Epidemiological and immunological studies of environmental mycobacteria

- with focus on *Mycobacterium abscessus* -

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ABSTRACT

Mycobacterium avium, *M. abscessus* and *M. gordonae* are three mycobacterial species that are ubiquitous in the environment. *M. gordonae* is non-pathogenic, while *M. avium* and *M. abscessus* can cause skin abscesses and airway disease, the latter mainly in patients with cystic fibrosis. The aim of this thesis was to examine the molecular epidemiology of *M. abscessus* and interactions of environmental mycobacteria with the human immune system. *M. abscessus* isolates from the airways of cystic fibrosis patients and from skin infections in patients injured during the tsunami catastrophe in Thailand 2004 were analysed by pulsed-field gel electrophoresis (PFGE). Almost all patients had unique strains, indicating that patient-to-patient transmission was rare. *M. abscessus* exhibits two colony variants; smooth and rough, the latter thought to be more virulent. Rough isolates dominated in chronic airway infection, while smooth strains caused wound infection or were transient colonizers of the airways, without associated symptoms. Environmental mycobacteria induced a unique cytokine pattern in human peripheral blood mononuclear cells (PBMC) characterized by very high levels of IL-17, high levels of IL-10, moderate levels of IL-23, relatively little IFN-γ, and no IL-12. The cytokine pattern induced by *M. abscessus*, *M. avium* and *M. gordonae* was identical, despite differences in pathogenic potential. Mycobacterial surface lipids and lipoarabinomannans induced very large amounts of IL-17, but down-regulated production of Th1 cytokines. Morphological analysis of PBMC interacting with *M. abscessus* showed that smooth isolates were readily phagocytosed, while rough strains formed multibacterial cords that escaped phagocytosis. Instead, monocytes appeared to entrap cord-forming bacteria in meshworks containing dsDNA and histones. These meshworks resembled NETs previously described in neutrophils, but to our knowledge, extracellular traps formed by monocytes have not previously been described. Rough isolates of *M. abscessus* induced significantly less IL-10 and IL-23, but more IL-1β than smooth isolates. Our results suggest that cord formation increases mycobacterial virulence and that mycobacterial cell wall lipids efficiently modulate innate responses and possess unique IL-17 inducing properties. The cellular basis for this IL-17 production remains to be determined.

Faculty Opponent: Professor Kristian Riesbeck
Department of Laboratory Medicine, Lund University, Malmö University Hospital, Sweden

LIST OF PUBLICATIONS

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals (I-V).

I. Jönsson B, Gilljam M, Lindblad A, Ridell M, Wold AE, Welinder-Olsson C. **Molecular epidemiology of *Mycobacterium abscessus*, with focus on cystic fibrosis.** J Clin Microbiol. 2007; 45(5): 1497-1504.

II. Appelgren P, Farnebo F, Dotevall L, Studahl M, Jönsson B, Petrini B. **Late-onset posttraumatic skin and soft-tissue infections caused by rapid-growing mycobacteria in tsunami survivors.** Clin Infect Dis. 2008; 15 Jul, 47(2): e11-6.

III. Jönsson B, Ridell M, Wold AE. **Environmental mycobacteria are uniquely strong inducers of IL-17 production – role of cell wall lipids.** Submitted.

IV. Jönsson B, Telemo E, Bylund J, Johansson BR, Wold AE. **Frustrated phagocytosis of mycobacterial aggregates and formation of monocyte extracellular meshwork.** In manuscript.

V. Jönsson B, Ridell M, Wold AE. **Frustrated phagocytosis and altered cytokine pattern in response to rough colony variants of *Mycobacterium abscessus*.** Submitted.

To Niklas & Ann-Margret

ABBREVIATIONS

aa	amino acid
AIDS	acquired immune deficiency syndrome
BCG	*Bacille Calmette-Guérin*
CD	cluster of differentiation
CRP	C-reactive protein
dsDNA	double-stranded DNA
FITC	fluorescein isothiocyanate
G+C	guanine+cytosine
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mRNA	messenger RNA
NK cell	natural killer cell
NKT cell	natural killer T cells
NOD	nucleotide-binding oligomerisation domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPD	purified protein derivative
RT	room temperature
Th	T helper
TNF	tumour necrosis factor

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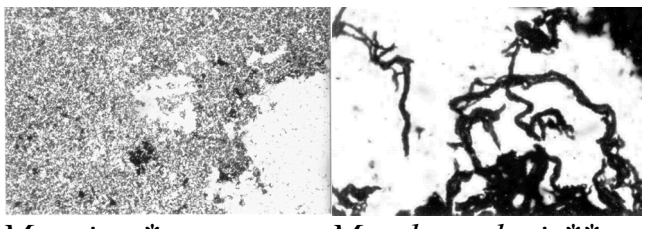
INTRODUCTION

THE MYCOBACTERIA

The family *Mycobacteriaceae,* with its single genus *Mycobacterium*, is included in the phylum *Actinobacteria*, which comprises Gram-positive bacteria with high G + C content (51-70%) in their DNA (172, 242). *Bifidobacterium*, *Nocardia*, *Corynebacterium*, and *Streptomyces* are examples of other genera belonging to this phylum (242). The three genera *Corynebacterium*, *Nocardia*, and *Mycobacterium* form a group within the *Actinobacteria,* characterized by a lipid rich cell wall containing mycolic acids (71). Within the genus *Mycobacterium* about 85 species of have been identified since the isolation of *M. leprae* in 1873 (33, 242). The majority of mycobacterial species are able to grow on simple substrates and optimal growth temperatures vary between 30° and 45 °C (172, 242). Their growth rate is however, slow, with generation times ranging from two to more than twenty hours depending on species (172).

Members of the genus *Mycobacterium* are impermeable to the dyes used in Gram stain, due to their lipid rich cell wall, but are instead stained with dyes such as fuchsin and Nachtblau. These dyes are retained by the mycolic acids after exposure to acid-alcohol, a phenomenon termed acid-fastness, the basis for special staining methods such as the Ziehl-Neelsen, Hallberg and auramine techniques (115, 172).

Mycobacteria are aerobic, non-spore-forming and non-motile rods, 1-10 μm long. Usually they appear as single cells, but within a few species like *M. tuberculosis*, the bacteria line up in organized multi-cellular aggregates, termed cords, when cultured *in vitro* (173).



*M. avium** *M. tuberculosis***

Figure 1. *Microscopic appearance of mycobacteria. Hallberg stain. *growing as single cell; **forming cords (1,000 x magnification)*

Most mycobacteria are free-living saprophytes in soil and water environments. Two species are obligate pathogens (*M. tuberculosis* and *M. leprae*) (172, 242), others are regarded as practically apathogenic, while still others are opportunistic pathogens, which can cause infections in susceptible hosts (78). Below some mycobacterial species will be described.

Mycobacterium tuberculosis

M. tuberculosis is a member of the *M. tuberculosis*-complex which also comprises the species *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. pinnipedii* (53, 172). *M. tuberculosis* was discovered as the cause of tuberculosis by the German microbiologist Robert Koch in 1882 (143), but has now been identified in prehistoric remains of humans, indicating a co-evolution of this bacterium with its human host over a long time (108). Today, it is estimated that nearly one third of the Worlds population is infected with tuberculosis (52).

The initial infection usually occurs by inhalation of droplets generated when patients with pulmonary tuberculosis cough (161). In the lungs, *M. tuberculosis* bacilli are engulfed by alveolar macrophages. Failure to kill *M. tuberculosis* leads to establishment of a chronic infection. Normally the cell-mediated immune defence of the host limits bacterial spread. Bacteria may, however, remain viable but dormant for decades. If the cell-mediated defence is weakened, as in HIV infection, tuberculosis bacilli may spread in the entire body and kill the host (161, 172).

Mycobacterium leprae

M. leprae as the infectious cause of leprosy was discovered in 1873 by Gerhard Hansen, a physician at the leprosy hospitals of Bergen, Norway (33, 167). Leprosy is a chronic, granulomatous disease, whose principal manifestations are anesthetic skin lesions and peripheral neuropathy with nerve thickening (3). Transmission of disease is thought to occur by shedding of bacilli from the nose (172). The global prevalence of leprosy is estimated to 0.2 million cases. Treatment with antibiotics have reduced leprosy by 90% over the last 20 years (246).

Environmental mycobacteria

Mycobacteria other than *M. tuberculosis* and *M. leprae* are termed "atypical" or "environmental" mycobacteria. Despite their ubiquitous occurrence in many ecosystems, environmental mycobacteria rarely cause overt disease. However, to date about 50 species of environmental mycobacteria have been reported to be opportunistic human pathogens (235). The incidence of infection by environmental mycobacteria is 1-2 cases per 100,000 individuals in industrialized countries (100). Environmental mycobacteria are thought to be acquired from environmental exposure, but the specific source of infection is seldom identified (250). The most common diseases are chronic pulmonary infections, infections in skin, soft tissue, bone and joints, and lymphadenitis in young children. More seldom disseminated infection may occur (125).

Risk factors for contracting disease from environmental mycobacteria are pre-existing pulmonary diseases such as silicosis and pneumoconiosis, thoracic structural abnormalities, cystic fibrosis, HIV-infection, malignancy, and immuno-suppression associated with organ transplantation. Individuals with mutations in the genes encoding the interferon-γ receptor, the IL-12 receptor or IL-12 also have increased susceptibility to environmental mycobacteria (40, 65, 78).

In this thesis, three species of environmental mycobacteria were studied: *M. avium* (member of the *M. avium*-complex), *M. abscessus* and *M. gordonae*. The former two are opportunistic pathogens causing disease in selected risk groups, while *M. gordonae* is regarded as practically apathogenic. The three species and their disease spectrum are described below.

The *Mycobacterium avium*-complex

The *M. avium*-complex (MAC) consists of two distinct species, *M. avium* and *M. intracellulare*, and probably, in addition, a third species referred to as the MAC-X strain. *M. avium* is in turn, divided into three subspecies – subsp. *avium*, subsp. *paratuberculosis*, and subsp. *silvaticum* (172). MAC are slow growers with visible colonies appearing only after 10-21 days on Middlebrook 7H10 agar (172). MAC has been isolated from water, aerosols, soil, plants and animals. They are important pathogens in poultry and swine (78, 172). Both *M. avium* and *M. intracellulare* are able to grow in phagocytic protozoa, and their virulence is enhanced by this intracellular passage (78).

Infections caused by MAC

MAC was first isolated in humans in the 1940s (172) but is now the most frequent environmental mycobacterium associated with human disease (78, 100, 172). A number of disease manifestations are recognized, including:

1. ***Cavitary lung disease.*** This is the traditional form of lung disease with apical location affecting males in their fifties with a history of smoking and excessive alcohol use (43, 100).

2. "Lady Windermere syndrome" localized bronchiectasis with nodular and interstitial nodular infiltrates in the right middle lobe or lingua. This occurs in otherwise healthy Caucasian or Asian postmenopausal, non-smoking females (43, 100).

3. Lung infection in patients with cystic fibrosis (186) and patients with other structural lung defects, such as prior granulomatous disease - usually tuberculosis - with bronchiectasis (20).

4. Lung infection and generalized disease in patients with AIDS. The incidence of generalized disease is now decreasing due to more potent anti-HIV treatments (235).

5. Hot-tube-associated hypersensitivity pneumonitis, so called hot tube lung (103, 136).

6. Unilateral cervical lymphadenitis in children, 1-5 years of age, (183, 235).

Isolates of *M. avium* causing disease in humans produce hemolysin and fibronectin-binding proteins, which may be regarded as putative virulence factors (138, 168, 201).

Mycobacterium abscessus

M. abscessus is closely related to *M. chelonae* and *M. immunogenum* which together form the *M. chelonae/abscessus* group (31, 172). Over the last decades, there has been confusion regarding whether *M. abscessus* is a species or not. *M. abscessus* was first isolated in 1953, from a knee abscess (hence the name) and described as a novel species (177). This isolate is the type strain of *M. abscessus* (ATCC 19977). Thereafter, many investigators regarded *M. abscessus* as belonging to *M. chelonae*, because of nearly identical biochemical profiles, and the two taxa were classified as *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus*, in 1972. Genomic DNA-DNA hybridization studies, however, showed them to be

separate species with <70% genomic homology and both *M. abscessus* and *M. chelonae* regained species status, in 1992 (31, 150). Their 16S rDNA gene differs by only four base pairs, however (150). It is often difficult to know whether reports refer to *M. chelonae* or *M. abscessus*, especially during the period between 1972 and 1992 when little efforts were done to separate them, despite their subspecies status (30, 31).

M. abscessus is a rapid grower yielding visible colonies on Middlebrook 7H10 agar after 3-7 days (172). Colonies have either of two phenotypes, one smooth and shiny and one dry and wrinkled. The latter phenotype, termed rough, has reduced amounts of glycopeptidolipids in the cell wall (118). A rough mutant was reported to form cords, in contrast to the smooth wild type (118), and a rough mutant of the type strain has been reported to be hypervirulent when injected into mice (41).

M. abscessus is a natural inhabitant of soil and water, including municipal tap water (39). Up to 90% of biofilms taken from piped water systems contain mycobacteria belonging to the *M. chelonae/abscessus* group (213). The members of this group are also able to grow in disinfectants and biocides like organo-mercurials, chlorine, and glutaraldehyde (31, 223).

Infections caused by *M. abscessus*

M. abscessus is considered to be an emerging opportunistic pathogen involved in several types of infections:

1. Community-acquired pulmonary disease. This is a common form of disease caused by *M. abscessus*. The disease is often indolent and slowly progressive over years (100). Known risk factors are bronchiectasis, cystic fibrosis, gastroesophageal reflux, or prior granulomatous diseases like tuberculosis or sarcoidosis. However, the largest patient group are white, female non-smokers older than 60 years, with no predisposing conditions or history of previous lung disease (31, 144).

There is controversy about whether *M. abscessus* could colonize the lung without causing significant symptoms, but repeated isolation of *M. abscessus* from sputum samples is usually associated with significant lung disease (31).

2. Disseminated disease. This form is characterized by generalized infection with multiple nodular skin lesions and positive blood cultures (235, 237).

3. *Localized infections – abscesses.* *M. abscessus* may cause abscesses after direct contact between traumatized skin and contaminated soil or water (31). Examples of localized infections are skin and soft-tissue abscesses, lymphadenitis, otitis media, mastoiditis, and infections in bone and joints (177, 235, 237).

4. *Nosocomial outbreaks.* Nearly half of localized *M. abscessus* infections are believed to be iatrogenic (31). A large outbreak was reported from the United States, where 87 persons contracted abscesses after injection of an unlicensed injectable medicine (90). Minor outbreaks have been reported due to contaminated benzalkonium chloride used as skin disinfectant prior to intra-articular steroid injections (228) and by contaminated hospital water-systems (255). Contaminated bronchoscopes have caused transient pulmonary colonization by *M. abscessus*, and contamination of samples in mycobacteriological laboratories simulating outbreaks, so called "pseudo-outbreaks", have also been reported (24, 151, 236). In many of these outbreaks and "pseudo-outbreaks" strain typing revealed that *M. abscessus* isolates from the affected patients were identical with the strain found in the contaminated water supply or suspension (255).

5. *M. abscessus disease in cystic fibrosis.* A patient group which appears to be at major risk for contracting lung infection by *M. abscessus* is patients with cystic fibrosis (CF). CF is often referred to as the most common life-shortening inherited disorder (188), with an incidence in Sweden of 1/5600 live-births (153). Earlier, most patients died in early childhood due to infections (165), but today lifespan is predicted to > 50 years of age due to much improved infection control (63).

CF is an autosomal recessive genetic disorder in which a protein of 1480 aa, named the cystic fibrosis transmembrane conductance regulator (CFTR), has mutated. This protein is a chloride channel situated in the apical membrane of epithelial cells in exocrine glands. CFTR transports and plays a role in both secretion and reabsorption of ions and fluid at epithelial surfaces (4). There are currently about 1600 known CFTR mutations (257), the most common being a single amino acid deletion (ΔF508) (79).

Most CF-patients have chronic pulmonary disease due to altered airway mucociliary clearance, and chronic pulmonary inflammation accounts for much of the morbidity and almost all mortality in CF (54, 188). "Normal" pulmonary pathogens such as pneumococci are not a problem in this patient group (188), who instead acquire infections by *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*, and less commonly *Burkholderia cepacia*, *Stenotrophomonas maltophilia,* and environmental mycobacteria (54).

Infections with environmental mycobacteria were uncommon in patients with CF before 1990. Today, prevalence ranges from 4 to 20%. The most commonly isolated species are MAC (76%) and *M. abscessus* (18%) (100).

Clinical manifestations include increased and/or purulent sputa and declining FEV1 (forced expiratory volume) despite conventional antibiotics. Systemic manifestations include fever, malaise, and weight loss. High resolution computer scanning (HRCT) show small peripheral nodules, "tree in bud" formations and/or cavitation, progressing over time (69). The disease spectrum range from mild asymptomatic infection to severe disease with deteriorating lung function, and in some cases fatal outcome (99, 100, 211). *M. abscessus* is naturally multi-resistant and difficult to treat, and the result of antimycobacterial drugs is decreased bacterial load, rather than eradication (54, 100).

M. abscessus infection prior to lung transplantation is a risk factor in developing lung infection or disseminated disease after transplantation. However, mortality after transplantation appears not to differ between those with and without mycobacterial disease (44).

Mycobacterium gordonae

M. gordonae is a bright orange slowly growing mycobacterium yielding visible colonies on Middlebrook 7H10 agar after 10-25 days (172). It is frequently found in soil and water, municipal tap water included (172).

M. gordonae is regarded as practically non-pathogenic (70). Some cases have, however, been reported in immuno-compromised individuals (6, 208). *M. gordonae* is the environmental mycobacterium most commonly isolated in sputum samples in clinical mycobacteriological laboratories. It is usually considered to be a contaminant occurring in the patient sample, due to its frequent presence in municipal tap-water (100). A hospital "pseudo"-outbreak was caused by heavy contamination of refrigerated fountains with drinking water, yielding culture positive sputum samples with no clinical relevance (152).

