pg/ml; IL-6 response – low: less than 30pg/ml, medium: 30-70pg/ml, high: more than 70pg/ml; Growth curves: slow growing: exponential phase after 2nd day, fast growing: clear exponential phase before 3rd day; Survival – high: above 50%, medium: 20-50%, low: less than 20%.

Patient	MAC Strain	TNF-α Response	IL-6 Response	Growth Curve	Survival (24h p. inf.)	PFGE
1	1-1	high	medium	slow growing	low	no band difference
	1-2	medium	not detectable	slow growing	low	
2	2-1	low	low	fast growing	medium	no band difference
	2-2	high	medium	fast growing	medium	
5	5-1	high	medium	fast growing	low	single band difference
	5-2	medium	not detectable	fast growing	low	
7	7-1	high	medium	fast growing	low	no band difference
	7-2	medium	low	fast growing	low	
8	8-1	high	low	fast growing	medium	no band difference
	8-2	low	medium	fast growing	low	
9	9-1	low	not detectable	fast growing	low	no band difference
	9-2	low	low	fast growing	low	
	9-3	low	low	fast growing	medium	
	9-4	medium	medium	fast growing	low	
	9-5	medium	medium	fast growing	low	
	9-6	low	low	slow growing	low	
10	10-1	medium	medium	slow growing	not detectable	no band difference
	10-2	medium	medium	slow growing	high	
	10-3	medium	medium	not growing	low	
11	11-1	high	high	fast growing	high	different strains
	11-2	medium	not detectable	slow growing	high	
12	12-1	low	not detectable	slow growing	low	no band difference
	12-2	low	not detectable	fast growing	low	
13	13-1	high	high	slow growing	medium	single band difference between MAC 13-2 and MAC 13-3
	13-2	low	low	fast growing	low	
	13-3	low	low	fast growing	low	
	13-4	medium	high	slow growing	medium	
14	14-1	high	medium	fast growing	not analysed	single band difference
	14-2	not analysed	not analysed	not growing		
15	15-1	medium	high	fast growing	low	different strains
	15-2	low	medium	slow growing	medium	

5. Discussion

M. avium is known to be a harmful pathogen especially in immunocompromised hosts. It is particularly associated with patients suffering from HIV infections and can cause a disseminated disease in this patient group[28]. In this work we characterized the immunostimulatory properties as well as genetic mutations occurring in patients over the period of infection.

The first phenotypic differences could be observed in the ability of the bacteria to grow without supplementation of Tween 80. It is known that mycobacteria tend to clump when cultured in liquid media like 7H9 and therefore a supplementation of the nonionic surface-active detergent Tween 80 is common. Some strains were unable to be cultured in 7H9 media without Tween 80 supplementation. The observed clumping of bacteria led to the use of Tween 80 in some of the experiments. The detergent is known to have an effect on colony morphology, growth and ultrastructure[110] of mycobacteria and all these effects are concentration dependent. *M. avium* utilizes Tween 80 by the help of Tween-hydrolyzing esterases[111]. Tween 80 is known to be harmful to macrophages, as it lyses cell membranes by solubilizing the membranous lipids and proteins[112]. Hence, a proper removal before infection of macrophages was needed. An additional washing step was included to reduce the concentration of Tween 80 and minimize possible side effects. Nevertheless, an influence on measurements when the detergent is used cannot be excluded completely and especially the results for strains where one is grown with Tween and the other is grown without should be interpreted with great care.

For the reproducibility of the infection experiments the mycobacterial strains were all grown to a comparable OD_{600} of approx. 0.5. Afterwards the strains were frozen in aliquots so that for each infection a new vial could be taken from the freezer. Mycobacteria are known to have a high survival of up to 100% when stored at -80°C[113]. After each infection the viability of the bacteria from the used vial was counted and no major differences could be determined so that the storage at -80°C does not have a major impact. Indeed all experiments were repeated at least once and the cytokine responses showed the same trend in every experiment.

The PFGE was carried out to define the identity of the *M. avium* isolates and to investigate the relatedness of the bacterial strains. It was used to determine whether the patients kept the same strain over the whole period of infection or got repeatedly infected with different *M. avium* isolates. The PFGE is a currently used standard method concerning epidemiological characterizations of bacterial strains[114]. It is a good tool to visualize genetic relatedness of bacterial isolates. A limitation is of course that only major DNA changes like big insertions, deletions or mutations that occurred directly in a restriction site can be detected. Therefore, a determination of genetic loci that are responsible for observable phenotypic changes is not possible.

If the patients with different isolates are repeatedly infected with new and different strains or only simultaneously infected with several strains, require further studies. Some strains displayed single band differences, but the reasons for the observed band differences are difficult to determine by the use of PFGE only. It is known from other bacterial species that pattern changes can arise due to the development of pathogenicity islands as well as the gain or loss of plasmids and genetic rearrangements[115]. If these reasons are important for the analyzed mycobacterial strains, will need further determination. At least for MTB it is shown that genetic changes like antibiotic resistances are mainly acquired through chromosomal mutations which either directly affect the binding site of the drug receptor or mutated bacterial enzymes involved in activating a pro-drug[116]. In addition, it was shown that usually more than one mutation leads to the development of drug resistances[117]. If the observed mutations were driven by antibiotic treatment and resulted in drug resistance will be verified later, when the patient history will be accessible. All genetic changes in the PFGE are observed in strains clearly identified as *M. avium*. The analyzed *M. intracellulare* strains did not display observable genetic changes. Whether *M. avium* tends to change more easily in the host or this is just a random observation needs to be determined with more isolates especially of *M. intracellulare*. Mycobacteria are known to display high genetic variability as well as regional genetic differences[118,119]. The pathogenicity of different *M. avium* isolates to humans is also determined by genetic differences observed between different strains and subspecies[23]. Therefore, the great variation of strains found in the PFGE analysis seems plausible. The PFGE has shown that, except of some patients (3 and 14, 4 and 6) with identical PFGE patterns, all patients were infected with individual strains. There are many possible infection sources known such as drinking water, shower heads or hot tubs as mycobacteria are extremely heat resistant[120].

The genomic diversity observed in our PFGE is also known for other bacteria. It was shown that *Escherichia coli* collected from the same infection site displayed phenotypic and genotypic diversity[121]. Also for *Pseudomonas aeruginosa* a genotypic and phenotypic development for a better survival in cystic fibrosis patients was investigated[122]. Recently, a genome wide analysis of sequential isolates of *Mycobacterium abscessus* demonstrated that the bacterium underwent certain mutations as an adaptive process to the host[123]. As genotypic variations during acquisition of drug resistance could be also shown for MTB[117], it was not unexpected that this study could confirm genotypic changes over the period of infection in the host for *M. avium*.

The bacterial growth analysis should further determine phenotypic differences. There are many factors known which influence growth and virulence of mycobacteria. One point that was not further considered in this work is the colony morphology of *M. avium* strains grown on agar plates. Studies have shown that the colony morphology can determine the virulence of the strain[124]. An aspect investigated in this work was the growth rate of the bacterial isolates. These results were strongly varying as there were fast growing strains entering the exponential growth phase already at day two of the measurement while other strains needed up to six days for a measurable growth rate. Also variations of the strains obtained from the same patient were clearly observable. Nevertheless, a direct correlation between bacterial growth and immunostimulatory properties could not be established.

The measurement of the intracellular survival rate in infected macrophages was mainly used to characterize the ability of the strains to avoid immune detection by the macrophages. A higher intracellular survival in the macrophage is a known pathogenic strategy of mycobacteria and this is just possible if the strain is able to circumvent immune detection by the host[125]. Our results were again strongly varying between the different strains and also the overall survival rate varied from up to 90 % down to no measurable survival 24h p. inf.. The survival rate measurements provided an overview of how effective the analyzed mycobacterial strains were in the evasion of macrophage-killing mechanisms. As the immune system is a multifactorial system involving complex interactions of innate and adaptive immune responses, the chosen method just provides information about the potential of immune evasion in the macrophage. For instance the influence of the adaptive immune system was not measured in this assay. To evaluate the total survival of the strains our results need to be validated in more complex experimental approaches involving more components of the immune system.

