Secreted proteins of mycobacteria and their role during infection

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Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

2011

Acknowledgements

The present work was performed at Section for microbiology and immunology, The Gade Insitute, Faculty of Medicine and Dentistry, University of Bergen. The research project was funded by the Research Council of Norway.

First of all I would like to thank my supervisors Dr. Tehmina Mustafa and Professor Harald Wiker for introducing me to the topic of tuberculosis, providing me with the opportunity to work and write a thesis on this exiting field, and for guiding me through the process of completing it. It has always been easy to drop by their office for discussing my results regardless if they were of the positive or negative kind. Their great experience in this field has been inspiring.

I thank Gustavo de Souza, for involving me in his project on mycobacterial membrane proteins, and for just being good guy to have around the lab. If you get his nod of approval, you know you're on the right track. The rest of the tuberculosis group, past and present, has been wonderful company throughout the years: Tina, Linda, Guri, Hiwa, Gisele, Lars, Heidi and Ruth.

I thank the Bergen Research School of Inflammation (BRSI) for contributing to a positive research environment and providing a forum open for discussion of ideas and science with peers and seniors.

I also thank the following people:

- All colleagues and students at the Gade Institute, who have all been incredibly friendly and helpful during my time here.
- Kalaiarasy Kugarajh and Karen Egeland for their great assistance with the work with animals.
- My co-authors: Swati Prasad, Inge Jonassen, and Lisbet Sviland, for their important contributions to the papers

- The staff at Vivarium, the Animal Research Facility
- Dr. Rogelio Hernandez-Pando, Diana Aguillar, Yuriria Cortés, and Dr. Francisco Javier Llamas Gutiérrez and all the other staff at UNAM, Mexico City, Mexico, for teaching me how to work with mycobacteria in mouse models, and taking very good care of me during my stay in their fantastic city.
- Philip Hogarth and Daryan Kaveh at TB Research Group, Veterinary Laboratories Agency, Surrey, UK, for their hospitality.

I thank my friends for positive distractions.

My family has been always been there for me, and I would never have made it to where I am to today without your support.

I thank Cecilie for being the one.

Summary

One-third of the world's population is infected with the causative agent of tuberculosis, *Mycobacterium tuberculosis*. A fraction of infected individuals have an active form of the disease, while the remainder is able to control, but not eradicate the bacilli. The progression and outcome of the disease is dependent on precise interactions between the bacterium and its host, and the balance between the two organisms reflects thousands of years of co-evolution and adaptations. The host immune system recognises structural features of the bacilli, and immediately initiates responses upon pathogen entry. This does in turn lead to the development of highly specific immune responses that prime infected cells to become efficient mycobacterial killers. The bacterium on the other hand, produces molecules that modulate the responses in its own favour, and is therefore able to survive within the host cells for a prolonged time. In this thesis, our primary focus has been to investigate various aspects of the proteins produced by the bacterium. A broader knowledge in this field might contribute to the development of new vaccines, diagnostic markers, and drug targets, as well as providing increased understanding about the biology of this lethal pathogen.

In paper I, we determined the best algorithm for predicting mycobacterial proteins that are secreted via the general secretory pathway, which is responsible for secretion of a majority of secreted proteins. Proteins processed by this system harbour an N-terminal signal peptide that is cleaved off as the protein is released on the exterior of the cell. The SignalP v3.0 hidden Markov model was found to be the best performing algorithm, and it identified the signal peptide of all proteins in a positive validation set, and did not generate any false positive results from a negative set. It also correctly identified the majority of the signal peptide cleavage sites.

In paper II, we identified 30 proteins with predicted signal peptides that contrary to the prediction were observed to be uncleaved. These proteins were found to be enriched in the membrane fraction of the bacterium. A comparison of amino acid

sequences showed these uncleaved signal peptides to be similar to peptides known to be cleaved, but with some variations in the amino acid composition, particularly in the -3 and -5 positions relative to the cleavage site. Some of the proteins had a transmembrane domain spanning the predicted cleavage site, and which might interfere with the signal peptide processing. A combination of features probably explains why most of these proteins are not processed by the signal peptidase. It is possible that these uncleaved signal peptides act as hydrophobic anchors for their respective proteins, and cause them to be retained in the membrane.