THE BACTERIAL CELL WALL

The most common way to classify bacteria is according to their Gram-staining properties. Bacteria are stained with crystal violet, fixated with a iodine solution, destained with ethanol or acetone, and counterstained with safranin (172). Gram-positive bacteria retain the crystal violet-iodine complex inside, while Gram-negative bacteria are decolourized (194).
This difference in staining properties is due to differences in the cell wall composition of Gram-positive and Gram-negative bacteria. The cell wall of Gram-positive bacteria is composed of a thick homogenous layer of peptidoglycan, which is a polymer of the sugars N-acetyl-glucosamine and N-acetylmuramic acid linked together by peptide inter-bridges. The crystal violet-complexes are entrapped by

these cell wall meshes. In addition, the accessory polymers teichoic and lipoteichoic acid are usually present (23).

The cell wall of Gram-negative bacteria has a much thinner peptidoglycan layer, with fewer peptide inter-bridges, and an outer membrane composed of phospholipids, proteins and lipopolysaccharide (LPS) a molecule unique to Gram-negative bacteria (23). The thin peptidoglycan layer is not able to retain the crystal violet-complexes.

Mycobacteria are Gram-positive bacteria, but their cell wall differs strikingly from classical Gram-positives, and they are not readily Gram stained (148, 172).

Mycobacteria have a unique cell wall. The mycobacterial cell wall is highly lipophilic and has low permeability. These properties contribute to the intrinsic resistance of mycobacteria to most antibiotics and its capacity to survive harsh environments (67).

Figure 2 shows a schematic picture of the mycobacterial cell wall. The core, or the inner leaflet, contains peptidoglycan. A branched polysaccharide, termed arabinogalactan, is attached to the peptidoglycan layer by ester bonds. The arabinogalactan is in turn covalently linked to mycolic acids. The outer leaflet is composed of extractable lipids, that are not covalently linked to the core (27, 116, 148).

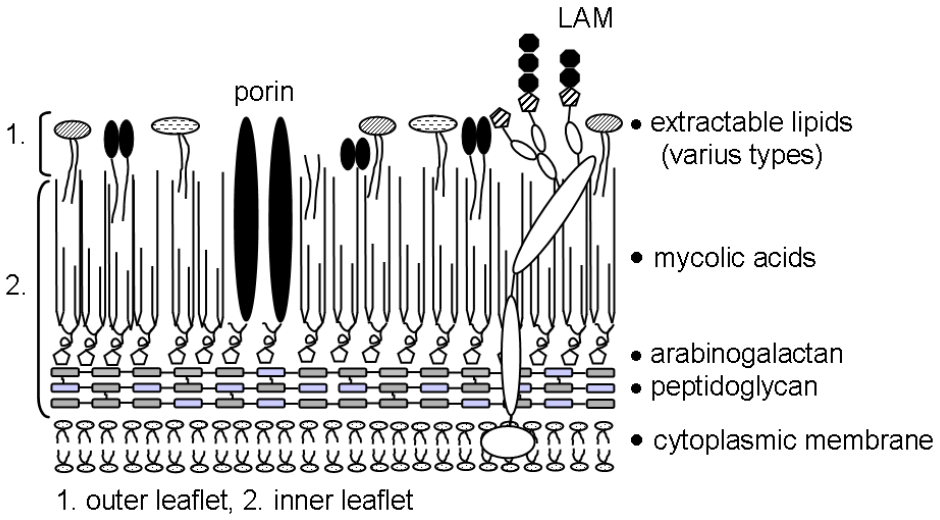


Figure 2. *Schematic picture of the mycobacterial cell wall. Adapted from references: (27, 67, 116, 148).*

Mycolic acids

The mycolic acids are major components of the mycobacterial cell wall forming a tightly packed layer. They are branched fatty acids with up to 56 carbon atoms in one branch and 20-24 carbon atoms in the other. Some mycolic acid types are of general occurrence among mycobacteria, while others are species specific (148, 175). Characterization of the mycolic acid composition of the cell wall by high pressure liquid chromatography can thus, be used to identify mycobacterial species (36).

Lipoarabinomannan

Lipoarabinomannans (LAM) are long branched lipoglycans composed of the sugars D-mannan and D-arabinan, attached to a phosphatidyl-*myo*-inositol unit, which anchors it to the plasma membrane. The other end is exposed on the cell wall surface. Different mycobacterial species have differentially modified LAMs. *M. tuberculosis*, *M. leprae*, and *M. avium* have short mannose containing oligosaccharide "caps" on the arabinosyl side chains (Fig. 3). This form is called ManLAM. In other species, such as *M. chelonae,* mannose caps are absent and the LAM is termed AraLAM. In *M. smegmatis,* arabinosyl side chains are terminated by inositol phosphate, and called PILAM (27, 148). A truncated form of LAM without the arabinan part, called lipomannan, is also present in the cell wall (22).

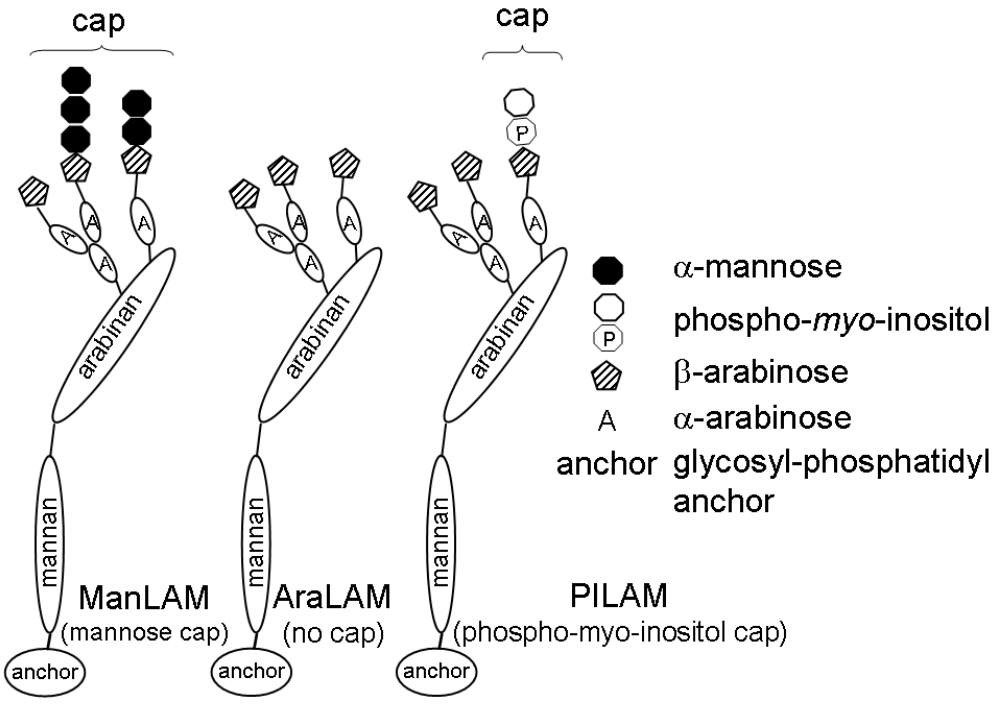


Figure 3. *Examples of lipoarabinomannans: ManLAM from M. tuberculosis, AraLAM from M. chelonae, and PILAM from M. smegmatis. Adapted from references: (57, 101).*

Extractable complex lipids

The outer leaflet of the mycobacterial cell wall consists of free lipids associated with the mycolic acid monolayer, thus forming a bilayer that acts as a permeability barrier against hydrophilic substances. An extraordinary variety of unique lipid structures are found within this group, including trehalose-6,6'-dimycolate, sulpholipids, phthiocerol-dimycocerosates, phenolic-glycolipids, and glycopeptidolipids. Both common mycobacterial and species specific types exist. The patterns of complex extractable lipids can, thus, be used for speciation after separation by gas-liquid chromatography (27, 148, 229). Examples of biological effects proposed to relate to these extractable mycobacterial cell wall lipids are summarised in Table I.

Trehalose 6,6' – dimycolate (TDM) consists of two mycolic acids linked to the disaccharide trehalose. It constitutes only a relatively small amount of the mycolic acids in the cell wall, but is released in high amounts by virulent *M. tuberculosis* (121, 148). Although this lipid is commonly known as the "cord factor", little evidence is found that it contributes to cord formation. However, it has been reported to fill the open spaces between bacteria in cord formations (122). It has also been proposed to induce production of cytokines and to trigger granuloma formation (102).

The sulpholipids consists of sulphated trehalose coupled to multi methyl-branched fatty acids (148). They have been reported to inhibit phagosomal maturation, but also to increase oxygen radical production (Table I).

Phthiocerol dimycocerosates are major mycobacterial waxes. They are unusual apolar lipids consisting of two fatty acids, esterified to a phthiocerol backbone (27, 148). Apart from acting as permeability barrier no biological functions has been ascribed this group.

The phenolic glycolipids are related to phthiocerol dimycocerosates, but the lipid core is terminated by an aromatic nucleus, probably derived from *para*-hydroxy benzoic acid, which is linked to short oligosaccharides. They are present in the cell wall of *M. bovis*, *M. bovis* BCG, *M. leprae*, and the *M. tuberculosis* "canetti" strain, while most *M. tuberculosis* (sensu stricto) are devoid of these lipids (148). They stimulate TNF and oxygen radical production (Table I).

The glycopeptidolipids also referred to as C-mycosides, are among the most unique of the mycobacterial glycolipids. They contain a tripeptide amino-alcohol core with two monosaccharide residues linked to long chain fatty acids. The glycopeptidolipids constitute the chemical basis of the 31 serovars of *M. avium*, but the lipids are also present in other mycobacterial species (48, 74, 202). Addition of

glycopeptidolipids to stimulated T cell cultures down-regulates IFN-γ production (Table I).

Table I. Examples of extractable mycobacterial cell wall lipids and some of their biological effects.

Lipid group	Biological effects	References
Trehalose dimycolate	Involved in granuloma formation and caseation Induces IL-1β TNF, IL-6, and IL-12 production in murine macrophages	(102, 121, 124, 191)
Sulpholipids	Proposed to inhibit phagosomal maturation; how is still unclear Induces production of oxygen radical production in neutrophils	(27, 254)
Phthiocerol dimycocerosate	Bacterial permeability barrier	(219)
Phenolic glycolipids (PGLs)	Induce TNF in human monocytes Induce antibody formation in leprosy patients Induces production of oxygen radical production in neutrophils	(47, 49, 77)
Glycopeptidolipids	Reduce IFN-γ production by human PBMC	(117)

STRAIN TYPING OF MYCOBACTERIA

Species identification of mycobacteria is done by both phenotypic and DNA-based methods. For epidemiological purposes, strain typing of bacterial isolates belonging to the same species is needed to reveal relatedness and indicate the route of transmission. A strain is a bacterial clone consisting of genetically identical individuals, with the exception of recent mutations. The members of a clone have a common origin.

Strain typing methods need to be accurate and highly discriminatory. They also have to be stable in performance, to enable epidemiological investigations over several years (172). During the last decades, several molecular strain typing methods have been developed, which can meet these demands, including restriction length polymorphism (RFLP) methods, spoligotyping, whole genome typing with pulsed-field gel electrophoresis (PFGE), and random amplified polymorphic DNA PCR (RAPD) (172).

1. IS*6110* RFLP

IS*6110* RFLP is considered to be the "gold standard" for typing strains of *M. tuberculosis*. It uses a probe binding to the repetitive insertion sequence IS*6110* which is only present in the *M. tuberculosis*-complex. Both copy number (0-25) and position on the chromosome varies between strains. A shift in one band occurs over a 3-4 year period in half of the investigated strains (59, 240). A related method, IS 1245 RFLP, has been used in strain typing of *M. avium* (14).

2. Spoligotyping

Spoligotyping i.e. "spacer oligo-typing", is based on polymorphism in 36-base pair repeats interspersed between non-repetitive spacers. Strains vary in number of repeated elements and are differentiated by their hybridization patterns. This method can only be used on strains belonging to the *M. tuberculosis*-complex, and is the method of choice for sub-typing of *M. bovis* (32, 82, 205).

3. Pulsed-field gel electrophoresis (PFGE)

PFGE is a whole genome technique, which means that no knowledge of specific gene sequences is needed. Restriction endonucleases that cut DNA infrequently are used to digest the genome, yielding large DNA fragments (50-750 bp). Such long fragments cannot be separated by conventional agarose gel electrophoresis. Instead, a special electrophoretic procedure with alternating electric fields is used to separate these large fragments. PFGE has been used for sub-typing of several mycobacterial species. Interlaboratory comparison is possible, but the method requires sophisticated software for image analyses (35, 170, 214, 238, 248).

4. Random amplified polymorphic DNA (RAPD) PCR

RAPD is a PCR based method in which one or a few short primers of arbitrary sequence are allowed to bind under low stringency conditions to various sites on both strands of the bacterial genome. Amplification yields PCR products of varying size, which yields band patterns after separation by electrophoresis. This method has been used to confirm nosocomial outbreaks caused by *M. abscessus*. Due to low reproducibility, isolates of interest need to be compared on the same gel. Further, the risk of misinterpretation is greater than for PFGE due to variation in band density. Its advantages are speed and low costs (151, 255).

IMMUNE DEFENCES IN MYCOBACTERIAL INFECTION

Innate immunity

The innate immune system is present in all animal phyla and has an intrinsic ability to recognize conserved microbial structures, so called danger signal, through pattern recognition receptors. Interaction between danger signals and their cell-bound or soluble receptors initiates production of a range of inflammatory mediators, collectively inducing the classical inflammatory response (83, 91, 96).

The main actors in innate immune defence are macrophages, polymorphonuclear granulocytes (neutrophils, eosinophils, and basophils), thrombocytes, natural killer cells, and soluble mediators such as complement and a variety of antimicrobial proteins (96).

The macrophage – a main actor in defence against mycobacteria

Phagocytosis
Macrophages (big eaters) play a key role in controlling infection. In the lungs resident alveolar macrophages are the first cells to encounter inhaled bacteria and all tissues contain vast numbers of resident macrophages. They derive from blood-borne monocytes that exit the blood stream and mature into different phenotypes under influence of tissue factors (159).

Phagocytosis is initiated after binding of a microbe to surface receptors, which induces rearrangement of the actin cytoskeleton and internalization of the particle in a vesicle (phagosome) (234). The actin filaments thereafter dissociate from the membrane surrounding the phagosome (1). Killing if the ingested microbes, requires secretion of toxic compounds, into the phagosome. The toxic reactive oxidant nitric oxide (NO) is produced by NO syntethase and protons are pumped in, acidifying the phagosome. Proton pumps and bactericidal enzymes are recruited from the endoplasmatic reticulum leading to “phagosome maturation”. A mature phagosome is formed which permits microbes to be killed (1). Non-pathogenic mycobacteria are invariably killed by macrophages (131, 146, 193).

When particles are too large to be ingested, so called “frustrated phagocytosis”, is seen. Morphological changes take place in the macrophage to increase its area of contact with the large prey. For example, the Golgi complex region is reorganized

and its membranes are recruited to the surface of the macrophage to increase the contact surface (9).

Mycobacteria are in general readily phagocytosed. Pathogenic mycobacteria like *M. tuberculosis* are, in contrast to non-pathogenic species, able to arrest phagosome maturation by inhibiting fusion of phagosome with vesicles from the endoplasmatic reticulum. Thus, the phagosome is not acidified and contains low levels of lysosomal enzymes (243). Instead are vesicles with iron loaded transferrin complexed to transferrin receptor, recruited to the phagosome, giving the mycobacteria access to iron. The capacity to inhibit phagosome maturation has been described both for *M. avium* and *M. tuberculosis* (50, 225, 243). The "early phagosome" becomes a perfect resort for thriving and hiding of mycobacteria, but the mechanisms responsible to this processes are still poorly understood (131).

Recent research has shown that while *M. tuberculosis*, *M. bovis* BCG and *M. leprae* all reside and multiply inside the phagosome, *M. tuberculosis* and *M. leprae,* but not *M. bovis* BCG, translocate into the cytosol after a few days (239).

Apoptosis or necrosis or necrosis after phagocytosis

Engulfment of microbes may lead to death of the phagocyte. Two different ways for the eukaryotic cell to die have been described. Necrosis occurs when the integrity of the cell membrane is compromised due to toxins, hypoxia, or attack by complement or porins. Cell contents leak out of the cell and provide danger signals triggering inflammation, in surrounding cells. In apoptosis, or programmed cell death, eukaryotic cells commit suicide in a controlled process, in which proteins and DNA are degraded without leaking of cell contents. Little or no inflammation is induced (96).

Non-pathogenic and pathogenic mycobacteria trigger different forms of cell death in macrophages. Non-pathogenic mycobacteria, including attenuated mutants of *M. tuberculosis,* trigger apoptosis of infected macrophages, resulting in an impermeable envelope preventing bacterial escape. Engulfment of apoptotic corpses by healthy macrophages leads to killing of bacteria and activation of specific T cell responses. In contrast, internalized virulent *M. tuberculosis* strains cause macrophage death by necrosis, which disrupts the macrophage cell membrane and enables bacterial escape and spread (84, 85, 195).

Production of cytokines

Cytokines are small proteins by which cells in the innate and acquired immune systems communicate. Cytokines bind to specific receptors on target cells and induce events such as proliferation, cytokine production, receptor-synthesis, and chemotaxis, dependent on which intracellular signalling pathway that is activated

by cytokine-receptor binding. Macrophages are the main producers of cytokines. Some of these orchestrate the inflammatory response. They are termed pro-inflammatory cytokines, and are described below. Others modulate the specific immune responses to microbes, and are described in the section covering acquired immunity (96, 224).

IL-1β and TNF

IL-1β and TNF are the prototypes of pro-inflammatory cytokines. They are produced by macrophages in response to microbes, but TNF is also produced by certain activated T cells. They have very similar effects, including activation of endothelial cells leading to expression of adhesion molecules (L-selectin and ICAM), production of IL-6 and IL-8, expression of tissue factor and pro-coagulant activity. IL-1 and TNF also induce recruitment of neutrophil granulocytes from the blood into the infected/inflamed tissue and in large quantities, tissue necrosis. TNF and IL-1 both induce systemic reactions like fever and increase the levels of acute phase proteins (e.g. CRP), blood glucose and cortisol. They also stimulate the bone marrow to production of leukocytes. When injected systemically in high amounts, IL-1 and TNF induce multiple organ failure and death (114, 192).