For the infection experiments the murine macrophage cell line RAW 264.7 was used. The decision for the usage of a cell line instead of primary human macrophages was mainly driven by the nearly unlimited availability of macrophages derived from a cell line compared to the availability of human primary macrophages. As this work was mainly intended for a characterization of so far untested bacterial isolates, the usage of a cell line was more convenient. In further studies a switch to primary human macrophages could be considered since it is known that due to the acquired immortality of the RAW 264.7 cells some signaling pathways are modified[126]. Besides, mouse macrophages have a slightly different immune response compared to humans[23,109] and the use of primary human macrophages would provide the possibility to test the immunostimulatory parameter's validity directly in the human host. For example RAW 264.7 macrophages are not expressing IL-12 which is known to be a potent pro-inflammatory mediator in the human immune response[127]. IL-10 can suppress the IL-12 expression[23], but as it is also not measurably secreted there must be another reason for the lack of IL-12 expression.

Another point that should be considered is the used infection ratio. A ratio of macrophage to mycobacteria of 1:50 is most likely higher compared to the bacterial load a host is challenged by the environment and in natural occurring infections. To infect especially an immunocompromised person the bacterial load can be lower. Nonetheless, other studies working with similar infection systems used similar high ratios[109] and no proper immune response could be detected with lower doses (compare Figure 12). Another reason for the high infection dose needed could be the differential regulation of MAPKs by pathogenic and nonpathogenic mycobacteria. The suppression of MAPKs by pathogenic mycobacteria also suppresses the cytokine secretion[128]. Moreover the time point for collection of supernatants was proven to be important. Though TNF-α is a rapidly expressed cytokine, a measurable cytokine response for IL-6 took longer. As a result of different secretion pathways for both cytokines and a stimulatory role for TNF-α on IL-6 secretion in addition to an inhibitory role for IL-6 on TNF-α secretion[83], the time differences in cytokine expression appear reasonable. Especially for TNF-α an influencing factor could be also the change of medium 4h p. inf. to remove free bacteria. This step was needed to prevent an effect of non-phagocytized bacteria on the ELISA measurements, but it unfortunately also removes some of the TNF-α already produced by the macrophage.

It was already shown that the human macrophage response to clinical isolates of MTB shows strong variation between the different isolates[108]. Consequently, the measurement of pro-inflammatory cytokines like TNF-α and IL-6 was used to characterize the innate immune response induced by the analyzed bacterial strains and differences in the intensity of cytokine secretion were also detected for MAC. As *M. avium* is an intra-macrophage pathogen, the macrophage response to infection, including synthesis of cytokines like TNF-α, is of great importance for the host immunity. Generally, it should be beneficial for mycobacteria to avoid the induction of a strong cytokine response to evade detection by the host immune system. The cytokine analysis showed strong differences in the ability to induce cytokine secretion. Whereas some strains, such as MAC 1-1, MAC 13-1 and MAC 14-1, forced a very strong immune response of up to 4,000pg/ml of TNF-α, other strains (MAC 9-1, MAC 9-3 and MAC 12-2) induced less than 300pg/ml. Thus, the detection by the host immune system is most likely influenced by the intensity of the cytokine secretion as well. As TNF-α can amplify its own production in a positive feedback loop[80] the fast and strong cytokine secretion is most likely driven by this mechanism. The strong decrease in the cytokine concentration in supernatants collected 24h past infection is mainly caused by the expression of anti-inflammatory cytokines such as TGF-β[80] as well as the expression of IL-6, which is known to inhibit TNF-α expression in mycobacterial infections[83].

The response pattern of IL-6 was quite similar to the responses seen for TNF-α, but on a general lower level. The cytokine secretion is also lower than shown in other studies where cytokine levels of up to 2000pg/ml were measured[109]. The high responding strains led to an expression of up to 250pg/ml by the macrophages, while for a lot of strains a measurable induction of cytokine secretion was not even detectable. This could be to some extent driven by the overall lower cytokine secretion of IL-6 and also the slower induction of cytokine expression. Nevertheless, supernatants taken 24h p. inf. did not show observable higher amounts of cytokine secretion.

A connection between intracellular survival rates and the quantity of a cytokine response, especially TNF-α expression, could be established only to some extent. The observed changes were not huge, but it could be seen for all patients infected with strains displaying a strongly varying immune response, that the survival rates of the higher responding strains were usually a bit lower than the rates for low responding strains. However, analysis of more strains would be needed to determine a connection between the ability to stimulate the cytokine production in macrophages and the intracellular survival of mycobacteria.

Mycobacteria are still a great challenge for global health and major improvements in treatment and vaccination strategies are still needed. This work has established a protocol for PFGE of *M. avium*/ *intracellulare* strains isolated from patients to allow a better characterization of epidemiologic parameters concerning these mycobacterial strains. Furthermore, a characterization of immunostimulatory properties of different patient-isolated MAC strains was carried out to give a better understanding of basic characteristics of these bacteria to be further used in the research for new treatment strategies in the future.

6. Conclusion and future perspectives

This study established a PFGE protocol for the analysis of epidemiologic relatedness of MAC isolates. It was shown that the strains can mutate in the host over the period of infection and that an infection with different strain is possible. If the strains are co-existing or the host got repeatedly infected needs to be further determined by picking several single colonies from the bacterial isolates.

The hypothesis that strains isolated from the same patient grow similar could be validated only to a small extent. Most of the strains displayed strongly varying growth characteristics. To which extend this is related to the genetic changes detected by the PFGE needs to be further characterized. An already planned sequencing of the mycobacterial strains may provide additional information relating to the varying growth characteristics. Also the influence of the host on the bacterial development needs to be verified comparing the strain characteristics with the patient history. An application is already submitted to the local regional ethics committee to get approval for contacting the patients in order to get permission to perform the investigation.

By the measurement of intracellular survival our results confirmed only in part our hypothesis that MAC strains evolve in the host over time so that they develop an increased survival in the macrophage during long-term infections. However, a connection between the intensity of pro-inflammatory cytokine secretion and the ability to survive was observed. It was shown that strains inducing a high cytokine response were killed more effectively by the macrophages then low inducing strains, but even these had a low survival.

The expected decrease in the induction of a pro-inflammatory cytokine response was also only to some extent confirmed. A strain that induced a high cytokine response usually displayed a low cytokine response in the following isolate.

Further projects should focus more on the involved signaling pathways and which effectors play an important role to determine new possible therapeutic approaches. RNA silencing as well as inhibitory antibodies could work as useful tools in the signal pathway analysis. Extended analysis on more immune parameters, other immune cells and especially their interactions with each other will provide a deeper insight into mechanisms of immune evasion by MAC strains and could consequently also be used to find new therapeutic targets.