In paper III, we studied a long term (up to 17 months) course of infection with two BCG strains in mice, and investigated the *in vivo* expression of secreted and somatic mycobacterial proteins. BCG Pasteur is phylogenetically more distant from the original BCG strain than BCG Russia. According to the general belief that serial passages of a laboratory strain causes successive attenuation, BCG Pasteur should be more attenuated. However, in our mouse model, BCG Pasteur was more persistent and induced greater pathology than BCG Russia. The increased virulence of BCG Pasteur might be related to a higher expression of proteins with antioxidant properties, which make the bacilli more able to resist killing mechanisms of the host cells. At 2 and 3 months after infection we investigated the expression of mycobacterial antigens by immunohistochemistry using 7 different polyclonal rabbit antibodies. The staining patterns revealed strong staining, particularly in macrophage-dominant areas, but also in lymphocyte-dominant areas and in bronchial associated lymphoid tissues. In addition, we observed scattered cells with high antigen load in the normal lung parenchyma. This likely reflects the ability of the bacilli to migrate from established foci to more distant cells and remain unnoticed by the host's danger signalling system preventing it to alert other immune cells of the spreading infection. At the time point investigated, the immunohistochemical analyses revealed great similarities in protein expression between the BCG strains.

In paper IV, we investigated the *M. tuberculosis*-expression and localisation of secreted and somatic proteins in human lungs and lymph node tissues. We found a

higher frequency of staining of the secreted proteins in loosely organised cell aggregates in the lungs, as compared to well-structured lymph node granulomas. In the latter, some major secreted proteins were only detected in the necrotic centres and not in the surrounding cells. Antibodies to the proteins of the antigen 85 complex gave generally low staining intensities, while anti-MPT64 produced strong staining of cells in all granulomas. Accumulation of proteins like MPT64 might eventually modulate immune responses to facilitate persistence of mycobacteria and pathogenesis of disease. Finally, staining with anti-Mce1A was found more frequently in organised granulomas, compared to regions without well-structured granulomas. This is in line with other studies that implicate a role for this mycobacterial protein in granuloma formation, and emphasises that both the host and the pathogen favour this structure.

Abbreviations

ATP	Adenosine triphosphate
BALT	Bronchi-associated lymphoid tissue
BCG	Mycobacterium bovis Bacillus Calmette-Guérin
CD	Cluster of differentiation
CFU	Colony forming units
CLRs	C-lectin type receptors
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-related kinase
FcRs	Immunoglobulin Fc receptors
GKO	Gene knock-out
HIV	Human immunodeficiency virus
ICAM	Intracellular adhesion molecule
IL	Interleukin
INF	Interferon
IS	Insertion sequence
JNK	Jun N-terminal kinase
LAM	Lipoarabinomannan
ManLAM	Mannosylated lipoarabinomannan
MAPK	Mitogen-activated protein kinase
Mce	Mammalian cell entry
MHC	Major histocompatibility complex
MIRU	Mycobacterial interspersed repetitive unit
mRNA	Messenger ribonucleic acid
NK	Natural killer
NO	Nitric oxide
PIM	Phosphatidylinositol mannoside
PPD	Purified protein derivative
PRR	Pattern recognition receptors
rBCG	Recombinant BCG
RD	Region of difference
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate

SNAREs	N-ethylmaleimide-sensitive factor-attachment protein receptors
SPase	Signal peptidase
sRNA	Small ribonucleic acid
SRP	Signal recognition particles
STAT	Signal transducer and activator of transcription
TACO	Tryptophan aspartate containing coat protein
TGF	Tumor growth factor
Th	T helper cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRIM	Tripartite motif-containing
VNTR	Variable number tandem repeats

List of publications

I. Leversen NA, de Souza GA, Målen H, Prasad S, Jonassen I, Wiker HG. Evaluation of signal peptide prediction algorithms for identification of mycobacterial signal peptides using sequence data from proteomic methods. Microbiology (Reading, Engl). 2009 Jul.;155(Pt 7):2375–2383.

II. de Souza GA, Leversen NA, Målen H, Wiker HG. Bacterial proteins with cleaved or uncleaved signal peptides of the general secretory pathway. Journal of proteomics. 2011 Dec.; 75(Issue 2):502-510

III. Leversen NA, Sviland S, Wiker HG, Mustafa T. Long-term persistence of BCG Pasteur in lungs of C57BL/6 mice following intranasal infection. Scandinavian Journal of Immunology 2011: *In press*

IV. Mustafa T, Leversen NA, Sviland L, Wiker HG. Differential *in vivo* expression of mycobacterial proteins in *Mycobacterium tuberculosis* infected lungs and lymph node tissues. 2011: *In manuscript*

Contents

1. Introduction

1.1 Tuberculosis at a glance

Tuberculosis is caused by the bacterium *Mycobacterium tuberculosis*. This organism has coexisted with us humans since our origin, and is likely to have killed more of us than any other microbial pathogen. Despite extensive efforts to fight this disease during the last century, it remains one of our most ferocious killers. Around 1.7 million people die from tuberculosis every year, which is a huge number in itself, but WHO has estimated that 2 billion people are infected with this pathogen (http://www.who.int/topics/tuberculosis/en/). That is one-third of the world's population; an enormous reservoir of the bacteria. This prevalence represents one of the greatest challenges in the fight against this disease.