An important difference between IL-1 and TNF, is that TNF also up-regulates the killing capacity of macrophages. IL-1, but not TNF, provides co-stimulation to naïve T cells that have recognized their specific antigen. TNF and IL-1 are secreted in different fashions. TNF is secreted as a larger precursor on the endoplasmatic reticulum and is cleaved by a membrane-associated proteolytic enzyme. The release of IL-1β is complicated and incompletely known. IL-1 exists in seven varieties, IL-1β being the most important secreted form. IL-1β is produced as a precursor, pro-IL-1β (31 kDa) in the cytoplasm in response to a first inflammatory signal. It is cleaved to IL- 1β (17 kDa) by caspase-1, which is activated by inflammasomes that are assembled in response to another inflammatory signal. Mature IL-1β is packed in vesicles and exported (61, 62).

IL-6

IL-6 is produced by macrophages activated by microbes, but also by certain T cells. This cytokine is a primary inducer of fever, production of acute phase proteins in the liver, and synthesis of ACTH (adrenocorticotropic hormone) in the pituitary gland, leading to production of cortisol. Its effect on the specific immune system include that it promotes B cell maturation into plasma cells and activates cytotoxic T cell activity (129).

IL-6 down- regulates IL-1 and TNF production, and thereby limits tissue damage. Injection of IL-6 gives no acute toxic effects as do IL-1 and TNF, but instead seems to increase survival. Thus, IL-6 has certain pro-inflammatory properties, but also

balances the harmful effects of inflammation despite often being grouped with the pro-inflammatory cytokines (129).

IL-8
IL-8 is a chemokine that attracts and activates neutrophils to the site of infection, but has few or no systemic effects (7, 8).

The pattern of pro-inflammatory cytokines
Mycobacterial virulence is reported to be inversely correlated with secretion of pro-inflammatory cytokines. *M. tuberculosis* induces less TNF, IL-6 and IL-8 than do opportunistic and non-pathogenic mycobacteria. No difference was seen in IL-1 induction (18, 80, 222).

Among isolated mycobacterial components, glycopeptidolipids and total lipids from *M. avium* induce TNF (12, 13). ManLAM from *M. tuberculosis* has been reported to induce monocyte production of TNF, IL-1, IL-6, and IL-8, while lipomannan but not LAM, from *M. chelonae*, induce TNF and IL-8 (11, 247). On the other hand, have other researchers reported that ManLAM inhibits TNF, while PILAM induces TNF (141, 182). The mycobacterial cell wall component trehalose 6,6´dimycolate (cord factor) has been reported to induce IL-6, TNF and IL-1β when injected intravenously into mice (123).

Receptors for microbial structures on macrophages
Macrophages possess a range of receptors recognizing microbial danger signals. These include C-lectins (including the mannose receptor), scavenger receptors, CD14, Toll-like (TLR) receptors, and NOD-receptors (Table II).
Depending on which receptors that are engaged by a certain microbe, different patterns of inflammatory mediators are produced (181).

Table II. Examples of receptors found on macrophages.

Macrophage receptors	**Specificity**	**Microbe**
C-lectins (e.g. mannose receptor)	carbohydrate structures found on microorganisms (e.g. mannose, fucose, NAG)	bacteria, fungi, virus
Scavenger receptors (class A-J)	negatively charged macromolecules such as phospholipids, LPS and teichoic acid	bacteria, virus
CD14	LPS protein- complex with LPS-binding	Gram-negative bacteria
TLR2	Lipoprotein	Gram-negative bacteria
	lipoteichoic acid	Gram-positive bacteria
TLR4	LPS and LPS binding - protein-CD14 complex	Gram-negative bacteria
NOD1	meso-diaminopimelinic acid in peptidoglycan of Gram-negative bacteria	Gram-negative bacteria
NOD2	muramyldipeptide in peptidoglycan of Gram-positive and Gram-negative bacteria	bacteria

Whereas recognition of LPS by CD14 and TLR4 accounts for most of the reactivity of the innate immune system to Gram-negative bacteria, less is known about recognition of Gram-positive bacteria. TLR2 probably recognizes lipoteichoic acid and NOD2 binds to the muramyldipeptide unit making up peptidoglycan in both Gram-positive and Gram-negative bacteria (19, 133, 233).

Human macrophages can bind and internalize virulent *M. tuberculosis* via mannose receptors and Toll-like receptors (127). The role of Toll-like receptors in immunity to mycobacteria is, however, controversial and it is unclear if binding of mycobacterial products to Toll-like receptors are essential for an effective immune response or not (128, 200, 247). NOD2 can recognize muramyldipeptide in the *M.*

tuberculosis cell wall, but mechanisms of intracellular killing of the mycobacteria is unknown (157, 251). CD14 has been reported to bind lipomannan but not lipoarabinomannan, from *M. chelonae and M. kansasii* inducing IL-8 secretion (247). Purified scavenger class A receptor can bind *M. tuberculosis* and mycobacterial cell wall sulpholipids (72).

Polymorphonuclear granulocytes (PMN) in mycobacterial defence

PMNs are recruited from the blood stream by macrophages in tissues that have been activated by danger signals. PMNs are efficient phagocytes and help the tissue macrophages in eliminating microbes and damaged tissue (96). A new mode for entrapping and killing microorganisms, has recently been described for neutrophils. The cells succumb in a form of active cell death distinct from necrosis and apoptosis resulting in extracellular spread of chromatine and granula contents. The bacteria are entrapped in these structures, termed NETs (Neutrophil Extracellular Traps) (28, 29, 88). A similar mechanism has recently been described in mast cells (249).

The role of PMN in host defence against tuberculosis is controversial. It has been suggested that PMNs may constitute an early defence line against this infection, while others refute this theory (reviewed in (146)).

Natural killer cells in mycobacterial defence

Natural killer (NK) cells are granular lymphocytes belonging to the innate system (158). NK cells produce IFN-γ that activates the killing capacity of macrophages. Human NK cells also produce granulysin, a small peptide reported to kill *M. tuberculosis* (149). Their role in mycobacterial defence is unclear.

Acquired immunity

In the acquired immune system which consists of T- and B-lymphocytes, each cell, in principle, carries its own unique antigen-specific receptor. In the case that microbes cannot be rapidly eliminated by the innate immune system, development of a specific immune response to microbial structures becomes fundamental. The acquired immune system aids the innate immune system in eliminating offending microbes, e.g. by production of antibodies that opsonize bacteria and activate

complement, or by activating macrophages by means of IFN-γ and other cytokines from activated T lymphocytes.

T cells recognize their specific antigen by a membrane bound receptor, the T cell receptor. This is composed of two protein chains, either αβ (most common) or γδ. Three subsets of T cells are known; $CD4^+$, $CD8^+$ and CD1 restricted T cells. CD 4 and CD 8 are membrane proteins binding to MHC class II and I, respectively (96).

T cells recognize their antigen on antigen presenting cells (monocytes, macrophages and dendritic cells). $CD4^+$ T cell recognizes peptide antigen on MHC II and in general develop into T helper cells while $CD8^+$ T cells recognizes peptide antigen on MHC I and develop into cytotoxic T cells (96, 160). CD1 restricted T cells recognize lipids and glycolipds presented on CD1 molecules, a group of glycoproteins distantly related to the MHC I protein family. They are divided into three groups; group 1 consisting of CD1a, CD1b, CD1c, group 2 of CD1d, and group 3 of CD1e. Mammals have all three CD1 groups. CD1 molecules display little polymorphisms, and CD1 molecules are present on dendritic cells but not on macrophages. CD1 restricted T cells may belong to the CD4+ , CD8+, and NKT subsets, and are likely to express the αβ-receptor type (17, 34, 119, 206, 217).

Immunoregulatory cytokines

The interaction between the antigen-presenting cell and a naïve T cell may either lead to activation, proliferation and maturation of the T cell into an effector cell, or to anergy. The fate of the T cell depends on additional signals provided by the antigen presenting cell, in addition to the antigen bound to MHC. Cytokines and membrane-bound co-stimulatory molecules provide such signals. These signals, including cytokines, also determine which maturation pathway the T cell will take. Currently three more or less distinct maturation pathways have been identified: Th1, Th2 and Th17 (189, 204). The current view of the Th1 and Th17 pathways is depicted in figure 4 and some of the cytokines involved in modulation of T cell lineage commitment are described below.

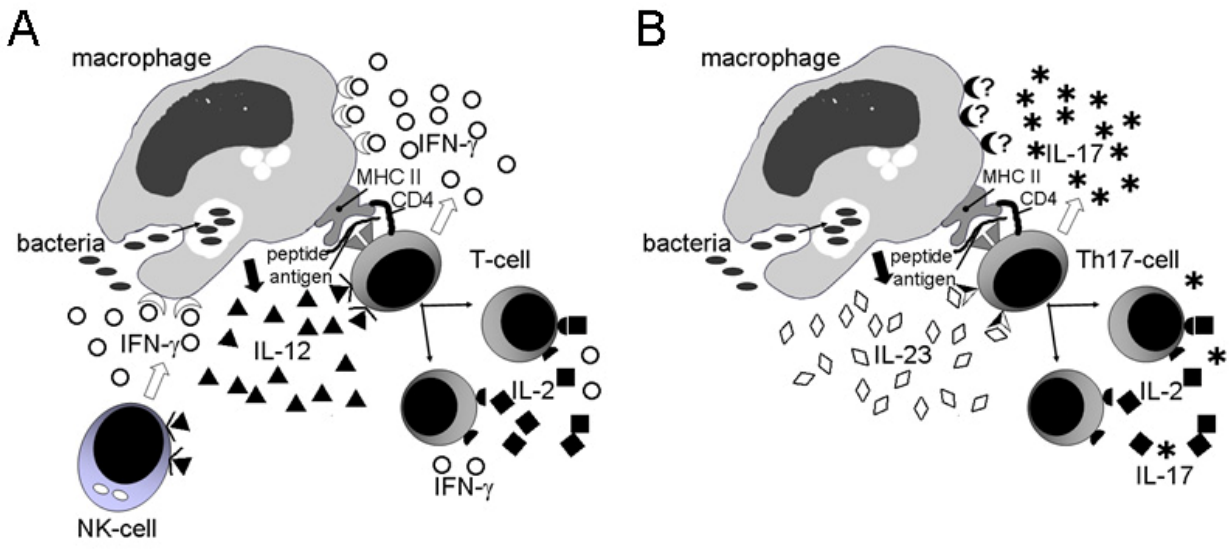


Figure 4. *Cytokines from the antigen presenting cell (here a macrophage) determine the maturation pathway of the T lymphocyte. A. Th1 pathway; B. Th17 pathway.*

IL-12

IL-12 consists of two subunits, p40 and p35, which together form the functional IL-12p70. IL-12 is produced by macrophages or other antigen presenting cells, and induces maturation of T cells into Th1 cells. IL-12 stimulates proliferation and production of IFN-γ in CD4+ T cells, and NK cells (21, 92, 142).

IFN-γ

IFN-γ is produced by $CD4^+$ and $CD8^+$ T cells and by NK and NKT cells. It activates macrophages leading to increase production of oxygen radicals, lysosomal enzymes and to increased surface expression of MHC II. This results in increased killing capacity of the macrophage. IFN-γ is fundamental in protection against bacteria, which like mycobacteria, may survive inside macrophages (105, 203, 204).

IL-10

IL-10 is produced by macrophages and certain activated T cells. IL-10 inhibits macrophage activation and antigen presentation, leading to decreased production of IL-12 and IFN-γ. Thus, IL-10 counteracts the Th1 pathway. IL-10 promotes maturation of B cells to antibody producing plasma cells (176).

IL-23

IL-23 is produced by macrophages and other antigen presenting cells. IL-23 is believed to induce maturation and/or activation of Th17 cells leading to production of IL-17. IL-23 shares the p40 unit with IL-12 but has a unique p19 unit (171, 187).

IL-17

IL-17 is produced by a certain CD4+ T cell subset termed Th17 cells. However, CD8+ T cells, γδ T cells, and NKT cells can also make IL-17. The IL-17A receptor is expressed in all tissues examined up to date and mRNA for the IL-17 receptor are found in epithelial cells, fibroblasts, endothelial cells, B and T cells, myelomonocytic cells, and marrow stromal cells (171, 178, 218). IL-17 activates macrophages to produce IL-1β, TNF and chemokines, thereby enabling both recruitment of neutrophils and increased killing capacity by macrophages (174). Furthermore, IL-17 enhances expression of antimicrobial peptides such as β-defensins (169, 174).

Production of immunoregulatory cytokines in response to classical Gram-positive and Gram-negative bacteria, as well as mycobacteria

Intact "classical" Gram-positive bacteria such as streptococci, staphylococci, bifidobacteria etc., induce large amounts of IL-12 and TNF from monocytes, and IFN-γ from T and NK cells (109). Thus, the Th1 pathway (cell mediated immunity) is promoted. Soluble components do not trigger IL-12 production and induce considerable less TNF than do intact Gram-positive bacteria. It appears as if the phagocytosis of intact Gram-positive bacteria and their persistence in the phagosome promotes production of cytokines in the Th1 pathway, leading to activation of microbial killing (10, 109-111).

Both Gram-positive and Gram-negative bacteria can induce IL-23 responses and Gram-positive peptidoglycan is also reported to induce IL-23 (81, 164, 220, 241). Gram-negative bacteria have been reported to induce production of IL-17, *in vivo*, in pulmonary infected mice, via IL-23 (104). In fungal infection, IL-23, rather than IL-12, has been reported to be critical in development of chronic inflammation (139).

Relatively little is known about the production of cytokines with immunoregulatory properties in response to mycobacteria and their components. Mycobacterial extractable total cell wall lipids have been reported to both stimulate and inhibit Th1 responses and IFN-γ production (117, 207). ManLAM from *M. tuberculosis* was found to induce IL-10 and inhibit Th1 responses, i.e. secretion of IL-12 and IFN-γ, while PILAM induced IL-12 and TNF (11, 93, 141, 182). Lipomannan from *M. tuberculosis* has both been reported to induce and not to induce IL-12, while ManLAM from *M. tuberculosis* and AraLAM from *M. smegmatis* did not induce IL-12 (57, 196). Trehalose 6,6′dimycolate (TDM, "Cord factor") has been reported to induce IL-12 and IFN-γ in the lungs of mice first immunized with TDM

and then challenged intravenously (184). No reports have been found regarding the ability of mycobacterial cell wall lipid extracts to induce IL-23 and IL-17.

Acquired immune responses in mycobacterial infection

In mycobacterial infection the key protective acquired immune responses is appearance of $CD4^+$ T cells with specificity to mycobacterial antigen. They secrete IFN-γ (and TNF) and activate macrophages so that they become able to kill internalized mycobacteria. However other T cell subsets seem to be involved in protection against of mycobacterial infection; $CD8^+$ T cells, γδ T cells, and CD1-restricted T cells. They all produce IFN-γ and all may be cytotoxic to macrophages (25). It has also been reported that $CD4^+$ T cells with cytotoxic activity may be involved in mycobacterial infection (140, 180).

It has been reported that $CD4^+$ T cells as well as CD8+ T cells are activated in *M. avium* infection but that only the $CD4^+$ T cells are effective since *M. avium* specific $CD8^+$ T cells undergo apoptosis early in infection (56).

Macrophage activation by IFN-γ secreted in the Th1 pathway (Fig. 4A.) is of fundamental importance in controlling mycobacterial infection since genetic disorders leading to IL-12 or IFN-γ deficiencies accelerate mycobacterial disease in infected individuals (40, 51, 87, 155). HIV infected individuals with declining numbers of $CD4^+$ T cells have a 10-fold risk of developing active tuberculosis, but are also at increased risk to acquire disseminating *M. avium* disease (215, 250).

An alternative potentially protective pathway, which recently has gained attention in mycobacterial defense is the IL-23/IL-17 route (Fig. 4B), in which activated macrophages secrete IL-23 (134) that activates T cells of the Th17 subset to produce IL-17. Absence of IL-23 and IL-17 in mice led to increased pulmonary inflammation and tissue damage but decreased fibrin deposition (2, 135).

In primary tuberculosis Th1 and Th17 responses are induced with the same kinetics, but ten times more Th1 than Th 17 cells are found. Hence, in experimental BCG infection, the Th17 cells are rapidly suppressed by the Th1 response. Thus, IFN-γ increases IL-12 production in BCG infected dendritic cells but reduces IL-23 secretion, while IL-17 limits IL-12 production and increases IL-23 secretion. IL-12 and IL-23 might, thus, cross-regulates each other, but the respective roles of Th1 and Th17 responses in mycobacterial infection remain unclear (134, 135).

Natural killer T (NKT) cells combine features of the innate and adaptive immune systems. NKT cells have antigen receptors like T cells and NK cells properties.

NKT cell receptors may recognize glycolipids that are part of the membrane of certain Gram-negative bacteria (230). NKT cells have been reported to recognize *Mycobacterium tuberculosis* infected macrophages in mice, and to kill intracellular bacteria (210).

CD1 (a-c) restricted T cells binds a number of lipid antigens derived from different mycobacterial species, including mycolic acids and lipoarabinomannans (38, 119, 212).

Production of IL-10 might contribute to mycobacterial persistence inside host macrophages because this cytokine inhibit cell-mediated immunity, including MHC-restricted cytotoxicity against infected macrophages (60). PBMCs from patients with long-standing tuberculosis produce more IL-10, equal amounts of IL-12, but less IFN-γ compared with cells from healthy controls, when stimulated with PPD (154).

Mycobacterial granulomas

Granulomas are concentrations of macrophages and lymphocytes in tissues surrounded by small lymphocytes and some plasma cells (Fig. 5A). Fused macrophages produce multinucleated giant cells. Other macrophages turn into epitheloid cells, so called because they morphologically resemble epithelial cells. Increased density of extracellular matrix, especially collagen, surrounds the granuloma. This reaction, termed fibrosis, might be viewed as a mean to encapsulate microbes and other structures that cannot be eliminated by the phagocyte defence system (58, 107).

Tuberculosis granulomas consist of a core of infected macrophages, surrounded by foamy macrophages, giant cells, and epitheloid cells. Outside is a mantle of lymphocytes surrounded by a fibrotic capsule. Neovascularisation of the granuloma also occurs, and in this stage no transmission of bacteria is seen. In later stages blood vessels in the granuloma centre disappear and caseous necrosis develops (Fig. 5B). Progressive tissue destruction in the caseous granuloma leads to liquefaction (Fig. 5C). Liquefaction may lead to granuloma rupture and release of bacteria into the surroundings, resulting in transmission. A cavity remains, that may be a focus for secondary infection. It has been reported that bacterial release, coincides with high levels of matrix metalloproteases, indicating that liquefaction is an active process (107, 209, 231).