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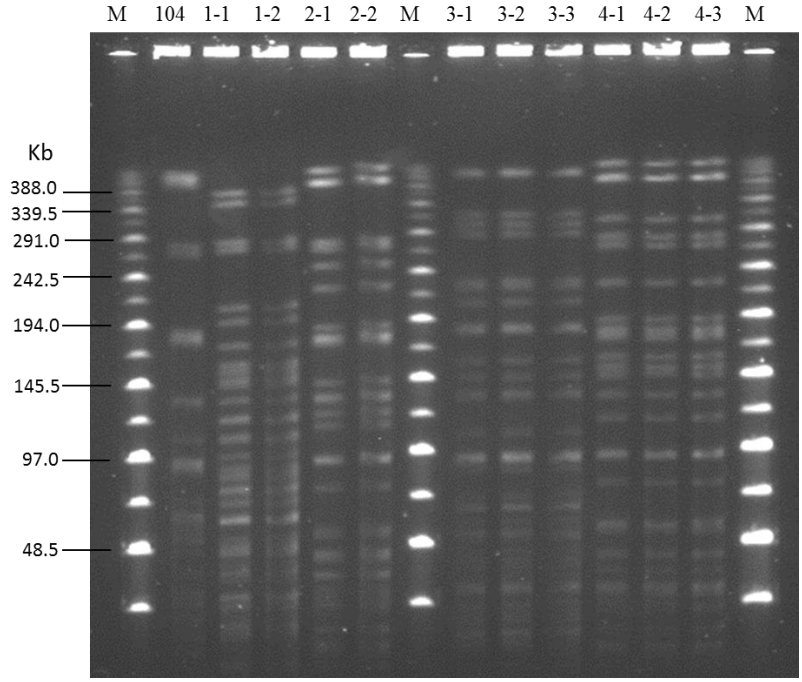
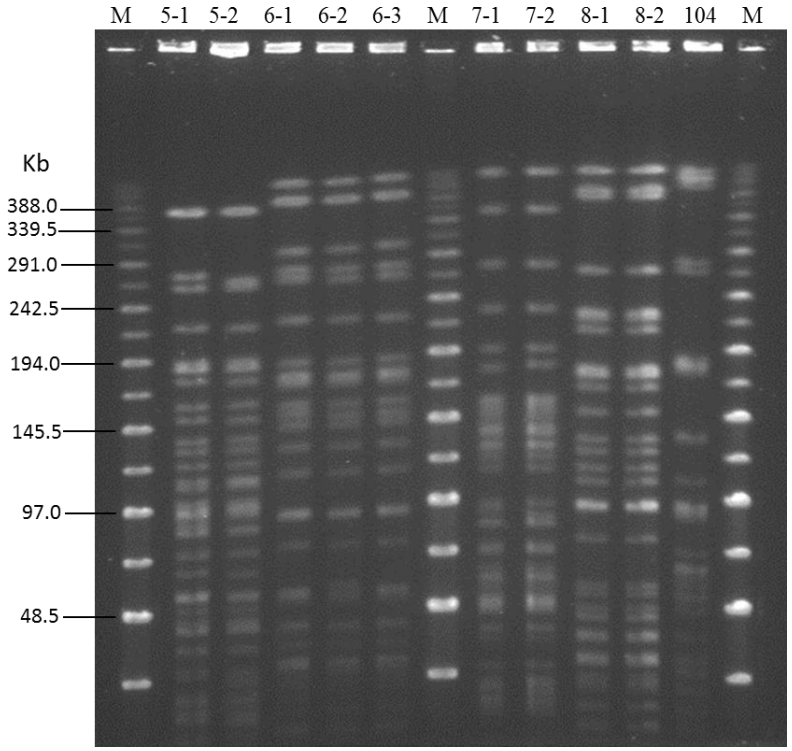
8. Appendices

Appendix I: PFGE profiles of all available isolates

A

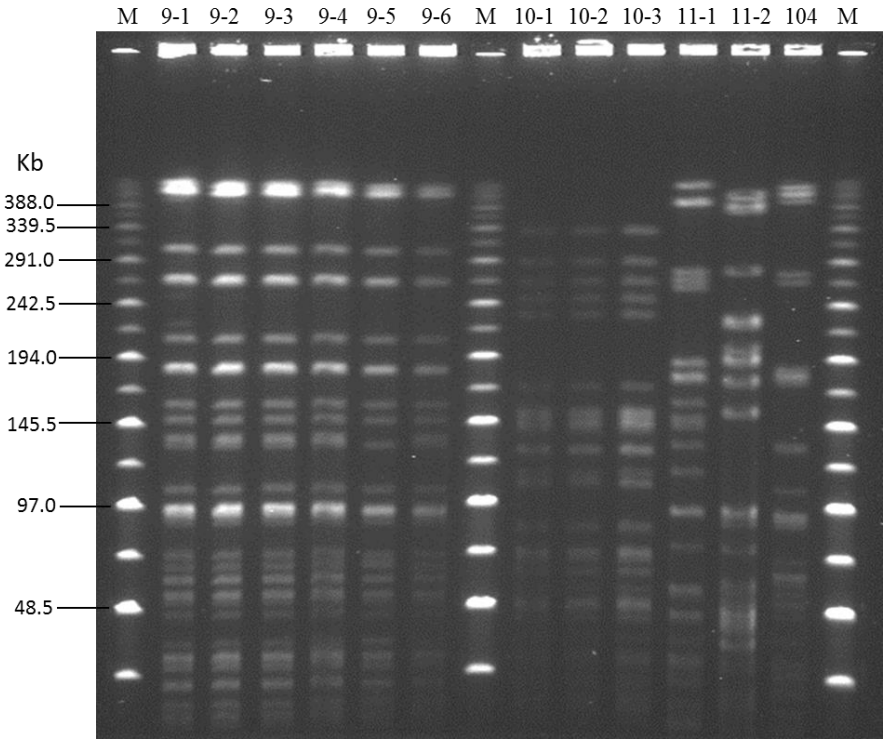


B

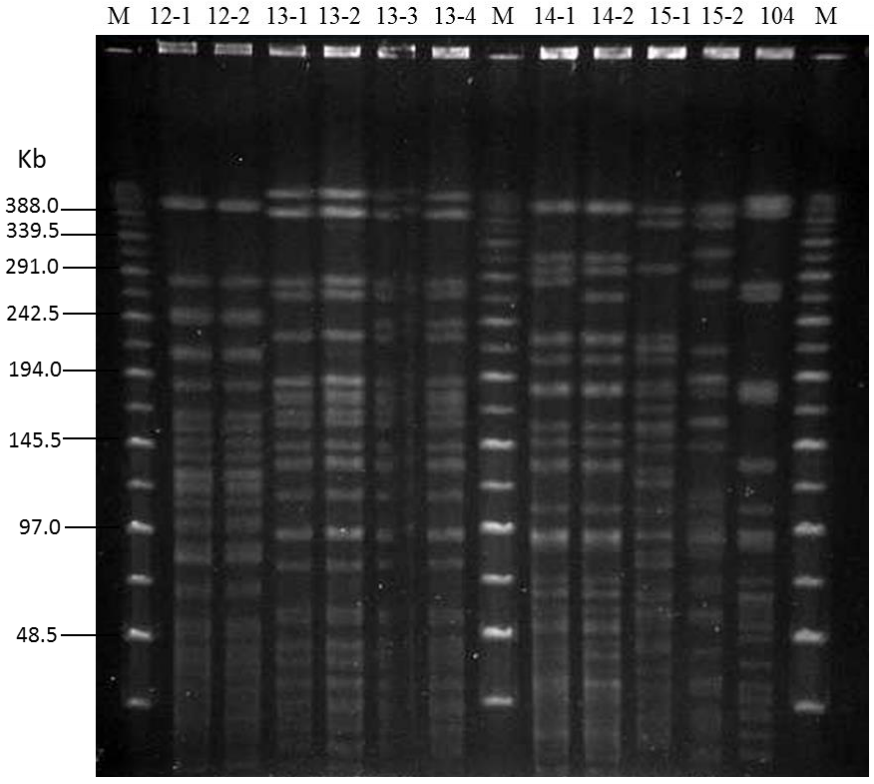


PFGE profiles of MAC clinical isolates with SnaBI; A) Patients 1-4; B) Patients 5-8; M = Molecular weight marker; 104 = reference strain M. avium 104.