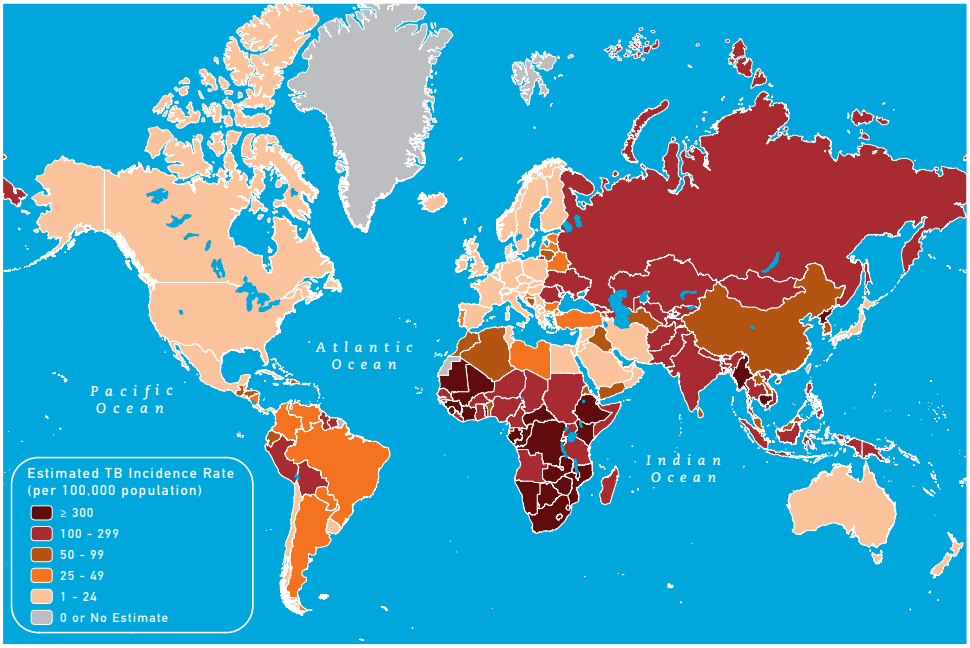

Figure 1. Estimated TB incidence rate for 2009 (Centers for Disease Control and Prevention, Atlanta, USA)

Diagnostic tools, treatment with drugs, vaccination programs, and improved living conditions and health care infrastructure, have almost eradicated the disease from the

industrialised world. The burden of epidemic disease now rests primarily on the developing world. It is tightly associated with poverty and poor living conditions, and tragically affects mostly young people in what should have been their most productive years. Twenty-two countries in Africa and Asia carries approximately 80% of the burden of this disease, with sub-Saharan Africa being one of the most intensively affected areas. Tuberculosis is also tragically linked to HIV, as coinfection with both pathological agents worsen the course of infection and outcome of both diseases (1).

Being infected with tuberculosis is not synonymous with clinical disease; the bacteria only cause active disease in an estimated 3-15% of those who are infected, the remainder have latent disease with no clinical signs of infection. Individuals with latent tuberculosis are able to control the disease, but not to the point of immediate eradication. Importantly, people with latent infection are not infectious, but they have a small chance of developing active disease later in life. The risk of reactivation is rapidly reduced as a function of time from the original infection, which strongly indicates that latency can be spontaneously cleared from the body (2). Active tuberculosis is partly characterised by symptoms like fever, a general malaise, and a long-lasting cough (3). The cough develops following inflammation and tissue necrosis of the lungs, and blood and sputum carries bacilli from the lung to the airways, and they are spread through droplet nuclei when the person coughs, sneezes, or talks. Worldwide, around 10 million people develop active tuberculosis every year.