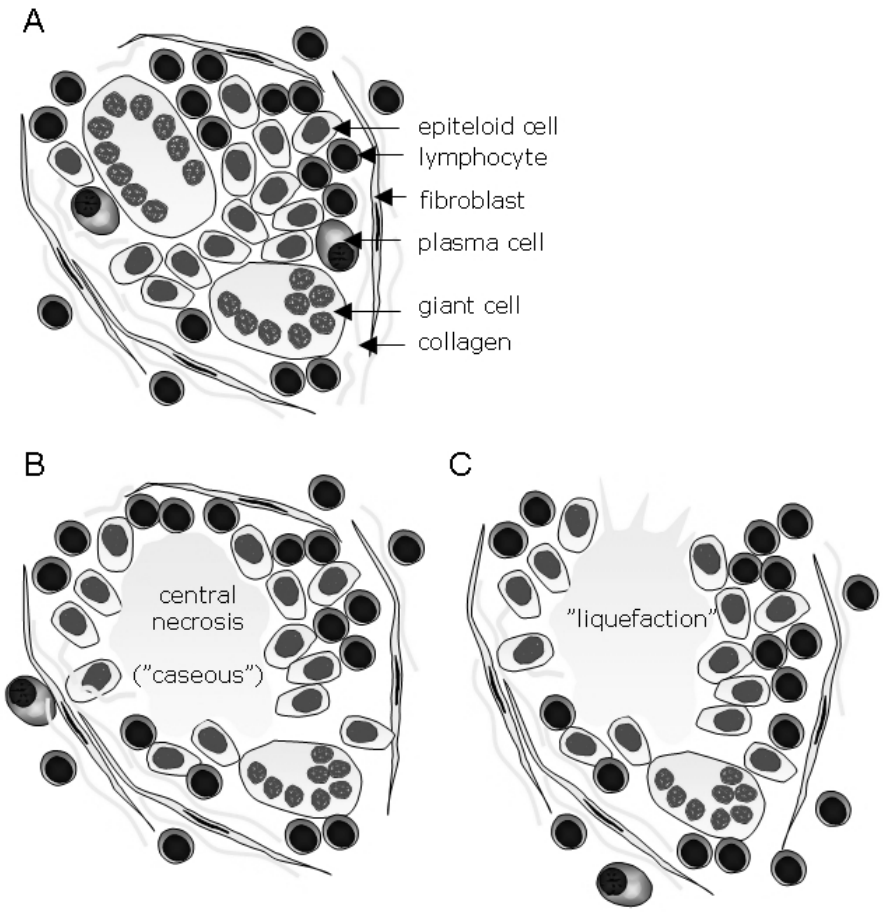


Figure 5. *Organisation of mature granuloma in M. tuberculosis infection. (A)Mature granuloma; (B) Granuloma with central necrosis; (C) Liquefaction and rupture. (Adapted from Inflammation; Mölne, J. and Wold, AE., 2007, with permission from the authors (181))*

Granuloma formation seems to be a hallmark of human interaction with mycobacteria and has been seen not only in *M. tuberculosis, M. abscessus* and *M. avium* infections, but also in the rare cases of infections with the generally non-pathogenic *M. gordonae* (6, 126, 237, 253). It has been suggested that the IL-23/IL-17 pathway participates in maintaining the integrity of granuloma, but how this would occur is unclear (134, 232).

The role of antibodies in mycobacterial infections

It has long been the dominant view that antibody protection has no place in the protection against tuberculosis (5). However, some reports have suggested that monoclonal antibodies directed towards the surface of *M. bovis* may increase the survival time of mice, infected with *M. bovis,* and that administration of an IgG3 monoclonal anti-arabinomannan antibody increases survival of mice infected in the lungs with *M. tuberculosis* (45, 226). The role of antibodies in human mycobacterial infection is still poorly understood (95).

AIMS

The aims of the present study were to:

- investigate the pattern of transmission of *M. abscessus*, with focus on respiratory infection in patients with cystic fibrosis and skin infections in survivors of the tsunami catastrophe

- characterize the cytokine responses to potentially pathogenic (*M. avium, M. abscessus*) and apathogenic (*M. gordonae*) environmental mycobacteria with focus on immuno-regulatory cytokines such as IL-12, IL-10 IFN-γ, IL-23 and IL-17

- investigate the importance of smooth versus rough colony morphology in *M. abscessus*, with respect to clinical manifestation and *in vitro* response of human mononuclear phagocytes

MATERIALS AND METHODS USED IN THE STUDY

EPIDEMIOLOGICAL STUDIES (Papers I and II)

Patient groups

Cystic fibrosis patients and other patient groups (Paper I)
In paper I, 28 patients, with cultures positive for *M. abscessus* were included. Fourteen of them had cystic fibrosis (CF); nine were cared for at the West Swedish CF centre at the Queen Silvia Children's Hospital in Göteborg, three were from the Stockholm CF centre, while two were from the Uppsala CF centre and referred to the Sahlgrenska University Hospital in Göteborg for lung transplantation.

Fourteen non-CF patients were included. Three had *M. abscessus* lung-infection/colonization (two women and one man, 56, 84 and 54 years old, respectively), one had *M. abscessus* mastoiditis and four contracted late-onset *M. abscessus* skin abscesses during the Asian tsunami catastrophe in December 26, 2004. Six additional patients had a single *M. abscessus* positive culture, which was regarded as the result of transient colonization without clinical significance.

Tsunami victims (Paper II)
In paper II, 15 patients (8 men and 7 women) injured during the tsunami catastrophe were included. They developed signs of skin and soft-tissue infection within 20-105 days of the trauma. Cultures were positive for rapidly growing mycobacteria: *M. abscessus* in 7 cases, *M. fortuitum* in 6 cases and *M. peregrinum* and *M. mageritense* in one case each. Strains from four of the *M. abscessus* infected patients were also included in paper I.

Isolation of mycobacteria from clinical specimens and identification of *M. abscessus*

Processing of clinical specimens were done according to the routine procedures at the Clinical Bacteriological Laboratories at Sahlgrenska University Hospital, Göteborg, Sweden (130).

For processing of sputum samples from CF-patients, special procedures were used. To kill irrelevant bacteria present in the samples, each sample was divided into three portions, which were treated by either: (i) SDS-NaOH (115) (ii) a cocktail of antibiotics: amphotericin B (250 μg/ml), carbenicillin (1.25 mg/ml), polymyxin B-

sulfate (5 mg/ml) and trimethoprim-lactate (500 μg/ml), or (iii) 5% oxalic acid foremost to suppress *Pseudomonas aeruginosa* (244, 245). The last method was introduced in 1993. All three decontamination methods was used on each sample.

Mycobacteria were cultured in flasks with Loewenstein-Jensen solid media. Colonies were confirmed as mycobacteria by the Hallberg staining technique (115). Slides with bacteria were heated to 80°C in a solution containing Nachtblau, cooled to room temperature, rinsed in distilled water, decolorized in 8% HNO_3 and 70% ethanol, and counterstained with fuchsine.

Identification of *M. abscessus* to the species level was performed using a biochemical test panel (tolerance to 5% NaCl, iron uptake, Tween hydrolysis, urease, nitrate reduction) (172) examination of the mycolic acid pattern by high-pressure liquid chromatographic analysis (36, 37) and sequencing of the 16S RNA gene (137). For isolates obtained from 2005 and onwards, these methods were complemented with the GenoType® Mycobacterium CM Test (HAIN Lifescience, Nehren, Germany) (162).

All isolates were stored at room temperature in vials with Loewenstein-Jensen medium until analyzed.

PFGE for strain typing of *M. abscessus*

Preparation and digestion of bacterial DNA
M. abscessus isolates were grown in Middlebrook 7H10 agar vials until rich growth was achieved, usually after one week. The colony morphology (rough or smooth) was noted. DNA was prepared according to a published method (89), but with minor modifications. A loopful of bacteria was transferred to 5 ml Middlebrook 7H9 broth (Difco, Detroit, MI) modified by the addition of 0.5 M sucrose, 0.05% (w/v) Tween 80, 0.2% (w/v) D-glucose and 10% oleic acid-albumin complex (Beckman-Dickinson, Cockeysville, MD), and incubated on a shaker for 72 h at 37ºC. This broth permitted adequate growth of isolates forming rough colonies on solid medium.

For disruption of bacteria, 400 μl of a solution containing 0.2 M glycine, 60 μg D-cycloserine/ml, 20 mM LiCl, 200 mg lysozyme/mL and 5 mM EDTA, was added to the cultures, which were incubated for 16 h. After centrifugation (2,300 g, 15 min, room temperature), the pellet was resuspended in 1.2 ml TS buffer (50 mM Tris, 0.5 M sucrose, pH 7.6) and 125 μl of the suspension was transferred to Eppendorf tubes and frozen at -20 ºC for 10 min, thawed in room temperature, heated to 75 ºC for 20 min, suspended in 125 μl of 2% low-melting-point agarose (Sea Plaque, Cambrex Bio Science Baltimore, Inc., MD), mixed with EC buffer (6

mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, pH 7.5) at 55 ºC, and finally cast into plugs. For lysis of remaining bacteria, the plugs were treated with 1 mg lysozyme /mL in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6), overnight at 37ºC, followed by incubation in 250 µl ESP-buffer (200 µl 0.5 M EDTA; pH 9.0, 25 µl sodium lauroyl sarcosine 10%, 25 µl proteinase K (5 mg/ml) for 24 h at 55 ºC. The ESP buffer was decanted and replaced by HE buffer (10 mM HEPES-NaOH pH 8.0, 1 mM EDTA) containing 0.1 mM phenylmethyl-sulphonyl fluoride (PMSF). After incubation at 4ºC for 1 h, the PMSF solution was decanted and plugs were washed 5 times × 30 min at 4ºC in HE buffer without PMSF. Using HE-buffer in the washing step, instead of Tris buffer as in the original method, gave better results. This modification had previously been described for PFGE-analysis of *Streptomyces lividans* (75). Plugs were stored in 0.2 M EDTA at 4ºC until used.

For digestion, plugs were washed in HE buffer 6 times × 15 min, at 4ºC. Hereafter genomic DNA was digested overnight at 37 ºC with 20 U of the restriction endonucleases *Ase*I or *Xba*I in buffer as recommended by the manufacturer (BioLabs Inc., New England, USA). The plugs were loaded into a 1% agarose gel prepared and run in HEPES buffer (16 mM HEPES-NaOH, pH 7.5, 16 mM sodium acetate, 0.8 mM EDTA). HEPES was used as electrophoresis buffer instead of Tris buffer due to problems with DNA degradation (198, 199).

Pulsed-field gel electrophoresis was performed with the Gene path system (Bio Rad Laboratories, Sundbyberg, Sweden) at 14 ºC and with a reduced voltage gradient, 4 V/cm, to compensate for the higher ionic strength of HEPES compared with Tris buffer (145). The programs used were modifications of the ones described by Wallace *et al.* (238). For plugs digested with *Ase*I, a combination of two programs was used. The first consisted of an initial switch time of 1 s, a final switch time of 23 s, a run time of 23 h, an angle of 120 º, and a linear ramping factor and was immediately followed by a second program consisting of an initial switch time of 10 s, a final switch time of 17 s, a run time 4 h, an angle 120 º, and a linear ramping factor. For plugs digested by *Xba*I, the program consisted of an initial switch time of 3 s, a final switch time of 12 s, a run time of 23 h, an angle of 120 º, and a linear ramping factor. In every run two plugs of genomic DNA of *Staphylococcus aureus* (NCTC 8325), digested with *Sma*I, were included for reference. Lambda ladder PFG marker (BioLabs Inc., New England, USA) was used as molecular weight standard.

Interpretation of PFGE patterns

Gel electrophoresis of the restriction endonuclease treated DNA yielded a band pattern specific for each bacterial clone. The PFGE types were interpreted according to Tenover *et al.*, i.e. isolates with patterns differing by 3 bands or less were considered as related or probably related (227). The gels were also digitized for computer-aided analysis using Fingerprinting™ II software (Bio-Rad

Laboratories, Hercules, CA). Bands sized 117-674 kb were considered. Calculation of the similarity matrix was done with the Jaccard algorithm and clustering was achieved with the unweighted pair group method (UPGMA). Two isolates were considered to belong to the same bacterial clone if the similarity was ≥ 90% according to the cluster analysis (Fig. 2, paper I).

IMMUNOLOGICAL STUDIES (Papers, III, IV and V)

Bacterial strains, culture and preparation

Mycobacteria used in Paper III
Three strains each of *M. avium*, *M. abscessus* and *M. gordonae* representing both human and veterinary isolates were studied (Table I, paper III).

Mycobacteria used in Paper IV
Four clinical isolates of *M. abscessus* were investigated, three of which exhibited invariant rough colony morphology, and one which always formed smooth colonies. The strains were human clinical isolates obtained from the the Bacteriological Laboratories at the Sahlgrenska University Hospital, Göteborg, Sweden. The rough *M. abscessus* strains were isolated from sputa of a cystic fibrosis patient (strain R1), a central venous device from another cystic fibrosis patient (strain R2), and sputa from a middle-aged female patient without known immunodeficiency, chronically colonized in the airways by *M. abscessus* (strain R3). The smooth strain was isolated from sputa of a third cystic fibrosis patient exhibiting chronic pulmonary *M. abscessus* disease (strain S1).

Mycobacteria used in Paper V
Eight isolates of *M. abscessus*, four rough and four smooth, were studied (Table I, paper V). The isolate termed MAB 1 is the type strain of *M. abscessus* (ATCC 19977), while the other 7 strains were clinical isolates obtained from the Bacteriological Laboratories, Sahlgrenska University Hospital. The isolates were shown to belong to different strains by pulsed-field gel electrophoresis (Paper I).

Enterococcus faecalis and *Streptococcus mitis* were used as representative Gram positive bacteria and *E. coli* as a representative Gram negative bacterium in papers III and V) (Table I, Papers III and V).

Culture conditions and preparation
Mycobacteria were cultured aerobically at 37°C for five days (for *M. abscessus*) or four weeks (for *M. avium* and *M. gordonae*) on Middlebrook 7H10 agar (Prepared in-house at the Bacteriological Laboratories, Sahlgrenska University Hospital, Göteborg, Sweden). Bacteria were harvested in Dulbecco's endotoxin-free PBS (PAA laboratories, GmbH, Pasching, Austria), and vortexed vigorously to obtain a homogenous suspension. Clumps were allowed to sediment for 15 min at room temperature. Hereafter bacterial suspensions were transferred to 6-well tissue culture plates and UV-irradiated for 1h (in preliminary experiments found to be required to obtain a negative viable count after 8 weeks of incubation on Middlebrook agar). *E. faecalis, S. mitis* and *E. coli* were cultured aerobically overnight at 37°C for 24 h on horse blood agar (Bacteriological Laboratories, Sahlgrenska University Hospital), and inactivated by exposure to UV-light for 15-18 min, which was confirmed by a negative viable count after culture on horse blood agar plates for 48 h. Inactivated bacteria were counted in a Bürker chamber and suspensions were adjusted to concentrations of $1x10^8$ or $1x10^9$ bacteria/ml, and stored frozen at -70, until used. Previous experiments showed similar response of human PBMC to freshly prepared frozen and thawed Gram-positive and Gram-negative bacteria (112).

Isolated mycobacterial cell wall components (Paper III)

Mycobacterial cell wall lipid extracts
Mycobacterial surface lipids were extracted by a simple purification method (207), with minor modifications**.** This method yields a mixture of apolar and polar lipids free of mycolic acids, protein and pyrogen (207). Suspensions of *M. avium* (CCUG 20992), *M. abscessus* (CCUG 20993) and *M. gordonae* (CCUG 21811) were inactivated at 60°C for 1.5 h. After washing, lipids were extracted by treating 2.50 – 2.75 g of mycobacteria (wet weight) with 6 mL of chloroform-methanol (2:1) at 55°C for 15 min. The extraction was repeated and the organic phases were pooled and washed twice with 1.25 mL of water. The solvent was evaporated and the dry lipid was weighed, dissolved in chloroform, and aliquoted into glass vials. After evaporation of the chloroform, vials were stored at 4°C until used. Before used to stimulate PBMC, the lipid material was hydrated in 1 mL of Dulbecco's endotoxin-free PBS (PAA laboratories) and sonicated for 60 min.

Lipoarabinomannan
Purified ManLAM isolated from the virulent type strain of *M. tuberculosis* (H37Rv) and AraLAM isolated from *M. smegmatis* were kindly provided from the Department of Microbiology, Colorado State University, Fort Collins, USA, as part of a NIH/NIAID Contract (see acknowledgements). The endotoxin concentrations in the preparations were 0.45 ng/mg (ManLAM) and 1.2 ng/mg (PILAM)

according to quality control information from Colorado State University. The LAMs were dissolved at 50 μg/mL in sterile deionized water, stored at -20°C, and sonicated for 10 min prior to use.

Isolation of peripheral blood mononuclear cells (PBMC), monocytes and polymorphonuclear granulocytes

Isolation of PBMC (Papers III, IV, V)
PBMCs were prepared from blood-donor buffy coats (Blood bank, Sahlgrenska University Hospital) by density gradient centrifugation (Lymphoprep, Nyegaard, Norway) at room temperature for 20 min at 820 x g. The mononuclear cells were washed x 3 (460 x g, 10 min) in ice-cold endotoxin-free RPMI 1640 medium with 2 mM glutamine (Invitrogen, Carlsbad, CA) and suspended in complete RPMI medium, i.e. medium supplemented with 2 mM glutamine, 0.01% gentamicin (Sigma, St. Louis, MO), and 5% inactivated endotoxin-free fetal calf serum (Invitrogen). The latter medium had an endotoxin level of <0.1 EU/mL, as assessed by the Limulus amoebocyte lysate assay (97). We found no commercial human serum that was endotoxin free (unpublished observations). In paper IV, autologous serum/plasma was used instead of fetal calf serum, because the latter was found to affect the experiments.