C

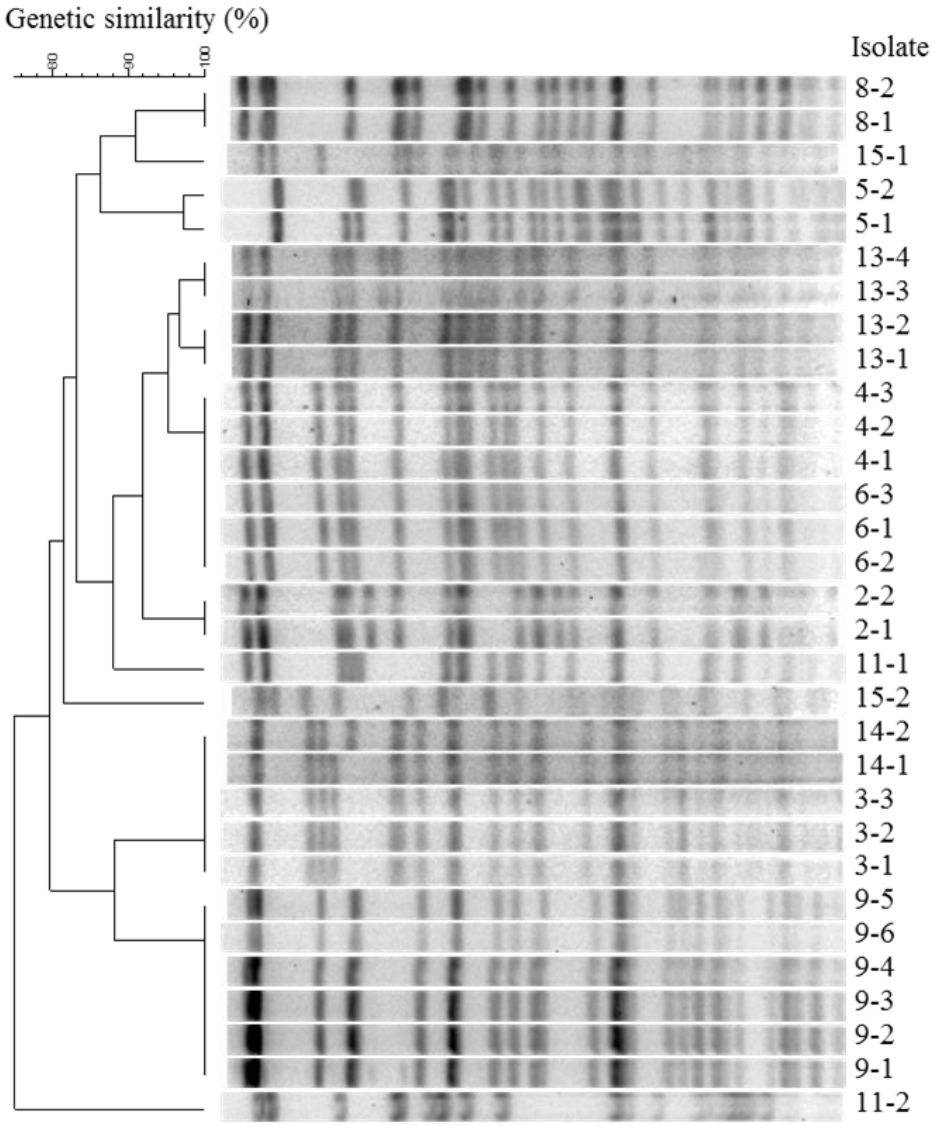


D



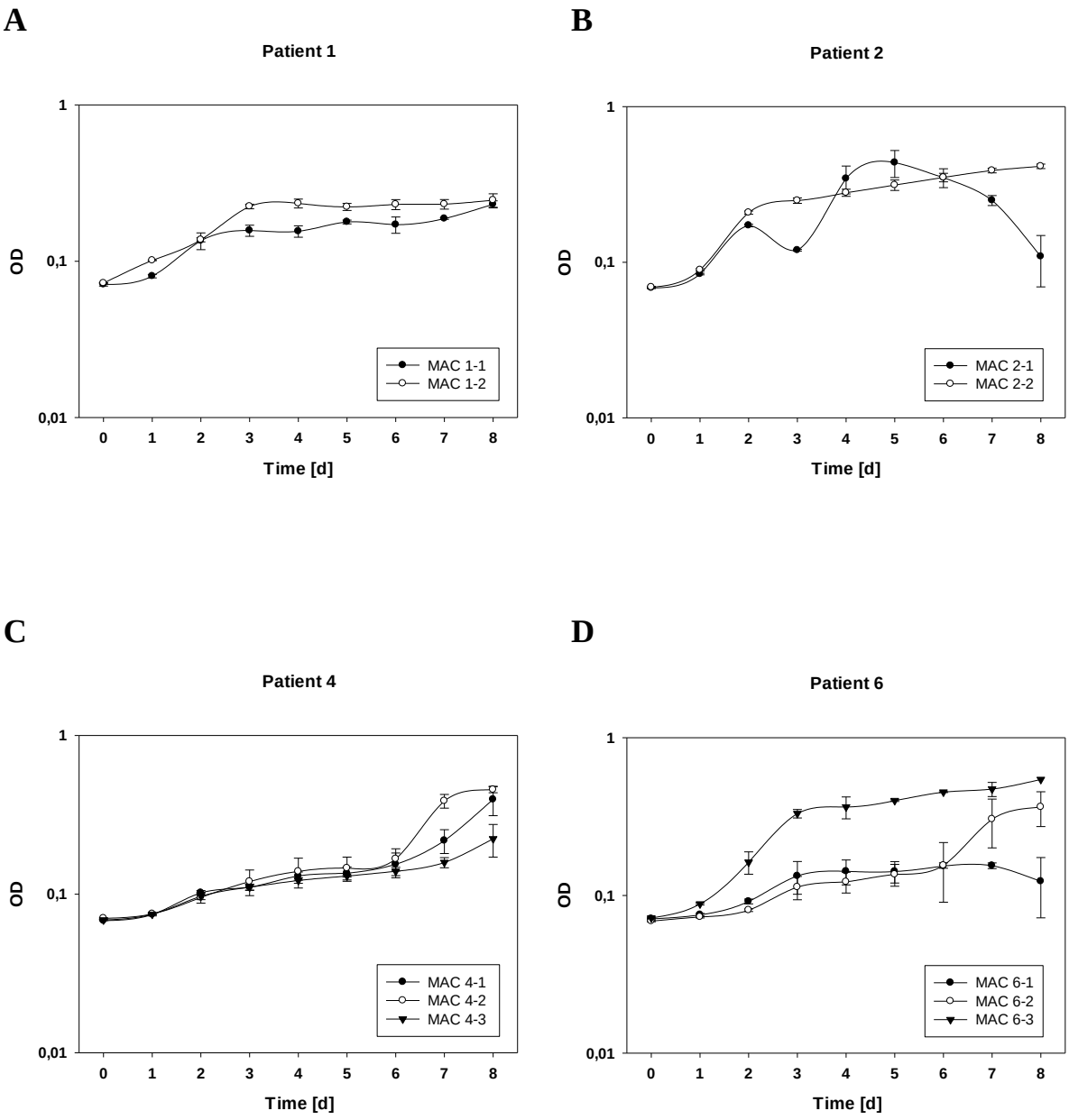
PFGE profiles of MAC clinical isolates with SnaBI; C) Patients 9-11; D) Patients 12-15; M = Molecular weight marker; 104 = reference strain M. avium 104.

Appendix II: Genetic similarity of MAC isolates



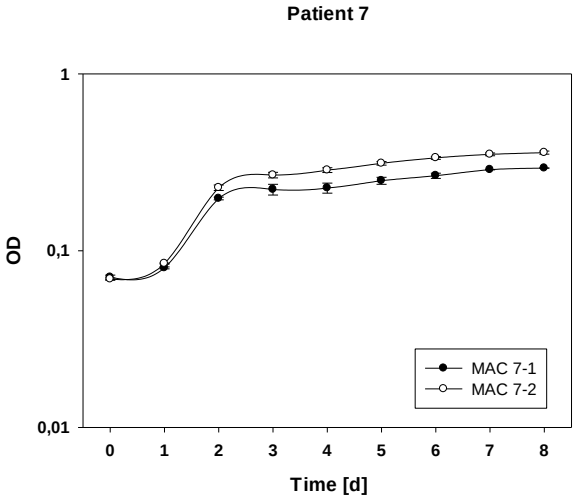
Genetic similarities of 31 *M. avium* clinical isolates and their associated SnaBI PFGE profiles; Dendrogram generated by the UPGMA method using the Dice similarity coefficient (Figure provided by Kathleen Lilleness).

Appendix III: Growth curves of MAC strains

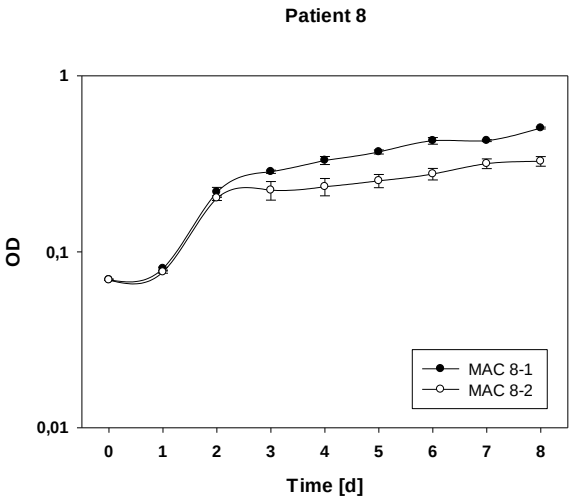


A-D) Growth curves of bacterial strains isolated from patients. The legend shows strains in chronological order of collection from the patients. The experiment was run in triplicates and the mean of the triplicates with the standard deviation is shown.

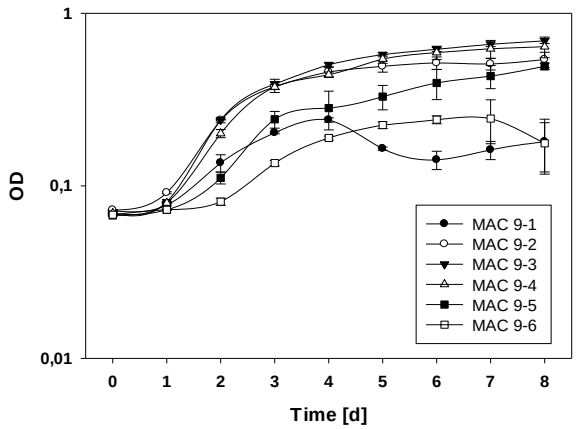
E



F



G



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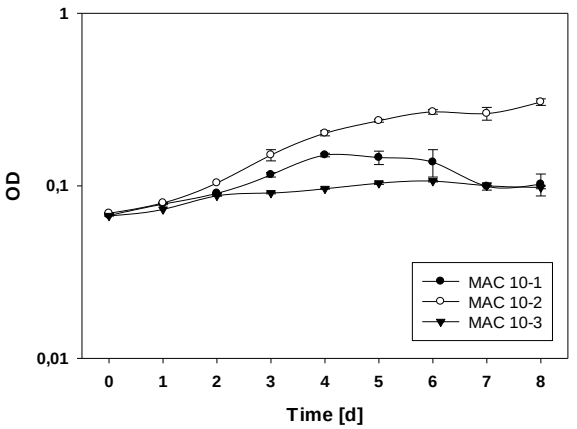
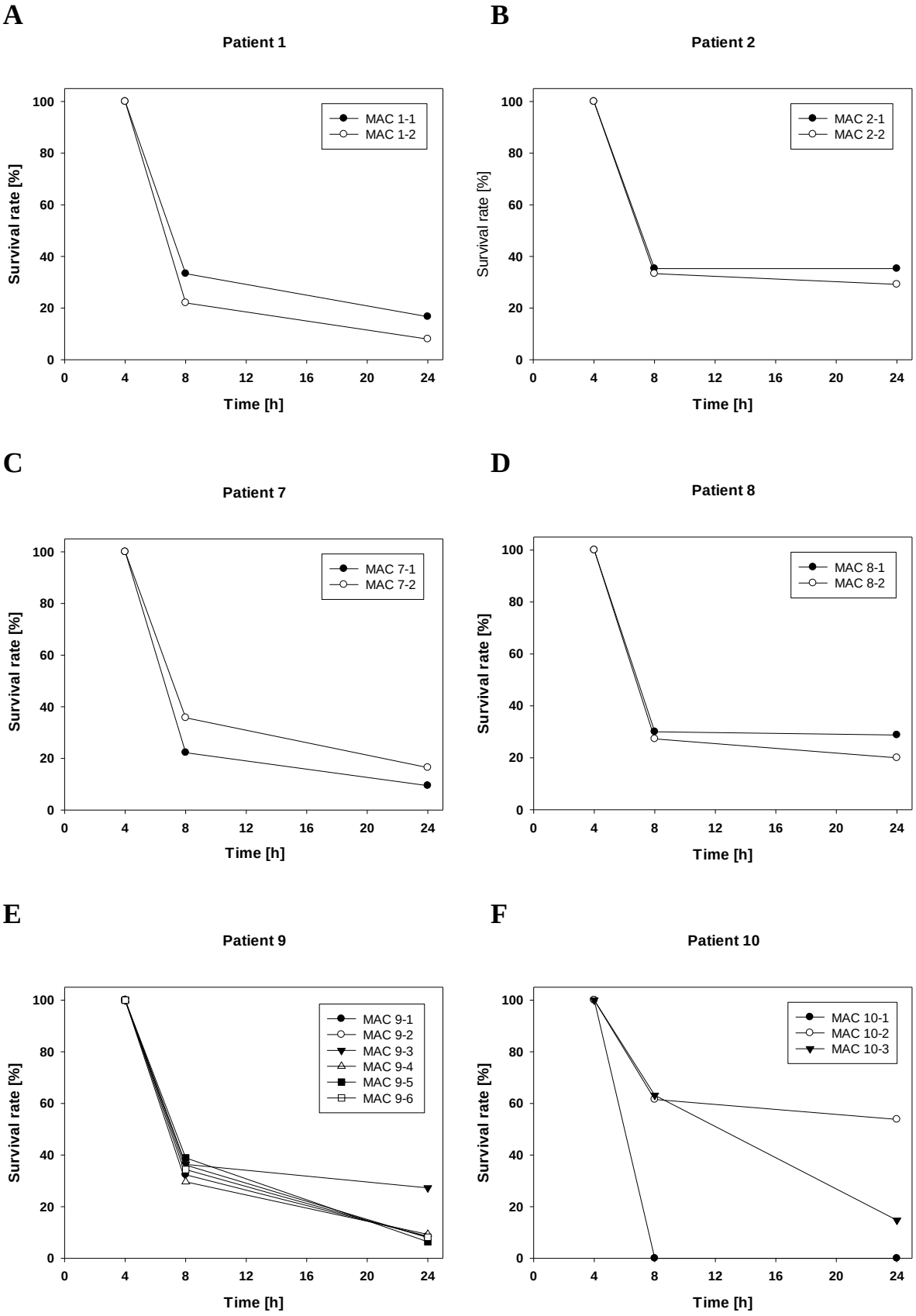


E-H) Growth curves of bacterial strains isolated from patients. The legend shows strains in chronological order of collection from the patients. The experiment was run in triplicates and the mean of the triplicates with the standard deviation is shown.

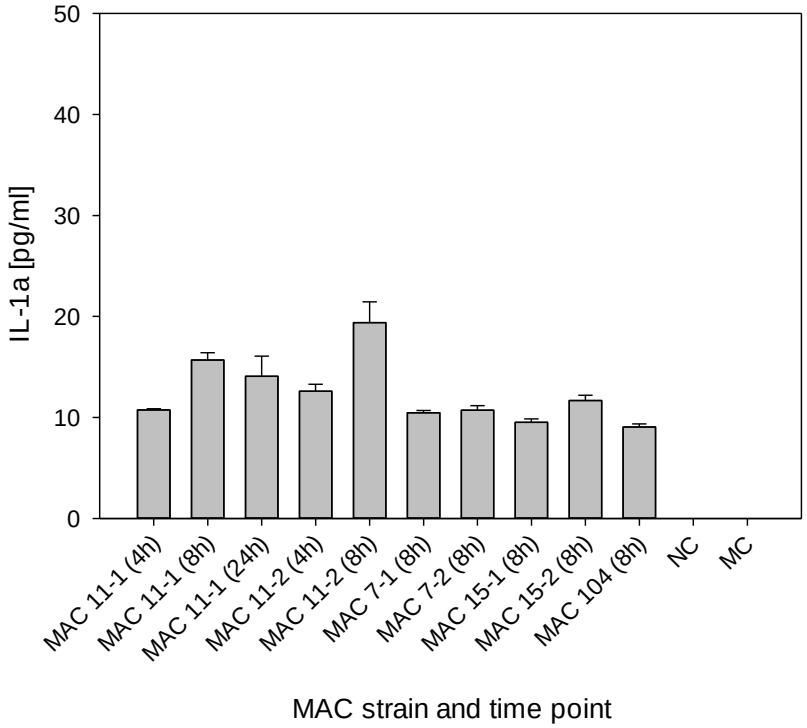
Appendix IV: Intracellular survival of MAC strains



The infection ratio macrophage to mycobacteria was 1:50. Not engulfed bacteria were removed 4h p. inf. and therefore this time point was chosen for a relative survival rate of 100%. The values at 8h and 24h p. inf. were calculated from the decrease in bacterial numbers compared to 4h p. inf.