Isolation of monocytes (Paper IV)
$CD14^+$ monocytes were purified from human peripheral blood mononuclear cells by positive selection with magnetic microbeads conjugated with monoclonal mouse anti-human CD14 antibodies, according to the manufacturer's recommendations (Mitenyi Biotec, Bergish Gladbach, Germany). Cells were incubated with beads in MACS buffer (PBS with 0.5% human serum albumin and 2 mM EDTA) for 15 min, washed and passed through the MACS column in a magnetic field. The column was rinsed three times with MACS buffer. After removing the magnet, $CD14^+$ cells were flushed out and washed three times (460 x g, 10 min) in ice-cold RPMI 1640 medium with 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Analyses by flow cytometry showed that 94-98% of the purified cells expressed CD14.

Isolation of polymorphonuclear leukocytes (Paper IV)
Human polymorphonuclear leukocytes were prepared from healthy blood donors, using dextran sedimentation and Ficoll-Paque gradient centrifugation (26). The cells were washed twice and resuspended in KRG (Krebs-Ringer phosphate buffer containing 10 mM glucose, 1 mM $Ca^{2+,}$ and 1.5 mM Mg^{2+}, pH 7.3) and washed once in RPMI 1640 medium with 2 mM glutamine (Invitrogen, Carlsbad, CA), before used. Cells were kept on ice during the entire separation process.

In vitro co-culture/stimulation of leukocytes with mycobacteria

Stimulation with intact UV inactivated bacteria (Papers III-V)
PBMC suspensions were aliquoted, $2x10^6$/mL (200µl/well), in flat bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark). Bacteria were added to achieve final concentrations of $5x10^5$, $5x10^6$ or $5x10^7$/mL, corresponding to 0.25, 2.5 and 25 bacteria per mononuclear cell, respectively. The cultures were incubated at 37ºC in a humidified atmosphere supplemented with 5% CO_2. Supernatants were harvested after 24 h (to measure IL-1β, IL-6, IL-8, IL-10, IL-12, IL-23, and TNF) or 5 days (to measure IFN-γ and IL-17) and stored frozen at - 20ºC until analyzed (Paper III and V). The time-points were previously found to be optimal based on stimulation of PBMCs with Gram-positive and Gram-negative bacteria (109). The kinetics of IL-23 and IL-17 production was investigated by collection of supernatants day 1, 2, 3, 4, and 5 (Paper III).

For morphological analyses (Paper IV), PBMCs, $CD14^+$ monocytes or PMNs, were stimulated with bacteria in 4-chambered slides (Lab-Tek™ II, Nunc, Roskilde, Denmark). The cells were suspended at 1 x 10^6 cells/mL in RPMI 1640 medium with 2 mM glutamine (Invitrogen, Carlsbad, CA) supplemented with 5% autologous serum/plasma (Fetal calf serum was found, in preliminary experiments to reduce mesh-formation). *M. abscessus* were added to achieve a final concentration of $1x10^7$ bacteria/mL. The mixture was incubated overnight, or for 5 days, at 37ºC in a humidified atmosphere supplemented with 5% CO_2. Wells without bacteria were used as medium control.

Neutrophils (polymorphonuclear leukocytes) were allowed to settle on the glass slips for 30 min before being incubated with bacteria and incubation was done for 30 min, 1h, 2h, 3h, 4h, and 16h.

Stimulation with lipids extracts and LAM (Paper III)
Crude lipid extracts were added to final concentrations of 4, 40 and 400 µg/mL. We calculated (considering losses during the extraction process) that 40 µg lipid roughly corresponded to $5x10^7$ mycobacteria. ManLAM and PILAM were both used in a concentration of 10 µg/mL. The cultures were incubated as described above.

Blocking experiments (Paper III)
For inhibition of human IL-10, anti human IL-10 (JES3-19F1), anti-human IL-10 receptor (CD210) (3F9), or an isotype control antibody (RTK2758) (all from BioLegend, San Diego, CA) were pre-incubated with cell cultures at final concentrations of 10 µg/mL for 45 minutes at 4 °C, prior to addition of bacteria.

Cytokine determination – ELISA (Papers III and V)

Concentrations of human IL-1β, IL-6, IL-8, TNF, IL-12 p70, IL-10, and IFN-γ in cell culture supernatants were determined by an in-house ELISA (132). Antibodies and standards were purchased from BD Pharmingen (San Diego, CA), except for antibodies and standards for IL-1β, which were from R&D (Minneapolis, Minnesota). Costar plates (Invitrogen) were coated overnight at 4°C with capture antibodies diluted in carbonate buffer (pH 9.6): anti-IL- 1β (Clone 2805), anti-IL-6 (Clone MQ2-13A5), anti-IL-8 (Clone G265-5), anti-TNF (Clone MAb1), anti-IL-12p70 (Clone 20C2), anti-IL-10 (Clone JES3-9D7), or anti-IFN-γ (Clone NIB42). The plates were washed three times in PBS and blocked for 1 h with 5% bovine serum albumin (Sigma-Aldrich) in PBS. After washing three times in PBS containing 0.01% Tween, 50 µl of appropriately diluted samples and standards were added and incubated overnight at 4°C. After washing, the following biotinylated detector antibodies were added and allowed to react for 1h: anti-IL-1β (Cat. No. BAF201), anti-IL-6 (Clone MQ2-39C3), anti-IL-8 (Clone G265-8), anti-TNF (Clone MAb11), anti-IL-12p70 (Clone C8.6), anti-IL-10 (Clone JES3-12G8), and anti-IFN-γ (Clone 4S.B3). The plates were washed and incubated with streptavidin-horseradish peroxidase (BD Pharmingen) for 30 min. Samples, standards, biotinylated antibodies and streptavidin- horseradish peroxidase were diluted in high performance ELISA dilution buffer (CLB, Amsterdam, The Netherlands). After washing, 50 µl of 3, 3'5, 5' -tetramethylbenzidine liquid substrate (Sigma- Aldrich) was added and the plates were incubated for 20 min in the dark. The colour reaction was stopped by adding 50 µl of 1 M H_2SO_4. The amount of substrate converted to coloured product was measured as the optical density at 450 nm in a spectrophotometer (Spectra Max Plus, Molecular Devices, Sunnyvale, CA). All post standard-sample incubations, were carried out on a shaker at room temperature.

Commercially available ELISA kits ("Ready-SET-Go!" from eBioscience, San Diego, CA) were used, according to the manufacturer's instructions, to determine human IL-23 (p19/p40) and IL-17 (=IL-17A). The limits of detection of the ELISA assays were 6, 6, 6, 16, 25, 12, 16, 62, and 16 pg/mL for IL-1β, IL-6, IL-8, TNF, IL-12p70, IL-10, IFN-γ, IL-23, and IL-17, respectively. Cut-off levels were determined according to the detection limit of the assay and the sample dilution used in the particular experiment. In general, medium without bacteria induced cytokine responses below cut-off level.

Phagocytosis assay (Papers III, IV and V)

PBMCs were prepared from blood donor buffy coats as described. Red blood cells were lysed by incubation in distilled water for 30 s. PBMC were washed and re-suspended in complete medium (see isolation and stimulation of PBMC above) at a concentration of 1 x 10^6 cells/mL in 24-well plates (Nunc). After addition of 1 x10^7 inactivated bacteria/mL, the mixture was incubated at 37ºC for 16 h in a humidified atmosphere supplemented with 5% CO_2. PBMC were washed and cyto-centrifuged onto glass slides (Cytospin, Shandon Southern, Runcorn, UK). Mycobacteria were visualized by acid fast staining by the Hallberg technique (115). Slides were examined in a microscope at 1,000 x magnification (Carl Zeiss AB, Stockholm, Sweden, supplied with Canon PowerShot G6 camera and software, Canon Inc., Tokyo, Japan). The presence of bacterial cords was noted. Forty monocytes on each slide were counted and the percentage of monocytes with internalized bacteria was calculated.

Scanning electron microscopy (Paper IV)

For scanning electron microscopy, PBMC-bacterial co-cultures on glass slides were fixed for 1 h with a mixture of 2% para-formaldehyde, 2.5% glutaraldehyde, and 0.02% sodium azide, in 0.05 M sodium cacodylate buffer. After double osmium tetroxide and thiocarbohydrazide treatment (OTO method (86)), specimens were dehydrated in ethanol and finally infiltrated with hexamethyldisilazane, which was allowed to evaporate in a fume hood. Dried specimens were sputter coated with palladium after conductive mounting on aluminum stubs. They were examined in a digitized Zeiss 982 Gemini field emission scanning electron microscope operated at mixed Everhart-Thornley and in-lens detector signals (Carl Zeiss AB, Stockholm, Sweden)

Immunohistochemistry (Paper IV)

Leukocyte-bacterial co-cultures performed on glass slips with removable chambers were fixed during 10 min with para-formaldehyde (4%) and washed twice in PBS prior to staining procedures, while still in the chambers.

Double-stranded (ds) DNA was stained blue, with 4,6-diamidino-2-phenylindole-dihydrochloride – DAPI (Invitrogen), during 2 min at RT. Alternatively, dsDNA was stained with SYTOX™ Green (Invitrogen), during 30 min at RT. A third method to visualize dsDNA was incubation during 30 min at RT with a 1/50 dilution of a patient serum with auto-antibodies to dsDNA (kindly provided, together with positive- and negative controls, by Dr Karlsson-Parra, Clinical

Immunological laboratories, Sahlgrenska University Hospital, Göteborg, Sweden) followed by incubation with anti-human FITC – conjugated IgG antibodies (ANA Test System, Zeus Scientific, Branchburg, NJ).

For visualization of histones, rabbit-anti-human-histone H2B monoclonal antibodies (1:100) (Epitomics, Burlingame, CA) were added and PBMCs were incubated at + 4°C overnight and washed x2 in PBS, followed by incubation with a donkey-anti-rabbit-IgG-FITC (1:100) (BioLegend, San Diego, CA) during 45 min at RT. An isotype rabbit IgG (Epitomics) was used as control.

PBMC cell membranes were visualized by incubation during 30 min at RT, with 2 mM Texas Red®-X- conjugated wheat germ agglutinin (Invitrogen), a lectin used to stain eukaryote cell membranes (147).

After the staining procedures, slides were washed x 2 in PBS, and x 1 in distilled water, mounted in ProLong Gold or ProLong Gold DAPI (Invitrogen) and examined in a fluorescence microscope using appropriate filters, (Leica HC DMR) supplied with attached camera (Leica CD 300 F or Leica DFC 420 C) and software (Leica Qwin Pro V 2.8 or Leica Application suite 2.8.1), from Leica Microsystems Ltd, Switzerland.

Bacterial cords could be visualized in the FITC or DAPI-filter due to a faint auto-fluorescence (yellow-green and very light blue, respectively).

For identification of monocytes and T cells, co-cultures of PBMCs and mycobacteria were not fixed with para-formaldehyde until after the staining procedure. Co-cultures were washed once in PBS, stained for 30 min with PE-Texas Red® conjugated human anti-CD14 antibody (undiluted) and Alexa Flour® 488 conjugated human anti-CD3 antibody (1:10), both from Invitrogen. Stained slides were then fixed for 10 min in para-formaldehyde (4%), washed x 2 in PBS, and x 1 in distilled water, and mounted in ProLong Gold or ProLong Gold DAPI.

DNase treatment (Paper IV)

To test if addition of DNase could disintegrate extracellular traps (NETs) or monocyte-derived DNA meshwork, overnight cultures of *M. abscessus* stimulated PBMCs were washed x1 in PBS and dried for 1 h at RT, stained with DAPI (Invitrogen) to visualize meshwork, and inactivated with 2,000 U of deoxyribonuclease I (Worthington, Lakewood, NJ). DNase activity was monitored in a fluorescence microscope. For monocyte meshworks photos were taken every 30 min for 2 h (the same field), and once after 24 h, using a fluorescence

microscope using appropriate filters, camera and software. For neutrophil NETs, photos were taken before DNA were added and after 3 minutes.

Statistical methods (Paper I, III, V)

In paper I, comparisons between groups were made using Fischer´s exact test. For descriptive statistics, the mean response to each bacterial strain, and the standard deviation based on the variation between blood donors, was calculated (Paper III). To compare cytokine production in response to different species of bacteria or preparations, repeated measures one-way ANOVA, followed by Tukey post-test, was used (Paper III, V). Paired *t*- test was used to analyze the effect of blocking antibodies on cytokine production (Paper III) and for statistical analysis regarding individual cytokines, blood donors and phagocytosis (Paper V). The above statistical analyses were performed using GraphPad Prism 4.0.

To compare the overall cytokine response pattern between the three mycobacterial species and the Gram-positive and Gram-negative control bacteria studied in paper III, principal component analysis (PCA) was performed (197). The PCA analysis was done using the SIMCA-P software (Umetrics AB, Umeå, Sweden).

RESULTS

M. abscessus in clinical samples and clinical correlates

Cystic fibrosis patients (Paper I)
There have been reports that *M. abscessus* has become a quite frequent colonizer of the respiratory tract of cystic fibrosis (CF) patients (69, 186, 216) *M. abscessus* has also been isolated with increasing incidence in the CF population of West Sweden. These patients are cared for at the Queen Silvia Children's Hospital, Göteborg, Sweden, and monitored by regular sputum cultures at the Bacteriological Laboratories, Sahlgrenska University Hospital, Göteborg, Sweden. The first case appeared in 1997. By the end of 2005, nine (6 %) out of 140 patients with cystic fibrosis (CF) included in the regular sputum screening programme had been positive for *M. abscessus* on at least one occasion. Details of these patients are shown in Table 2, paper I. Five of the nine patients were considered to have clinical disease caused by *M. abscessus* and were, thus, treated. Another patient was given anti-mycobacterial treatment, although the contribution of *M. abscessus* to the clinical disease was uncertain. A seventh CF-patient had been persistently colonized by *M. abscessus* since 1997, without clear clinical impact, after having successfully been treated for an *M. avium*-complex (MAC) infection in 1996. Two CF-patients were thought to harbour *M. abscessus* as transient colonizers and received no anti-mycobacterial treatment.

Two CF-patients from the Stockholm/Uppsala region were referred to Göteborg for lung transplantation. They had been chronically colonized by *M. abscessus* since 2000 and 2002, respectively. Both developed disseminated *M. abscessus* disease after transplantation, for which they received treatment. Three CF-patients, cared for at the Stockholm CF-centre, were interpreted as suffering from clinically relevant airway colonization with *M. abscessus*. One of these three patients had been treated for a MAC infection ten years previously. Whereas the MAC strain was eradicated, *M. abscessus* appeared in sputa during this treatment. The *M. abscessus* strain developed *in vitro* resistance to all drugs tolerated by the patient. Finally, the patient was given IFN-γ, which led to clinical improvement. Though the *M. abscessus* strain was never eradicated, this patient has managed without mycobacterial treatment for the past five years.

Tsunami victims (Paper II)
During the tsunami catastrophe in Thailand in 2004, several thousand Swedish tourists were injured. Crush trauma to the lower extremities infected by contaminated sea water was the most common cause of illness among survivors.

On their arrival to Sweden, patients were screened by medical staff and 126 persons were admitted to the Karolinska University Hospital in Stockholm or the Sahlgrenska University Hospital in Göteborg for care. Of these 126 patients, eight men and seven women developed late-onset skin and soft-tissue infections. New wounds or abscesses appeared 20-105 days after the primary trauma, with a median début after 60 days. These new lesions were located in undamaged skin near primary sutured wounds or skin grafts (Fig. 1-2, paper II). In these 15 patients, rapidly growing mycobacteria were isolated from swab specimens (seven cases) or biopsies (eight cases), namely *M. abscessus* (n=7), *M. fortuitum* (n=6), *M. peregrinem* (n=1*)*, or *M. mageritense* (n=1). On histopathological examination, granulomas were found in all eight biopsies. Nine of the patients were treated with anti-mycobacterial drugs, and all infections healed within twelve months. Concomitant subcutaneous infections due to other microorganisms, such as *Burkholderia pseudomallei*, methicillin resistant *Staphylococcus aureus*, *Actinomyces neuii*, or various fungi appeared both early and late after the trauma. Clinical data on the fifteen patients are shown in Table 1, paper II.

Smooth and rough phenotype of *M. abscessus*

Colonies of *M. abscessus* displayed either of two phenotypes when cultured on horse blood or Middlebrook 7H10 agar (Fig. 1, paper I). One phenotype was smooth and shiny and resembled that of the type strain ATCC 19977. The other form had larger rough and markedly waxier colonies. The two different forms were stable during subculture.

Strain-typing of *M. abscessus* isolates with PFGE (Papers I and II)

PFGE analysis of *M. abscessus* isolates (Paper I)
As colonization by *M. abscessus* had increased in the CF community in Western Sweden, we asked whether spread of clones (strains) had occurred within this group, e.g. due to contaminated equipment or social mixing at summer camps etc.

Seventy-one *M. abscessus* isolates from 28 patients, were examined by PFGE after digestion of bacterial DNA by *Ase*I to distinguish individual strains (Fig. 2). The *M. abscessus* type strain was included for comparison. Ten patients yielded several isolates of *M. abscessus* from successive specimens. The longest interval between the first and last isolates analyzed was almost four years (CF-patient no 2, Table 2, paper I). All isolates analysed from a single patient had the same PFGE pattern.

Figure 2, paper I shows the relatedness of the 28 patient strains after digestion with *Ase*I. Twenty-two of the patients had isolates with unique PFGE-patterns. Six

patients harboured strains that appeared identical or near identical. We termed these strains “cluster A”. Within a group of five patients (the four CF patients 1, 2 10 and 11, and the non-CF patient 20), all isolates exhibited a rough phenotype and all had identical PFGE pattern. This sub-cluster was named A1, while the strain colonizing a sixth patient (CF patient no 6) differed by a single band from A1 and was termed A2. Using another enzyme, *Xba*I, to digest DNA before DNA analysis, four of the A1 still yielded identical PFGE pattern, and were termed cluster A1a. Strain A2 and one of the A1 isolates (termed A1b) differed by one additional band from the A1 cluster, and by two bands from one another (Fig. 3 A-C, paper I).