Appendix V: Multiplex immunoassay for all tested cytokines

A



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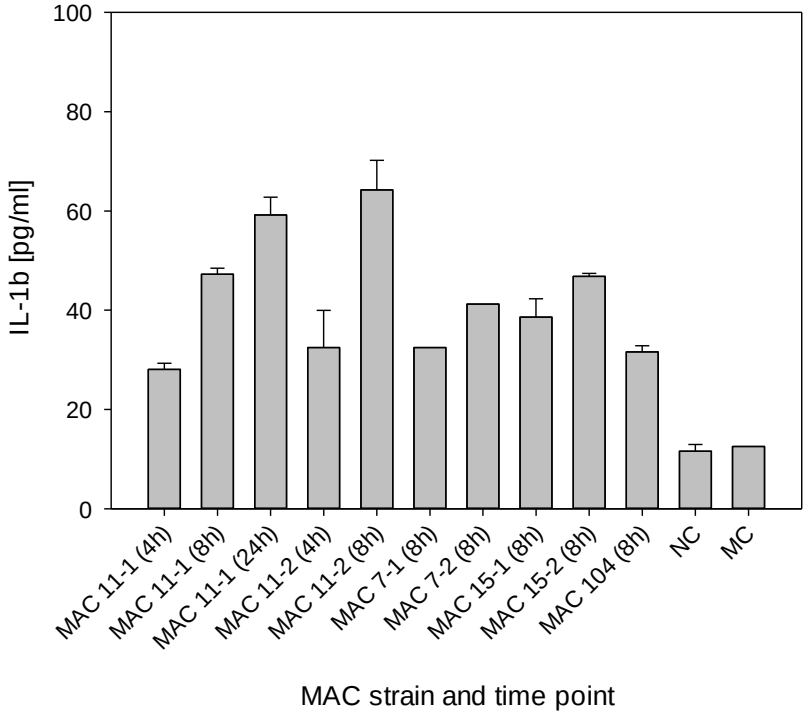
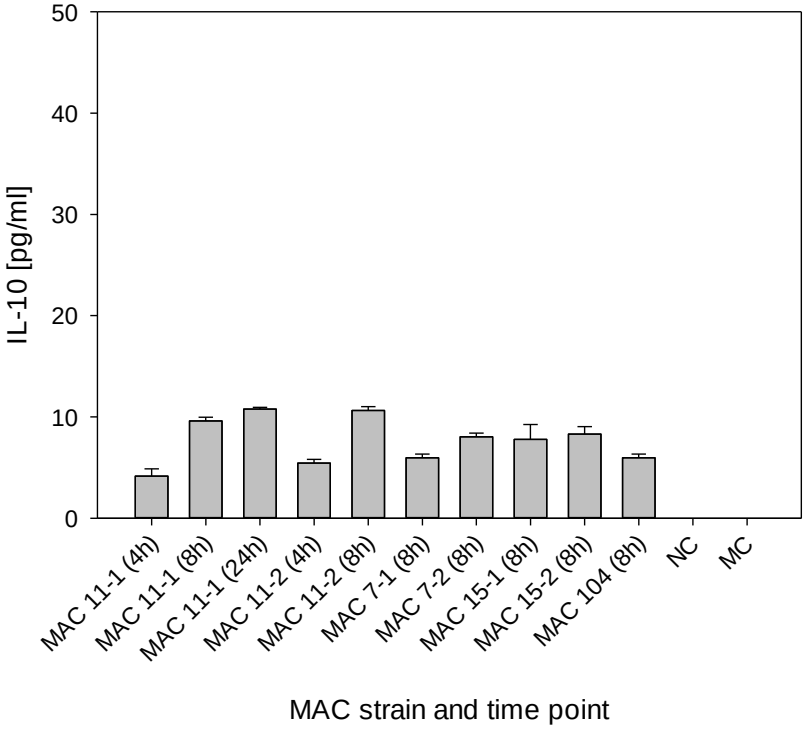
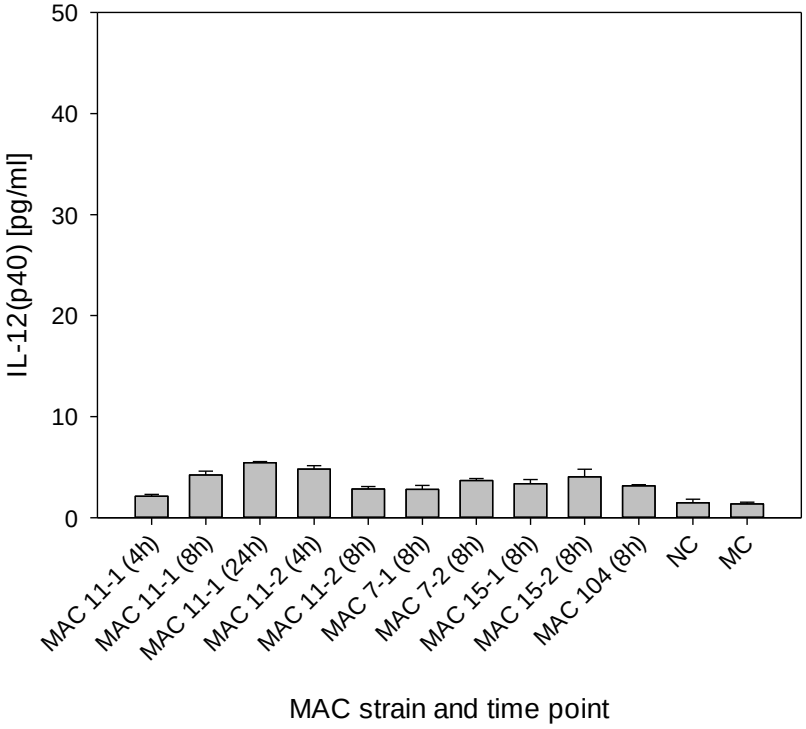


Supernatants were collected from RAW 264.7 macrophages infected with different M. avium strains at several time points after initial infection. The infection ratio macrophage to mycobacteria was 1:50. A) IL-1α concentration in pg/ml; B) IL-1β concentration in pg/ml; NC – negative control; MC – media control; The samples were run in duplicates and the standard deviation of these duplicates is shown.

C

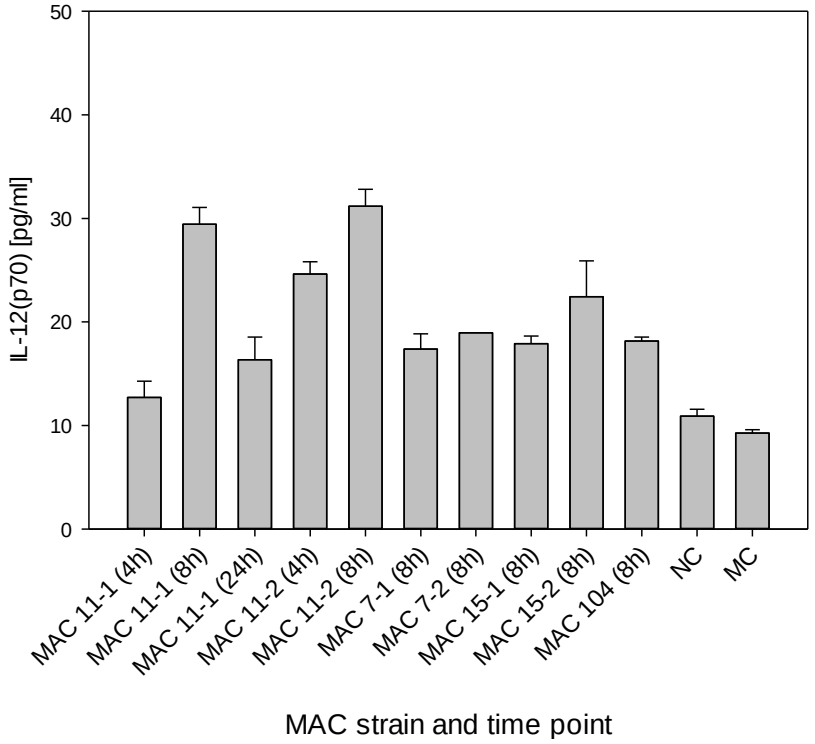


D

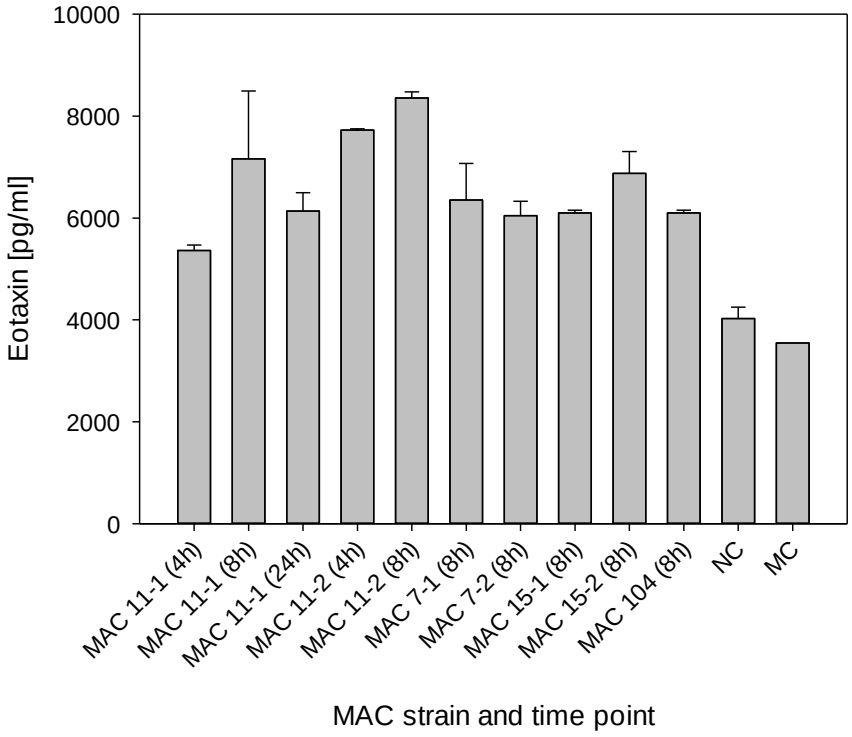


Supernatants were collected from RAW 264.7 macrophages infected with different M. avium strains at several time points after initial infection. The infection ratio macrophage to mycobacteria was 1:50. C) IL-10 concentration in pg/ml; D) IL-12(p40) concentration in pg/ml; NC – negative control; MC – media control; The samples were run in duplicates and the standard deviation of these duplicates is shown.

E

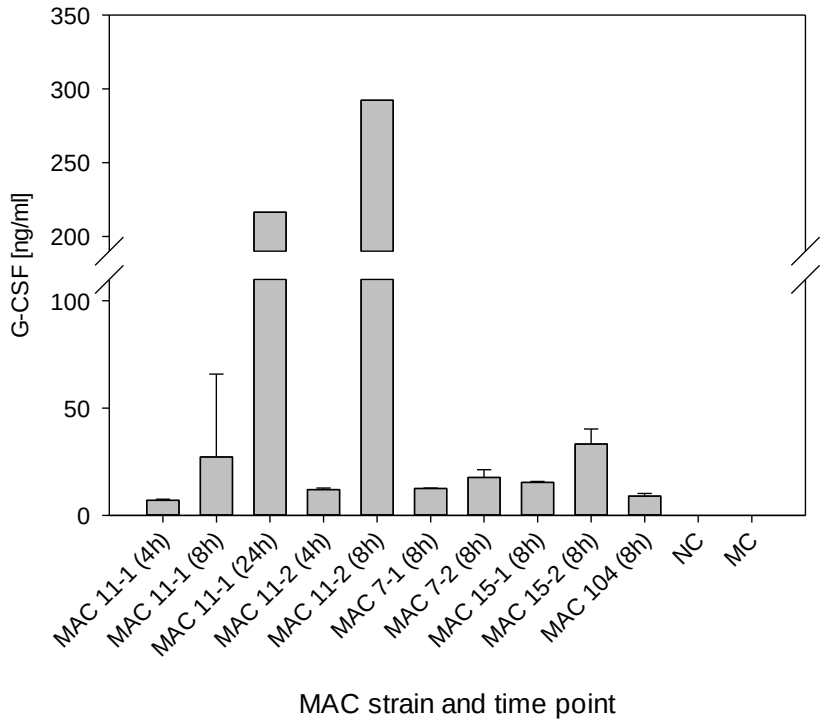


F

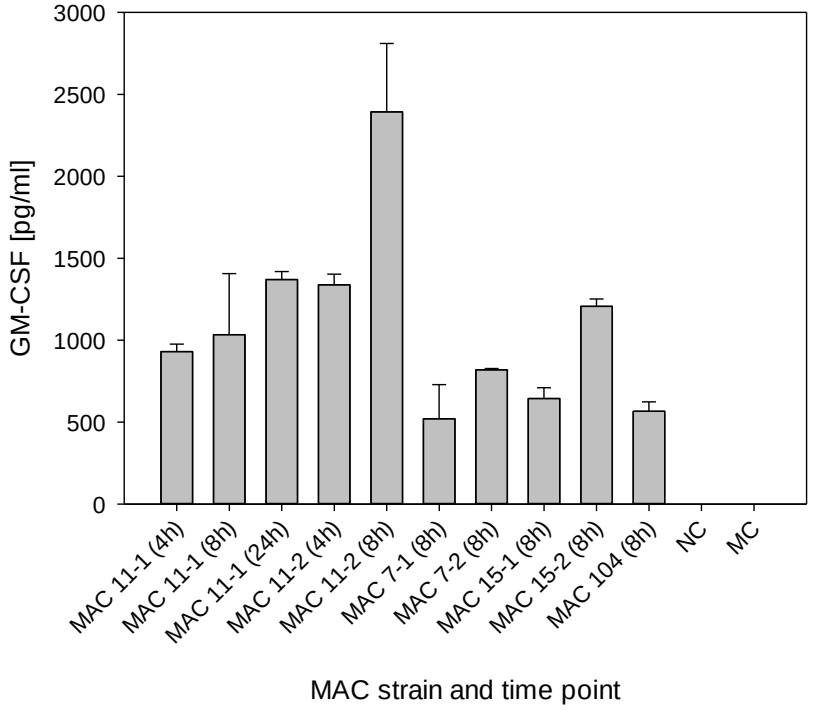


Supernatants were collected from RAW 264.7 macrophages infected with different M. avium strains at several time points after initial infection. The infection ratio macrophage to mycobacteria was 1:50. E) IL-12(p70) concentration in pg/ml; F) Eotaxin concentration in pg/ml; NC – negative control; MC – media control; The samples were run in duplicates and the standard deviation of these duplicates is shown.