Two of the four patients (no 1 and 11) harbouring cluster A1a were siblings, both suffering from CF. The older sibling (no 1) had been permanently colonized by *M. abscessus* since 2000 and had clinical disease resulting from this colonization (Table 2, paper I). The younger sibling (no 11) was positive for *M. abscessus* in sputum on one occasion only, in 2001. All subsequent screening cultures during the study period were negative. She never received anti-mycobacterial treatment. The third patient with a strain belonging to cluster A1a (no 10) is a CF-patient, living in Western Sweden, but no longer cared for at the CF-centre in Göteborg. She only had one *M. abscessus* positive culture. To our knowledge, she has had no social contacts with the CF-siblings. The fourth patient (no 20) having an A1a cluster strain, was an elderly woman from another town. She was investigated at the local hospital for unspecified pleuritis and *M. abscessus* was isolated from the pleural fluid on a single occasion in 2002, but was not interpreted as causing her condition. No other patient samples yielding the cluster strain was received from this hospital in 2002. Patient no 2, whose strain belonged to cluster A, and termed A1b, was a CF-patient also colonized by *Burkholderia cepacia*. Due to this colonization, she has been kept segregated from other CF-patients (Fig. 6)

Except for the A1 cluster, the *M. abscessus* isolates from CF-patients living in the Göteborg area were no more related to one another than to isolates from CF-patients living in the Stockholm/Uppsala area, or to skin wound isolates acquired in Thailand (Fig. 2, paper I). Furthermore, isolates causing a certain clinical condition (e.g. lung infection in CF-patients) were no more related to one another than to isolates causing other clinical conditions (e.g. wound isolates).

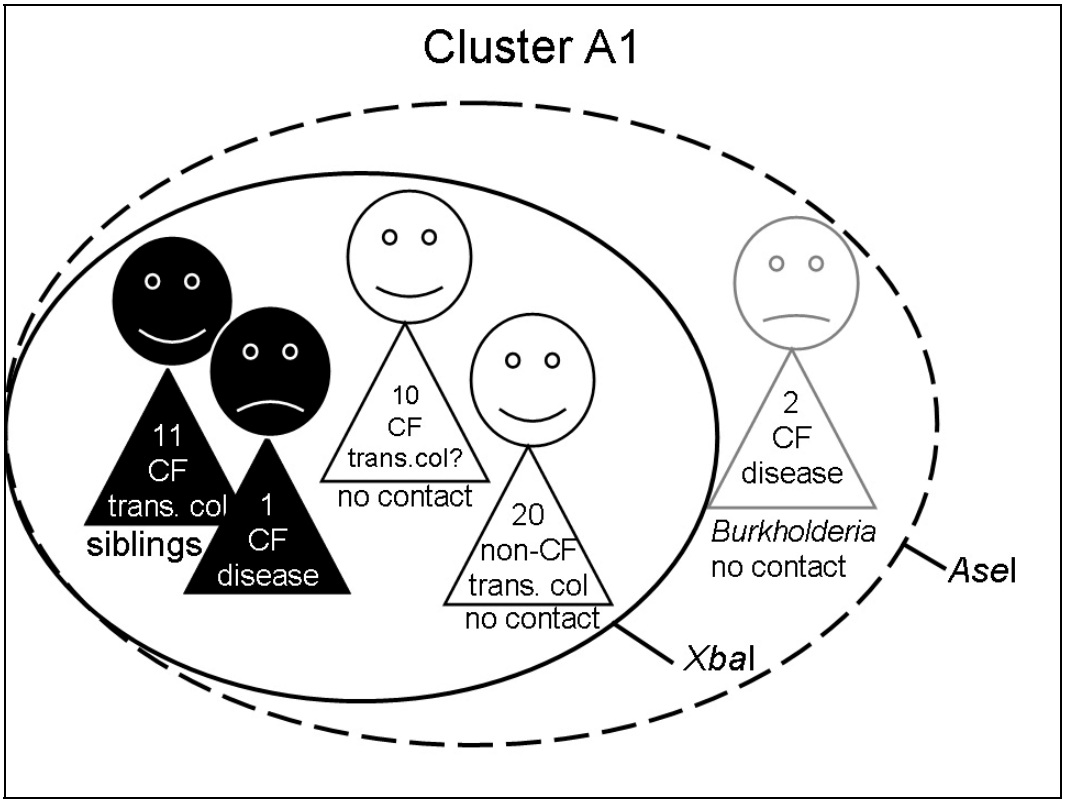


Figure 6. *M. abscessus cluster strain – Patients and clinical relevance.*

PFGE analysis of *M. abscessus* isolates from tsunami victims, paper II
Seven *M. abscessus* isolates from seven patients were examined by PFGE after digestion of bacterial DNA by *Ase*I to determine their strain identity. All patients had isolates displaying unique PFGE patterns, indicating an environmental origin (Fig. 3, paper II).

Colony morphology of *M. abscessus* in relation to clinical picture (Papers I and II)

M. abscessus exhibited either smooth or rough phenotype upon culture. We asked whether these morphotypes were linked to the clinical conditions of the infected patient.

The clinical origin of smooth and rough isolates is shown in Table 3, paper I. Sixteen patients yielded only rough isolates, most of whom were CF-patients and

non-CF-patients chronically colonized in the airways. A few rough isolates were considered to be transient colonizers of the respiratory tract. Two patients yielded a mixture of rough and smooth isolates. PFGE analysis revealed that both rough and smooth isolates from one patient belonged to the same strain. These patients had clinical respiratory disease due to *M. abscessus* (Table 3, Paper I).

Thirteen patients yielded *M. abscessus* isolates with a smooth phenotype (Papers I and II). One of these was a CF-patient (no 3) with severe respiratory disease (Table II, paper I). Five isolates were found transiently in airway samples, without related symptoms. Follow-up samples were negative suggesting transient colonization from environmental sources without clinical relevance (Table III, paper I). Seven isolates were from patients who acquired wounds contaminated from environmental sources during the tsunami catastrophe in Thailand in 2004 (Papers I and II).

These findings suggested that a rough morphotype was connected to airway pathogenicity. Thus, 81% (13/16) of the rough isolates were derived from patients with chronic colonization/disease in the airways, while only 10% (1/10) of the smooth ones were from this patient group, regarding the results in paper I ($p=0.0014$, Fischer's exact test).

Phagocytosis of smooth and rough phenotypes of *M. abscessus* (Papers IV and V)

All examined rough strains formed serpentine cords composed of many thousand bacteria (Fig. 2B, paper V). These formations resembled the cords formed by *M. tuberculosis*, in which this behaviour is regarded as an important virulence trait. Cord formation has previously been described in rough strains of *M. abscessus*, but a casual relation has not been noted.

Four rough and four smooth *M. abscessus* strains were examined for susceptibility to phagocytosis using PBMCs from five blood donors. Smooth strains were readily phagocytosed (Fig 2A, paper V). In contrast, the cords formed by rough strains could not be ingested by monocytes (Fig 2B, paper V). As a result, a significantly greater proportion of monocytes contained bacteria, when incubated with smooth, as compared to rough strains ($p = 0.002$, Fig 2C, paper V). We regard the cord formation as a potentially important virulence trait linked to the rough phenotype. Supposedly, cord forming bacteria may have greater capacity to resist defences and persist in the airways.

Cytokine production by human PBMCs in response to intact UV-inactivated environmental mycobacteria (Paper III)

Alveolar macrophages interacting with inhaled bacteria produce a range of inflammatory mediators that activate and modulate innate and acquired immune responses. Mycobacteria are Gram-positive bacteria, but their cell wall contains an abundance of lipids, not found in conventional Gram-positives.

We studied cytokine production of PBMC in response to UV-inactivated mycobacteria, as well as the two Gram-positive control bacteria *E. faecalis* and *S. mitis* and the Gram-negative *E. coli*. Three strains each of *M. avium*, *M. abscessus* and *M. gordonae* were inactivated by UV-light and used at $5x10^5$, $5x10^6$ and $5x10^7$ bacterial cells/mL (corresponding to 0.25, 2.5, 25 bacteria/ cell, respectively) to stimulate PBMC from healthy blood donors. None of the bacterial species used in the lowest concentration ($5x10^5$ bacteria/mL) were able to induce any of the cytokines examined in the study, and these results are therefore not further discussed.

IFN-γ and IL-17 production

IFN-γ is a key activator of the bactericidal capacity of macrophages and production of IFN-γ is fundamental in defence against mycobacteria (66).

Intact Gram-positive bacteria are in general very strong inducers of IFN-γ production in human PBMC, while Gram-negative bacteria are much less efficient (109). As seen in Figure 2A, paper III, mycobacteria induced IFN-γ levels intermediate between the Gram-positive enterococci and streptococci and the Gram-negative *E. coli*.

IL-17 is produced by $CD4^+$ T cells, "Th17 cells", but also by $CD8^+$ T cells and NKT cells (174). IL-17 responses were measured after 1, 2, 3, 4, and 5 days of stimulation of PBMC with UV-inactivated bacteria and the polyclonal T cell activator phytohaemagglutin (PHA). In 24 h cultures, only PHA induced IL-17. Significant production of IL-17 in response to the mycobacteria was evident from day 2, and increased up to day 5 (Fig. 2B, paper III), while typical Gram-positive and Gram-negative bacteria induced low IL-17 levels at all time points. Strong IL-17 induction was a trait shared by all tested strains of environmental mycobacteria, but with large variations between the blood donors (Fig. 2C, paper III). *E. faecalis* and *S. mitis* were poor IL-17 inducers, while *E. coli* induced some IL-17 (Responses at 5 days shown in Figure 2C, paper III). Thus, mycobacteria were uniquely effective IL-17 inducers.

IL-23, IL-12, and IL-10 production

IL-17 production has been reported to be stimulated by IL-23 produced by macrophages, but little is known regarding IL-23 responses to different microbes. We speculated that the strong IL-17 production seen in response to mycobacteria could be triggered by IL-23 released by macrophages. Blood donor PBMCs were stimulated with inactivated mycobacteria and Gram-positive/Gram-negative control bacteria. IL-23 responses were maximal on day 1 and thereafter decreased until day 5 (Fig. 7). The mycobacteria were efficient inducers of IL-23, particularly at the lower concentration ($5x10^6$/mL) (Fig 4A, paper III). The variability among blood donors was quite high. Gram-positive control bacteria also induced considerable levels of IL-23, while *E. coli* was a less efficient IL-23 inducer (Fig 4A, paper III).

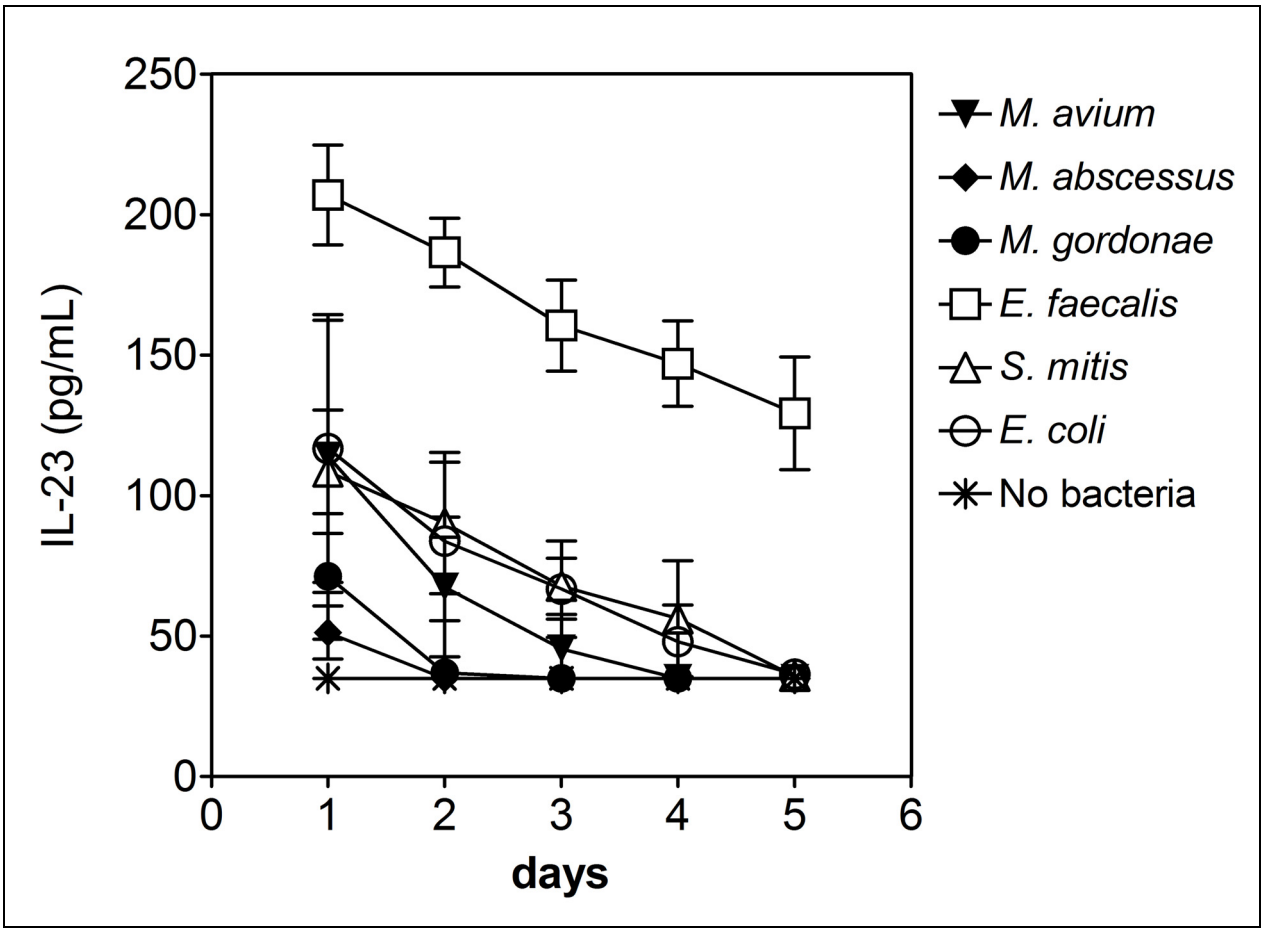


Figure 7. *The kinetics of IL-23 responses of human PBMCs to environmental mycobacteria. The figure shows the mean responses of eight blood donors to one strain each of M. avium, M. abscessus, M. gordonae and the "control bacteria" E. faecalis, S. mitis and E .coli. Error bars indicate the standard deviation between donors.*

IL-12 from macrophages induces IFN-γ production in T cells and NK cells. Intact Gram-positive bacteria are efficient inducers of IL-12, while gram-negative bacteria induce only little IL-12 from freshly isolated human monocytes. IL-12 production of PBMC in response to mycobacteria, Gram-positive and Gram-negative bacteria was measured after 24 h, a period previously found to be optimal for a variety of Gram- positive and Gram- negative bacteria. None of the tested mycobacteria induced IL-12 above the cut off level - 100 pg/mL (Fig. 4B, paper III). The Gram- positive species *E. faecalis* and *S. mitis* were very strong inducers of IL-12 production, inducing nanogram levels of IL-12 in response to $5x10^6$ bacteria/mL (2.5 bacteria/cell). Thus, the mycobacteria induced at least 50 times less IL-12 than either *E. faecalis* or *S. mitis* ($p< 0.001$ for both comparisons). The Gram-negative *E. coli* did not induce any IL-12 in freshly isolated PBMC, in accordance with previous findings of our research group (109).

IL-10 down-regulates IFN-γ production and is produced in large amounts by monocytes interacting with Gram-negative bacteria. IL-10 production of PBMC in response to mycobacteria, Gram-positive and Gram-negative bacteria were measured in culture supernatants after 24 h. Environmental mycobacteria induced variable amounts of IL-10 (Fig. 4C, paper III), the highest levels seen in response to the non-pathogenic species *M. gordonae*. When used at the higher bacterial concentration ($5x10^7$/mL), *M. gordonae* induced almost as much IL-10 as did *E. coli.* The IL-10 response to *M. avium* was strain-dependent, with some strains inducing high levels and others low levels. *M. abscessus* induced intermediate IL-10 levels among the mycobacteria. The Gram-positive bacteria, here represented by *E. faecalis* and *S. mitis*, induced little IL-10. The response to mycobacteria varied strongly between blood donors while the responses to the typical Gram-positive and Gram-negative control bacteria were much more stable. Thus, IL-10 responses to mycobacteria varied according to both the bacterial strain and individual donor.

Role of IL-10 as modulator of cytokine responses (Paper III)

We speculated that the fact that mycobacteria were potent inducers of IL-10 might explain their failure to induce IL-12, and relative inefficiency in triggering IFN-γ production. To investigate this hypothesis, neutralizing antibodies to IL-10 or its receptor (CD210) were added to the PBMC cultures before stimulation with bacteria ($5x10^6$ cells/mL). IL-12, IFN-γ, IL-23, and IL-17 were measured in the supernatants after 24 h (IL-12 and IL-23) and 5 days´(IFN-γ and IL-17). As seen in figure 5A, paper III, some IL-12 was produced in response to *M. avium*, *M. abscessus*, and *M. gordonae* when the action of IL-10 was neutralized, especially if the IL-10R was blocked. However, the amounts were still negligible compared to those induced by the Gram-positive bacteria *E. faecalis* and *S. mitis,* which induced even more IL-12 when IL -10 or the IL-10 receptor was blocked. Thus, the poor IL-

12 inducing capacity was an inherent trait of the mycobacteria not solely dependent on their efficient induction of IL-10. The IL-12 inducing capacity of the Gram-negative species *E. coli* was also improved by the addition of anti-IL-10 or anti-IL-10 receptor to the cultures.

Blocking of IL-10 or the IL-10 receptor augmented IFN-γ production in response to both mycobacteria and Gram-positive/Gram-negative control bacteria (Fig. 5B, paper III).

All mycobacteria were strong inducers of IL-23 as well as of IL-10. We therefore did not expect IL-10 to negatively affect IL-23 production. However, blocking of IL-10 or its receptor dramatically increased the IL-23 response to all tested mycobacteria, as well as the Gram-negative and Gram-positive control bacteria (Fig 5C, paper III). In contrast, blocking of IL-10 or its receptors either decreased or did not affect IL-17 levels (Fig. 5D, III).

Cytokine production by human PBMCs in response mycobacterial cell wall lipids (Paper III)

Crude surface lipid extracts were prepared from *M. avium, M. abscessus* and *M. gordonae*, and used in three concentrations (4, 40 and 400 μg/mL) to stimulate PBMC (7 donors). The responses were compared with the responses to $5x10^7$ per mL of intact UV-inactivated mycobacteria (roughly corresponding to 40 μg/mL of crude lipids). Surface lipids induced substantial amounts of IFN-γ, although intact bacteria were somewhat more effective (Fig 3B, paper III). Strikingly large amounts of IL-17 were produced in response to 4 and 40 μg of crude lipid extracts, while addition of more lipid inhibited IL-17 responses (Fig. 3A, paper III). In fact, as much IL-17 was produced in response to lipid extracts as to intact mycobacteria. Purified lipoarabinomannan (LAM) induced no IFN-γ but very large quantities of IL-17. Thus, ManLAM from *M. tuberculosis* induced 600 pg/mL of IL-17, and PILAM from *M. smegmatis* 200 pg/mL (Fig. 3C, paper III). These preparations contained some endotoxin, but *E. coli* induced very little IL-17 suggesting endotoxin contamination could not explain the strong IL-17 inducing capacity of the LAMs. Furthermore LPS does not induce IFN-γ (110).

In contrast to intact UV-inactivated mycobacteria, the surface lipid extracts, ManLAM and PILAM induced either no IL-10, or only levels just above background (probably a result of endotoxin contamination as LPS is an effective IL-10 inducer) in human PBMCs. The surface lipids induced only low levels of IL-23, whereas both ManLAM and PILAM induced moderate amounts of IL-23. The mycobacterial lipids were equally poor in inducing IL-12 as intact mycobacteria (data not shown).

Role of mycobacterial cell wall lipids in modulating IL-12 and IFN-γ responses (Paper III)

As the production of IL-10 in response to mycobacteria did not seem to fully explain their pronounced inefficiency in eliciting Th1 responses, we next examined the contribution of mycobacterial surface lipids in down-regulation of Th1 cytokine production. *E. faecalis*, which induces a typical Th1 dominated cytokine profile, was used to stimulate PBMCs from 6 blood donors in the absence or presence of crude surface lipid extracts from *M. avium, M. abscessus* and *M. gordonae*. The dose of lipids used, 40 μg/mL, was equivalent to approximately $5x10^7$ mycobacterial cells per mL. When used alone, *E. faecalis* was a potent inducer of IL-12 and IFN-γ, but when mycobacterial surface lipids were admixed, production of IL-12 was dramatically reduced (Fig. 6A, paper III). IFN-γ responses were equally reduced, but due to the larger inter-donor variability in response, the effect was not statistically significant (Fig. 6B, paper III). In contrast, admixture of mycobacterial surface lipids did not significantly alter IL-10, IL-23 or IL-17 responses to the Gram-positive bacterium *E. faecalis* (data not shown). As opposed to the surface lipids, neither ManLAM nor AraLAM could down-regulate IL-12 and IFN-γ responses to Gram-positive bacteria (data not shown). We conclude that mycobacterial surface lipids, albeit inducing quite weak IL-10 responses, had a dramatic inhibitory action on the classical Th1 response.

Principal component analysis picturing the overall cytokine pattern to mycobacteria and classical Gram-positive and Gram-negative bacteria (Paper III)

Principal component analysis (PCA) was applied to represent the overall cytokine pattern induced by mycobacteria, as compared to that induced by the two classical Gram-positive bacteria (*E. faecalis* and *S. mitis*) and the Gram-negative type bacterium *E. coli*. The PCA-procedure calculates new synthetic variables (principal components), which are linear combinations of the original variables, and that account for as much of the variance of the original data as possible (197). Here, each unit of observation (= one bacterial strain) is represented by totally 117 variables, representing the individual cytokine response of each donor to each concentration of the particular strain. From these 117 variables, 2 principal components were calculated. Figure 7A, paper III, shows the position of each strain in these two dimensions (the 1st principal component represented by the X-axis and the 2nd by the Y axis). As seen in the figure, the nine mycobacterial strains examined (three *M. avium*, three *M. abscessus* and three *M. gordonae* strains) clustered together without forming species specific groups, but they were clearly separated from both Gram-positive and Gram-negative control bacteria.

The variables contributing to the separation of the bacteria ("loadings") are shown in figure 7B, paper III. This plot shows that IL-12 and IFN-γ are strongly correlated and dominate the response to the Gram-positive *E. faecalis* and *S. mitis,* and that these cytokines relate inversely to both IL-10 and IL-17, while IL-23 is unrelated to IL-12, IFN-γ and IL-17, but inversely related to IL-10.

Monocytes form extracellular meshworks in response to cord-forming *M. abscessus* (Paper IV)

We observed that rough strains of *M. abscessus* formed large structures, cords, which could not be ingested by monocytes. When we further examined the interaction of human PBMCs and rough cord forming *M. abscessus* strains, by co-culturing bacteria and PBMCs for 16h, we found that mononuclear cells aggregated around bacterial cords. The nuclei of cells in contact with the mycobacterial cords, probably monocytes, were often distorted (Fig. 1D, paper IV). The close-up in figure 1E, paper IV shows that blood cells in contact with a *M. abscessus* cord produced meshworks that appeared to originate in the monocyte cytoplasm.

Scanning electron microscopy revealed that monocytes interacting with the smooth *M. abscessus* strain were evenly spread over the slide (Fig. 2A, paper IV). In higher magnification, monocytes were seen to attach and spread on the surface by pseudopodia and to send out very long protrusions to entrap bacteria (Fig. 2B, paper IV), i.e. a normal picture of a phagocyte ingesting bacterial particles. When interacting with rough cord forming *M. abscessus* PBMCs adhered to the cords that appeared to be partly covered by fibrous material (Fig. 2C, paper IV). Some cells in the conglomerate appeared round and regular (presumably lymphocytes), whereas others were more flattened and irregular (presumably monocytes) (Fig 2C, paper IV). On detailed examination the fibrous material seemed to originate from the surface of blood cells (Fig. 2D-F, paper IV).

The meshwork appeared in light microscopy to be formed mainly by monocytes (Fig. 1D, paper IV), but smaller, round cells, seemed to sit on top of the meshwork (Fig. 1D, paper IV). To identify which type of cells in the PBMC fraction that formed and associated with the meshwork, PBMC cultures were stained with fluorochrome conjugated anti-CD14 and anti-CD3 antibodies, surface markers for monocytes and T cells, respectively. Slides were counter stained with DAPI to visualize PBMC nuclei (blue). Bacterial cords auto-fluoresce in green when examined through the FITC-filter. Figure 3A, paper IV, shows that bacterial cords are surrounded by both $CD3^+$ T cells (green) and $CD14^+$ monocytes (red), confirming the scanning electron microscopy findings.

To determine if blood monocytes could form meshwork on their own or needed activation by lymphocytes, we isolated $CD14^+$ monocytes and stimulated them with smooth and rough *M. abscessus* overnight, or for 5 days. Isolated $CD14^+$ monocytes were able to form rudimentary DNA meshes (Fig. 3B, paper IV). However, unfractioned PBMCs from the same blood donor formed a thicker and more structured meshwork, when incubated with bacteria (Fig. 3C, paper IV). Our results suggest that lymphocytes (presumably T cells) activate the monocytes and thereby contribute to mesh formation. We also examined the cultures after 5 days of incubation and found that the morphological picture was the same as in overnight incubations. Un-stimulated PBMC controls did not form threads/meshes, neither after 1 nor after 5 days.

Monocyte meshwork contains DNA and histones

Extracellular DNA-containing material, termed NETs, has been shown to be released by PMNs interacting with bacteria (28). We examined whether the meshwork formed by monocytes also contained DNA by staining slide co-cultures of bacteria and human PBMC with DAPI to visualize double-stranded DNA (dsDNA), and observed that the meshwork was stained blue by DAPI, as were the nuclei of PBMCs, which were homogenously colored (Fig 4A-B, paper IV). We also observed that monocytes formed syncytial-like aggregates close to bacterial cords, and that cell nuclei seemed to be connected (Fig. 4C, paper IV). To confirm the presence of dsDNA in the meshes, serum from a patient with dsDNA antibodies was applied to the slide cultures followed by a FITC stained secondary antibody. The meshworks reacted with these anti-dsDNA antibodies (Fig 4D, paper IV). The third piece of evidence was that SYTOX™ Green, a third DNA staining dye also stained the meshwork (Fig. 4E, paper IV). We concluded that the meshwork formed by PBMCs interacting with rough cord forming *M. abscessus* contained dsDNA.

NETs formed by PMNs also contain histones (88). To investigate if histones were present in the monocyte meshwork, PBMC-*M. abscessus* co-cultures were stained with FITC-conjugated anti-human-H2B antibodies and counterstained with DAPI (blue, binding DNA). We found that histone H2B were, indeed, present on the meshwork, indicated by green colored dots on the mesh and weaker green treads (Fig. 4F, paper IV). The control antibody did not bind to the meshwork, and no signal was seen in the FITC-filter, except the usual auto-fluorescence from the bacterial cords, as expected when using this filter (Fig. 4G, paper IV).

DNA meshes are not contained in plasma membrane

To examine if meshes were contained in plasma membrane, PBMC-*M. abscessus* co-cultures were stained with fluorochrome conjugated wheat germ agglutinin (WGA), a lectin used to stain eukaryote cell membranes, and counter stained with DAPI to visualize dsDNA (Fig. 6D-F, paper IV). An overlay images of figures 6D

and 6E showed, that no WGA staining of DNA-containing meshes were seen (Fig. 6F). This suggests that the dsDNA was extracellular and not surrounded by membranes.

Comparison with NETs formed by neutrophils

We incubated neutrophils purified from human blood with rough cord forming *M. abscessus*, during 30 min - 4h, stained with DAPI to visualize dsDNA, and examined the preparations in a fluorescence microscope. We found that neutrophils in contact with a cord forming rough *M. abscessus* isolate indeed formed NET like structures on the surface of bacterial cords, containing dsDNA (Fig. 5A-B, paper IV). The smooth *M. abscessus* strain also did elicit some NET formation by PMNs, but they were rudimentary and scarce compared with those induced by the rough phenotype (data not shown).

NETs formed by neutrophils are readily dissolved by DNase treatment (28). We compared monocyte meshwork and neutrophil NETs with respect to susceptibility to DNase degradation. DNase was applied to slide cultures of PBMCs as well as neutrophils, stimulated with a cord forming rough *M. abscessus* strain. Neutrophil NETs disappeared 2 min after addition of DNase (data not shown). PBMC meshes were also degraded by DNase, but first after about 1h (Fig. 6A-C, paper IV).

Differences in Cytokine response to smooth and rough phenotypes of *M. abscessus* (Paper V)

IL-12, IFN-γ, IL-23, IL-10 and IL-17 production by human PBMC, in response to intact smooth and rough *M. abscessus*

Smooth isolates of *M. abscessus* were readily phagocytosed, while rough isolates formed cords that resisted phagocytosis, but induced formation of extra-cellular meshwork from PBMC. We investigated whether monocyte and T cell cytokines were differently produced in response to smooth and rough *M. abscessus*. Four rough and four smooth isolates of each phenotype were inactivated by UV-light, and used in a concentration of $5x10^6$ bacteria/mL to stimulate PBMC ($2x10^6$ cells/mL) from 12 blood donors.

IL-12, IL-10 and IL-23 responses were measured after 24 h, while IFN-γ and IL-17 responses were measured after 5 days. As the cytokine response varied greatly between donors, comparisons of reactivity to smooth and rough isolates of *M. abscessus* was performed as paired analysis, comparing the average reactivity of each donor to the four smooth isolates with the average response of the same donor to the four rough isolates. The result of this analysis is shown in figure 4, paper V. Neither smooth nor rough variants of *M. abscessus* induced any IL-12, with the

exception of one (rough) strain that induced modest amounts (data not shown). Only small amounts of IFN-γ were produced in response to *M. abscessus* and no difference was seen between rough and smooth isolates (data not shown).

IL-17 was analysed in 5 days´ supernatants, since this time point was found to be optimal for measuring IL-17 responses to mycobacteria, as shown above. *M. abscessus* induced production of very large amounts of IL-17 (up to 700 pg/mL). IL-17 was induced in similar or slightly larger quantities in response to rough, as compared to smooth strains (Fig. 4C, paper V).

Rough isolates of *M. abscessus* induced significantly less IL-10 than did smooth strains ($p = 0.0013$, Fig. 4A, paper V). On average, the blood donors produced four times more IL-10 in response to smooth, as compared to rough strains. Further, the blood donors produced significantly less IL-23 (2x) in response to rough, as compared to smooth *M. abscessus* strains ($p= 0.037$, Fig 4B, paper V).

Production of the pro-inflammatory cytokines IL-1β, TNF, IL-6, and IL-8 by human PBMC, in response to intact smooth and rough *M. abscessus*

Regarding pro-inflammatory cytokines, *M. abscessus* induced IL- 1β production from most donors, but the levels were much lower compared to those induced by both Gram-positive and Gram-negative control bacteria (Fig. 5A, paper V). The TNF response to *M. abscessus* was similar in magnitude to the response induced by *E. coli*, but much lower than the levels induced by Gram-positive bacteria (Fig 5B, paper V). IL-6 was produced in somewhat lower quantities in response to mycobacteria compared with Gram-positive and Gram-negative controls (Fig. 5C, paper V). IL-8 was produced in significant quantities in response to *M. abscessus* (Fig. 5D, paper V).

In paired analysis (8 blood donors), rough strains was shown to induce more IL-1β than did smooth strains in the same donor (2x, $p= 0.016$, Figure 6A, paper V), whereas TNF responses tended to be higher to the smooth strains (2x, $p=0.056$, Fig. 6B, paper V). No significant differences were seen in IL-6 or IL-8 responses to smooth and rough strains (data not shown).

DISCUSSION

In the present thesis, the molecular epidemiology of *M. abscessus*, and the interaction of environmental mycobacteria, particularly *M. abscessus*, with cells of the human immune system was studied. The work is based on questions that were raised during work with clinical samples in the routine diagnostic laboratory. The *M. abscessus* strains examined here were isolated at the Bacteriological Laboratories, Sahlgrenska University Hospital, Göteborg, Sweden.

In 1997, the first cystic fibrosis (CF) patient colonized by *M. abscessus* in the airways was discovered in Göteborg. Since then at least one new case has been diagnosed yearly, in this patient group which consists of 140 patients living in South West Sweden. By the end of 2005, 6% of the individuals in the West Swedish CF-population had been positive for *M. abscessus* in sputum, on at least one occasion. Other mycobacteria isolated from CF-patients included the *M. avium*-complex (3%) and *M. lentiflavum* (1%). Thus, *M. abscessus* has become the quantitatively dominant mycobacterial species recovered from CF patients in Western Sweden.

As the sputum samples have been prepared and cultured almost in the same manner since 1993 and as staff turnover at the mycobacteriological laboratory has been minimal over the past fifteen years, we find it hard to believe that increased awareness or better methodology is responsible for the increased rate of isolation of *M. abscessus*. *M. abscessus* is not likely to be missed, as it grows more rapidly than most other environmental mycobacteria. Instead, we believe that colonization of the airways of CF patients by *M. abscessus* has, indeed, increased. A similar increase has been reported from several CF Centres in Europe and the US (73, 106, 113, 185, 186, 216).

For comparison, we examined the species distribution of environmental mycobacteria in clinical samples of other patient groups. A total of 62 patients yielded environmental mycobacteria in airway samples processed at the Bacteriological Laboratories in Göteborg, during 2005. The *Mycobacterium avium*-complex dominated strongly (53% of isolates) while *M. abscessus* was much less common (6% of isolates). Thus, *M. abscessus* appears to display a strong predilection for the airways in patients with CF.

Our observations prompted the question whether patient-to-patient transmission of *M. abscessus* had occurred within our CF population in the last 10-15 years. Such spread could have occurred via social contacts in the CF community or through contaminated equipment used for clinical examination at the CF clinic. Two methods, PFGE and RAPD, have previously been used to retrospectively analyze *M. abscessus* outbreaks, all of which were found to involve a single strain usually

originating from water supply systems, with no spread between patients (238, 255, 256). We initially chose the PFGE method, since it was already used by our laboratory for sub-typing of other bacteria. Unfortunately we initially encountered problems with degradation of DNA in some strains during electrophoresis. A solution to this problem was to supplement the tris-borate buffer with tiourea (256), but this is a mutagenic substance whose use was prohibited by The Swedish Work Environment Authority. The same type of DNA degradation had also been reported for *Streptococcus lividans* and some strains of *Salmonella,* and the problem had been solved by using HEPES buffer in the electrophoretic step (145, 198, 199). When we changed to HEPES buffer during electrophoresis, DNA degradation ceased and the PFGE method proved suitable for identification of individual *M. abscessus* strains. Although PFGE is time consuming and expensive it has high reproducibility, permitting band patterns to be stored in a database for future comparisons. This is a significant advantage as *M. abscessus* infections are rare and the patient population of interest small. This PFGE method is now in routine use at our laboratory.

We also tried to use RAPD, a faster and less laborious method that had successfully been used to identify *M. abscessus* strains causing outbreaks and pseudo-outbreaks (151, 255). However, in our hands, this method had too low reproducibility since the same isolate did not yield the same band pattern, when analysed repeatedly. Due to poor reproducibility, isolates for comparison had to be run on the same gel, and comparisons with prior analyses were impossible. We were never able to optimize this method properly for *M. abscessus* and it was therefore abandoned as the PFGE method was modified and started functioning.

Typing of *M. abscessus* isolates by PFGE revealed that most CF- patients carried their own unique strain. The only exception was a cluster of four patients who harboured isolates that were indistinguishable by PFGE (Cluster A1a) and therefore belonged to a single strain, according to this method. Two of the patients sharing this strain were siblings with CF. The third patient was a CF-patient not cared for in Göteborg, who had no known contacts with the siblings, and the fourth patient an elderly woman in another town two hundred kilometres north of Göteborg. She was cared for at the local hospital for other causes, and she was culture positive for *M. abscessus* on a single occasion, without associated symptoms. The samples from which the cluster isolates derived were processed months apart, including the samples from the two siblings, making contamination via the laboratory highly unlikely.

One of the colonized siblings with CF has yielded *M. abscessus* in sputa continuously since 2000. The younger sibling was positive on a single occasion only (2001), during the 5-year period which was focus of the study. However, last year (2008), the younger sibling was culture positive for *M. abscessus* on several occasions. We are currently analysing these new isolates by PFGE. It is not

unreasonable to believe that the first colonized sibling may have passed on her strain to her sister in 2001, but on the other hand they shared the same household at the time, and thereby the same "bacterial environment". Transfer of *Pseudomonas aeruginosa* strains between siblings with CF has previously been observed (221).