G

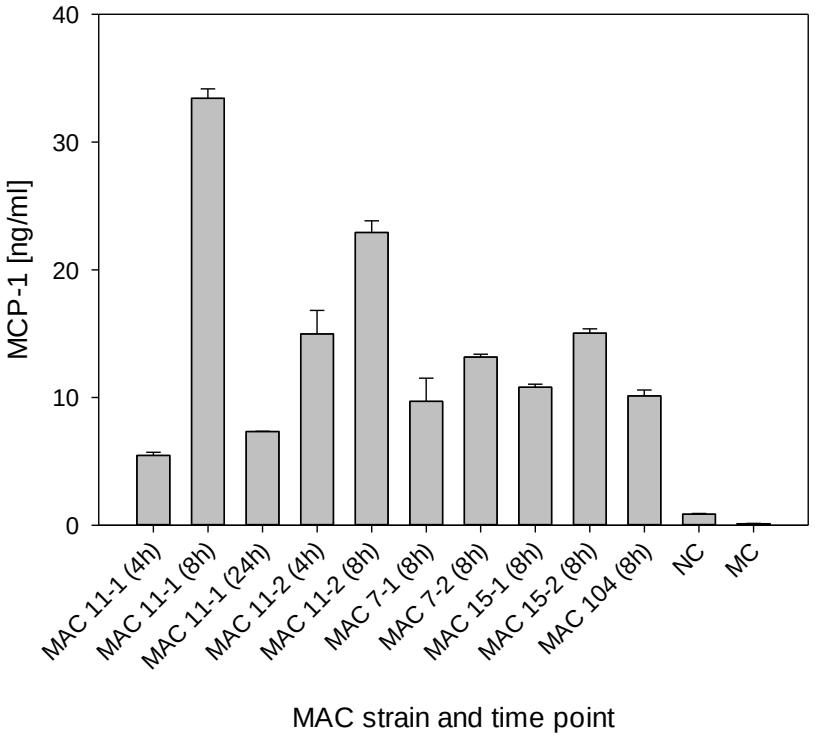


H

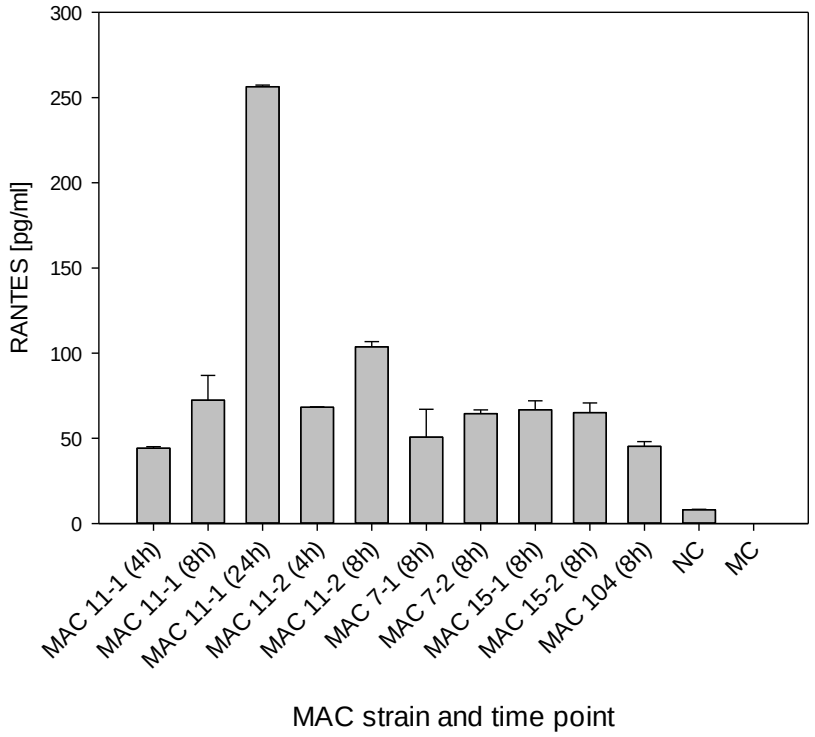


Supernatants were collected from RAW 264.7 macrophages infected with different M. avium strains at several time points after initial infection. The infection ratio macrophage to mycobacteria was 1:50. G) G-CSF concentration in ng/ml; H) GM-CSF concentration in pg/ml; NC – negative control; MC – media control; The samples were run in duplicates and the standard deviation of these duplicates is shown.

I

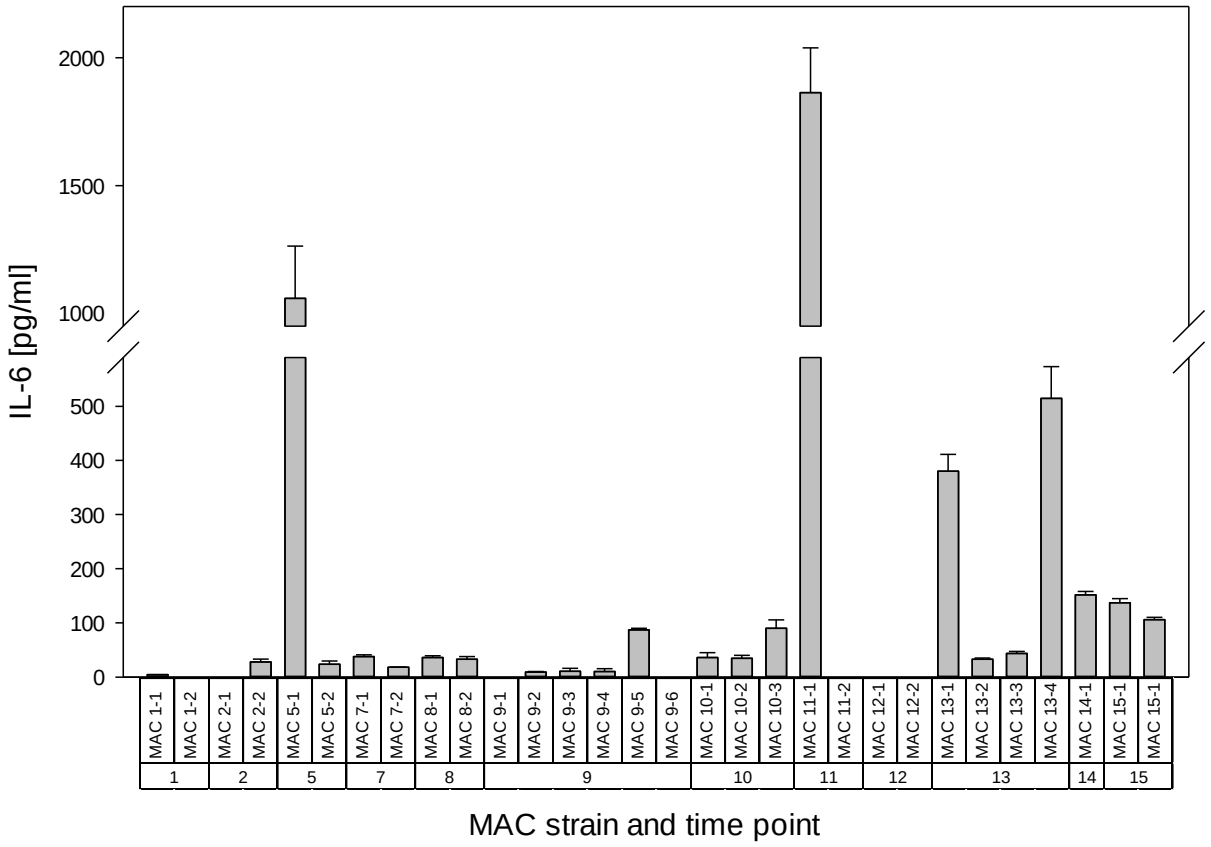


J



Supernatants were collected from RAW 264.7 macrophages infected with different M. avium strains at several time points after initial infection. The infection ratio macrophage to mycobacteria was 1:50. I) MCP-1 concentration in ng/ml; J) RANTES concentration in pg/ml; NC – negative control; MC – media control; The samples were run in duplicates and the standard deviation of these duplicates is shown.

Appendix VI: IL-6 ELISA with 24h supernatants



ELISA was carried out with a 1:50 macrophage – mycobacteria ratio. Supernatants were collected 24h p. inf. The samples were run in triplicates and the standard deviation from these triplicates is shown. Data from one of two experiments is shown.