Except for the possible transmission of clone A1a between the two siblings, we found no evidence of spread of *M. abscessus* between CF-patients cared for at the West Swedish CF centre. Furthermore, isolates from Western Sweden were no more similar to one another than isolates from other geographical locations. As *M. abscessus* is ubiquitous in nature, our results suggest that this bacterium is acquired from the environment by individual patients and that spread between patients is uncommon. In a single patient, the source of infection has seldom been found (216).

Two CF-patients both colonized/infected with *M. abscessus* underwent lung transplantation, and clearly, immuno-suppression is involved in aggravation of *M. abscessus* disease and both developed disseminated disease post-operatively, but survived. Both fatal and non-fatal infections with *M. abscessus* have been described after lung transplantation, but the overall mortality after transplantation is unrelated to *M. abscessus* colonization (44, 46, 76, 163).

One reason for the increased colonization in CF patients may be that better control of "classical" respiratory pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* might pave way for opportunists of lower inherent pathogenicity, *M. abscessus* included. Whether any particular abnormalities characterize individuals prone to respiratory colonization remains to be shown. CF is, in general, not associated with deficiencies in the innate or acquired immune systems. This does not exclude that the sub-group which becomes colonized with *M. abscessus* might have subtle still unknown deficiencies in their defence systems, besides the structural airway defects caused by the CF disease itself.

The finding that *M. abscessus* is now a significant pathogen in CF patients increases the need of improved surveillance for this species to enable prompt diagnosis and treatment. There are indications that eradication is possible only when treatment is started early in infection (94). However, treatment is problematic due to the inherent resistance of this species to a wide range of antibiotics.

The three non-CF patients with chronic pulmonary *M. abscessus* colonization/infection studied had no known immunological deficiencies, besides old age in one case. One patient had previously suffered from MAC disease and had developed bronchiectatic changes and *Aspergillus fumigatus* colonization. Allergic broncho-pulmonary aspergillosis and systemic steroids have been identified as risk factors for mycobacterial disease (179). The second patient was a smoker with chronic obstructive pulmonary disease (COPD), while the third was a

healthy non-smoking middle-aged woman. All three patients had underlying identified risk factors for pulmonary *M. abscessus* disease (patient previously treated for mycobacterial disease, COPD patients and middle-aged female non-smokers) (55).

We also studied fifteen patients, surviving the tsunami catastrophe in Thailand, in 2004, whose wounds were infected with rapidly growing environmental mycobacteria leading to late-onset wound infections. Of these, seven were culture positive for *M. abscessus*. The bacteria probably entered injured body parts with contaminated water or soil during the tsunami, although skin or soft-tissue abscesses developed first after a latency period. All tsunami patients harboured their own unique *M. abscessus* strain as judged by the PFGE pattern, suggesting an environmental source rather than nosocomial spread. PFGE also showed that the seven strains from Thailand were no more related to one another than to Swedish strains from patients with or without cystic fibrosis. Furthermore, PFGE revealed no apparent relation between strains causing a certain disease manifestation.

Instead, we observed a striking relation between colony morphotype and disease spectrum. All isolates, but one retrieved from chronically colonized airways of CF-patients displayed a rough phenotype when cultured on solid media, as opposed to the smooth and shiny appearance of the *M. abscessus* ATCC type strain. The wound isolates and all but one of the isolates interpreted as contaminants/transient colonizers of the airways were also smooth like the type strain. The only smooth isolate apparently causing airway disease was an isolate from a newly infected CF patient, who presented abruptly with clinical pulmonary disease and heavy mycobacterial growth in sputum. This suggests either a pronounced virulence of this particular smooth strain, or some particular defect in the antimicrobial defences of the infected individual. In two case reports of fatal pulmonary infection due to *M. abscessus* in patients with cystic fibrosis, the bacterial strain was noted to exhibit a rough phenotype (42, 211), supporting our findings of an increased capacity of rough strains to cause airway disease.

Two patients in our study had a mixture of smooth and rough isolates. In both cases, all isolates from the same patient belonged to a single strain, regardless of colony morphology, which shows that strains may switch between smooth and rough phenotype, *in vivo*. Both phenotypes were stable upon subculture. It has recently been reported that a rough mutant arising in an otherwise smooth population of *M. abscessus* was able to cause persistent, invasive infection in human monocytes and in the lungs of mice, which the smooth wild type did not (118). This conversion of *M. abscessus* from a smooth to a rough phenotype was associated with reduction of the glycopeptidolipid contents of the cell wall (118). In *M. chelonae*, a species closely related to *M. abscessus*, rough colony mutants were found to be more hydrophobic than the smooth wild type and they had also reduced susceptibility to glutaraldehyde and ethambutol (166). We speculate that a rough

phenotype might facilitate biofilm formation, a growth form thought to be advantageous in airway colonization of CF-patients (190), which is in concordance with a report demonstrating that rough phenotypes of *Pseudomonas aeruginosa* formed biofilm faster than the smooth wild type when cultivated in flow chambers (68).

We also found that all rough isolates of *M. abscessus*, but none of the smooth ones formed cords, which are multi-cellular aggregates with lined-up bacteria. Cord formation has been described for one rough *M. abscessus* isolate by another group (118) and is since long known as the normal behaviour and a virulence factor in *M. tuberculosis* (173). The exact biochemical background for cord formation is unknown and whether bacteria also grow as cords in infected patients is not known. However, we found that bacterial cords could not be phagocytosed, which most likely confers a significant increase in pathogenic potential.

Inhaled mycobacteria interact with pulmonary macrophages. These cells express a multitude of surface receptors that recognize microbial structures. Encounter of bacteria leads to production of cytokines and other inflammatory mediators that activate and modulate the innate and acquired immune responses to the microbe in question. Relatively little is known about the cytokine responses to environmental mycobacteria. We first studied production of human PBMCs stimulated with UV-inactivated bacteria belonging to three species of environmental mycobacteria: *M. avium, M. abscessus,* and *M. gordonae*. This study focused on IL-17 and IFN-γ, produced chiefly by T cells, and IL-12, IL-23, and IL-10 produced by monocytes/macrophages.

We found that all environmental mycobacteria examined induced remarkably high levels of IL-17, much higher than "conventional" Gram-positive and Gram-negative bacteria (*E. faecalis*, *S. mitis* and *E. coli*). Thus, mycobacterial antigens seem to especially stimulate T cell production of IL-17. Our results suggest that mycobacterial lipids may be the principal antigens eliciting IL-17 responses, as crude surface lipid extracts from the three mycobacterial species, as well as ManLAM from *M. tuberculosis* and PILAM from *M. smegmatis*, induced as much IL-17 from human PBMC, as did intact mycobacteria. IL-17 is produced by so called Th1 cells, i.e. $CD4^+$ T cells, but $CD8^+$ T cells, γδ T cells and NKT cells may also produce IL-17. We favor the hypothesis that T cells, rather than NKT cells were the chief producers of IL-17 in response to mycobacteria, as IL-17 concentrations in the cell supernatants increased from day 2 to day 5.

CD1 molecules are known to present lipid antigens to T cells (16, 17), but experiments we performed to inhibit IL-17 production by blocking CD1 were inconclusive, whereas blocking of MHC II reduced IL-17 production (unpublished data). This suggests that $CD4^+$ T cells were at least involved in the response. The

cellular source of the IL-17 produced in response to mycobacteria has to be further investigated.

Surface lipids could also be involved in IFN-γ induction, as mycobacterial crude lipid extracts induced modest IFN-γ responses, as did intact bacteria. IFN-γ is produced by $CD4^+$ and $CD8^+$ T cells, by NK cells and NKT cells. There was a considerable variation between blood donors in IFN-γ responses to the mycobacteria, which might reflect differences in prior immune encounter of these bacteria that are ubiquitous in the environment. IFN- γ is important in stimulating the bactericidal capacity of macrophages and combating mycobacterial infection, as IFN-γ deficiencies may underlie susceptibility to mycobacteria (64). The modest IFN-γ production in response to mycobacteria may be one factor contributing to the capacity of mycobacteria to survive within macrophages. However, the poor Th1 triggering capacity characterized all environmental mycobacterial species, without link to pathogenicity. Thus, the apathogenic *M. gordonae* and the opportunistic pathogens *M. avium* and *M. abscessus* induced approximately equal amounts of IFN-γ.

IFN-γ production from T cells and NK cells is promoted by IL-12 produced by mononuclear phagocytes. Mycobacteria induced no IL-12 production in freshly isolated human monocytes. All three species of environmental mycobacteria instead triggered production of massive amounts of IL-10, a cytokine known to counteract IL-12 and IFN-γ production (109). When IL-10 or the IL-10 receptor was blocked with a neutralizing antibody, mycobacteria were able to induce small amounts of IL-12, especially when the IL-10 receptor was blocked. IL-10, has been shown to promote survival of *M. tuberculosis* infected mice (15). Interestingly, however, strong IL-10 induction was a feature of all mycobacteria tested in this study, regardless of their pathogenic potential. Actually, *M. gordonae*, which has only rarely been linked to pathological conditions and whose presence in clinical samples is generally a sign of environmental contamination, was the most efficient inducer of IL-10. Mycobacteria were almost as efficient as Gram-negative bacteria in IL-10 inducing capacity, but the microbial patterns that trigger IL-10 production have not yet been identified. The mycobacterial cell wall differs widely from that of classical Gram-positive bacteria like streptococci and enterococci. Although mycobacteria are, in theory, Gram-positive, the surface lipids form a bilayer that partly resembles that of Gram-negative bacteria, albeit devoid of lipopolysaccharide (LPS). We found the soluble lipids, however, to be unable to induce IL-10, in contrast to LPS (110).

To study the role of mycobacterial cell wall components in modulating IL-12 and IFN-γ responses we cultured human PBMCs with an admixture of Gram-positive bacteria (*E. faecalis*) and surface lipids from mycobacteria and found that the lipid extracts strongly inhibited the IL-12 and IFN-γ inducing capacity of the Gram-

positive bacteria. Hence, these lipids are likely to play a significant role in reducing IL-12 and IFN-γ responses to mycobacteria, but the mechanism for this effect remains to be identified.

IL-23 is a cytokine akin to IL-12 in that they share the p40 subunit (120). Here, we show that intact environmental mycobacteria, as well as both Gram-positive and Gram-negative bacteria were capable of inducing IL-23. IL-23 stimulates Th17 cells, but has been reported to induce both proliferation and IFN-γ production in human activated T cells (187). The role of IL-23 in the defense against mycobacteria is still unclear, as neutralization of neither IL-23 nor IL-17 increases susceptibility to *M. bovis* BCG infection in mice, although IL-23 was found to control bacterial growth and promote granuloma formation in the absence of IL-12 (187). Clearly, the unique IL-17 inducing capacity of mycobacteria was not due only to their IL-23 stimulating capacity, as we found that Gram-positive bacteria, which were equally efficient in triggering IL-23 production, induced only minimal amounts of IL-17.

We performed principal component analysis to determine if the three species of environmental mycobacteria (*M. avium, M. abscessus,* and *M. gordonae*) examined, induced a unique cytokine profile different from other Gram-positive bacteria (*E. faecalis, S. mitis*) and from the Gram-negative *E. coli*, and whether the cytokine pattern differed between the three species. PCA-analysis placed the environmental mycobacteria in a distinct group clearly separated from both the conventional Gram-positive bacteria (*E. faecalis* and *Streptococcus mitis*) and *E. coli*. Thus, the environmental mycobacteria examined induced a unique cytokine pattern, clearly distinct from that induced by either conventional Gram-positive or Gram-negative bacteria. We could not reveal any substantial difference in cytokine responses to the three mycobacterial species, two of which are pathogenic in selected patient groups (*M. abscessus* and *M. avium*) and one regarded as apathogenic (*M. gordonae*). The cytokine response to mycobacteria was characterized by a very strong IL-17 response, strong IL-10 and IL-23 responses, weak IFN-γ responses and absence of IL-12 induction. Despite the claims that IL-23 stimulates IL-17 and IFN-γ responses, IL-23 was related to neither of these cytokines in PCA analysis.

The above studies were performed using smooth strains representing *M. avium*, *M. abscessus* and *M. gordonae*. As we had noted that rough varieties of *M. abscessus* exhibited increased capacity to colonize the respiratory tract and cause disease, we were interested in studying the interaction between human mononuclear phagocytes and rough forms of *M. abscessus*. We found that human PBMC and/or monocytes upon interaction with rough cord forming *M. abscessus*, too large to be phagocytosed, produced meshwork of threads that encapsulated the bacterial cords. The monocyte derived meshworks contained double-stranded DNA, as evidenced

by DAPI and SYTOX™ Green staining, and interaction with human antibodies to double-stranded DNA. We also found that meshes were decorated with human histone-proteins, a phenomenon also described for NETs (28). Meshes were not visualized by fluorochrome conjugated wheat germ agglutinin (WGA), a stain used to visualize plasma membranes, suggesting that they were mostly extracellular. However, the meshwork might contain both extracellular DNA from dead cells and cellular protrusions from living monocytes. The mesh structures resembled the extracellular fibre traps (NETs) released from neutrophils reported by Brinkmann et al. (28, 29) some years ago. We found that *M. abscessus*, especially the cord forming rough phenotype, was also capable of inducing neutrophil NETs. The fibres formed by PBMCs appeared to be thicker and were more resistant to DNase treatment than the previously described NETs originating from PMNs. This indicates that other still unknown stabilizing molecules may be present in monocyte mesh structures. These meshes were only induced in response to cord forming rough mycobacteria, while the smooth non-cord forming isolate of the same mycobacterial species did not elicit this response, probably because they were readily internalized by phagocytosis. These observations suggest that this meshwork formation is a defense mechanism which serves to limit spread of bacteria that cannot be ingested. As we inactivated the bacteria by UV-light before using them to stimulate PBMC, we conclude that the bacteria do not need to be alive to induce meshwork.

Staining of mononuclear cells surrounding cord formations with antibodies against surface markers for monocytes (CD14), T cells (CD3) and B cells (CD19), revealed that both monocytes and T cells participated in the cell aggregation around the cords. This coincided with the picture seen in scanning electron microscopy, where large irregular cells (probably monocytes) and smaller round cells (probably T cells) were both sitting on the cords. Only very few B cells were seen in the aggregates (unpublished data). Although both monocytes and T cells were found on the meshwork, purified $CD14^+$ monocytes were able to form meshes by themselves. However, these meshes seemed to be weaker, which suggest that interactions between T cells and monocytes may enhance mesh formation.

We also noted that monocytes formed syncytial-like aggregates in the vicinity of bacterial cords, and DAPI staining revealed that cell nuclei seemed to be connected to each other via bridges of DNA. It is possible that these mesh structure is the first step in granuloma formation *in vivo*.

Despite the marked differences in internalization by monocytes, cytokine responses did not differ dramatically in response to smooth and rough strains. For example, IL-12 was not induced, or induced only in small quantities, by both smooth and rough isolates. However, some distinct differences were noted. Rough strains induced much less IL-10 (4x), and also less IL-23 (2x), and TNF (2x) than did smooth strains, but more IL-1β (2x). IL-10 may be produced from both T cells and

monocytes, but we consider monocytes to be the most likely producers in this case, since we measured IL-10 after 24 h, a time point when contribution from T-cells would probably not yet be significant. TNF and IL-23 are both produced by monocytes. The lower production of IL-10, TNF and IL-23 in response to rough strains might be due either to the reduced amount of glycopeptidolipids in the bacterial cell envelope or to a less efficient cytokine triggering effect by extracellular, as opposed to internalized bacteria. TNF, for example, is triggered more efficiently by intact bacteria that can be phagocytosed, than by soluble bacterial components, which can only interact with the surface of macrophages (10).

High amounts of IL-17, but only moderate levels of IFN-γ were produced by human PBMC in response to both rough and smooth strains. So, despite reduced capacity of rough strains to induce IL-23 they were equally effective in inducing IL-17 (and equally inefficient in inducing IFN-γ). This confirms our findings that there is no simple relation between production of IL-23 from macrophages and IL-17 from T cells.

Interestingly, IL-1β was produced in greater amounts in response to rough isolates of *M. abscessus*, as compared with smooth. IL-1β has recently been implicated as a stimulator of IL-17 responses (156). Conversely, TNF was produced in greater amounts in response to smooth bacteria. TNF and IL-1β have overlapping effects, (62, 98). Intriguingly, IL-1β is produced in a two-step process. A first signal triggers the production of pro-IL-1β, which is stored intracellulary. This pro-IL-1β is cleaved and exported after a second signal that induces assembly of an inflammasome inside the cell (252). Thus, injury to the macrophage may be a necessary signal for release of IL-1β. One may speculate that the frustrated phagocytosis, mesh formations and/or the cords formed by rough *M. abscessus* leads to partial disintegration of macrophages. However, compared with Gram-positive and Gram-negative control bacteria, both rough and smooth strains of *M. abscessus* were quite poor IL-1β inducers. This could imply a limited innate inflammatory response to these bacteria, which facilitates their survival in an infected host. It is striking that the formation of extruded fibres from monocytes can take place without more pronounced release of endogenous danger signals and IL-1β production. This implies that the mesh formation occurs by a process that is not related to necrosis, which elicits violent inflammatory responses. NET formation in neutrophils is regarded as a death process distinct from apoptosis and necrosis (88).

In summary, the studies included in this thesis focus on the molecular epidemiology and immune defence to environmental mycobacteria that are opportunistic pathogens in some selected patient groups. We have reported that *M. abscessus* has become a significant pathogen in the West Swedish CF population,

but that no patient-to-patient transmission of *M. abscessus* had occurred. Furthermore, we have made the original observation that strains isolated from the airways mainly exhibit a rough colony phenotype, while strains isolated from wounds were mainly smooth, and that cords formed by rough bacteria, resist phagocytosis, but instead trigger extrusion of DNA and histone containing meshes from monocytes that entrap cords. We also report that mycobacteria are unique inducers of IL-17, and that mycobacterial cell wall lipids might contribute to this response. Protective immunity to environmental mycobacteria and tuberculosis is still incompletely understood and is today an active and challenging research field world wide, but the microbes are always one step ahead.

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