The greatest challenge for diagnosing tuberculosis is to distinguish between active- and latent tuberculosis. The sheer number of latently infected individuals makes it impossible to treat all cases and the focus of treatment is therefore patients with active and infectious form of the disease. Unfortunately, the current diagnostic tools are not optimised to specifically identify this stage, and clinicians primarily rely on clinical symptoms to identify contagious individuals. However, at this point the patient may already have infected several people in its near surroundings. The treatment of tuberculosis is cumbersome; the bacilli have a cell membrane that is

difficult to penetrate and they primarily reside inside host cells within dense cell structures called granulomas. Treatment therefore requires a long and strict course of multiple antibiotics. Failure to comply with the recommended treatment regimen is causing problems that might have a dramatic global impact; an emerging bacterial resistance to one or more of the available drugs presents a tough new challenge for controlling tuberculosis. The only available vaccine against tuberculosis, *M. bovis* BCG (Bacillus Calmette-Guérin, BCG), was made available in the 1920s, and has been widely used all over the world, in particular after World War II. This vaccine has undoubtedly saved many lives, but it also has its shortcomings; many adults living in areas with high prevalence of tuberculosis do not acquire complete protection from the disease, and can develop both latent and active tuberculosis despite prior vaccination with BCG.

1.2 The history of tuberculosis

The origin of the genus *Mycobacterium* has been hypothesised to be more than 150 million years old (4), but the modern strains of *M. tuberculosis* seem to have arisen from a common ancestor around 20,000-25,000 years ago (5). These strains seem to have their origin in East Africa, and migrating humans from this area appear to have taken tuberculosis with them across to all parts of the globe. The disease probably didn't reach epic proportions until humans started settling down in communities at permanent sites around 8000 BC, and investigations have revealed evidence of human tuberculosis in skeletons from one of the first villages with evidence of agriculture and domestication (6). Written accounts from India of the disease date back 3000 years, and it was recognised as a cause of death primarily in young adults in classical Greece (7).

In Europe, the tuberculosis epidemic is likely to have started at the beginning of the seventeenth century. A combination of high population density and poor sanitary conditions in the largest cities provided the necessary condition for the spread of this pathogen, and it was considered inevitable that people that became sick with

tuberculosis would eventually die from the disease. Although tuberculosis is likely to have pre-existed in all continents, a disease of epidemic proportions seems to have spread from Europe to America, Asia and Africa by exploration and colonisation (8). At this time there was little knowledge about the disease. Although physicians described changes and tubercular structures in the lungs and in other organs, many believed the disease to be hereditary rather than infectious. In the nineteenth century, pathological studies of tuberculosis victims lay grounds for a better understanding of the disease. René Théophile Hyacinthe Laennec (1781-1826) described most of the physical signs of pulmonary tuberculosis, and invented terms to describe his findings that are still in use today (9). The infectious nature of the disease was proven by the French military surgeon Jean-Antoine Villemin (1827-1892) in 1865, when he inoculated liquid taken from a tuberculous cavity into a rabbit, and described extensive tuberculosis in the animal when it was sacrificed 3 months later (10).

It is, however, the work of Robert Koch (1843-1910), which he presented to the Berlin Physiological Society in 1882, that represents the biggest shift in the modern understanding of the etiological agent of tuberculosis (11). Koch developed methods for staining the bacteria, and was able to identify bacilli in tissues, both inside and outside host cells. Furthermore, he developed a special growth medium and was able to isolate the bacteria from animal and patient samples and grow them in his laboratory. He then infected a large number of guinea pigs and rabbits with the *in vitro*-cultivated bacteria, and established that these cultures were the source of tuberculosis. This stepwise method of showing that the bacteria isolated from diseased tissue is the actual cause of the disease, rather than an opportunistic organism accompanying an unrelated disease, was originally proposed by Friedrich Gustav Jakob Henle (1809-1885) in 1840, but was refined by his student Robert Koch and is now referred to as the Koch-Henle postulates (12). Koch also created a substance derived from his *in vitro* cultures he called tuberculin, and he hoped to use this to treat tuberculosis patients. Although it was quickly discredited as a treatment, the reaction it induced in people and animals with prior contact with tuberculosis,

revealed itself useful for diagnostic purposes (13). A paediatrician in Vienna called Clemens Freiherr von Pirquet (1874-1929) heard about Koch's tuberculin in 1907, and used a vaccination lancet to introduce the substance subcutaneously, studied the subsequent swelling, and defined a cut-off for what he considered to be a positive response. He observed that some children had a positive reaction despite showing no clinical signs of tuberculosis, and coined the term latent tuberculosis to describe the phenomenon (14). In Norway, Johan Kloster (1901-1977) later modified this test by adding a drop of 1% adrenalin to make the reaction more prominent (15). An alternative method was introduced by Charles Mantoux (1877-1947) in 1908, who used a needle to inject tuberculin intracutaneously. In the 1930s, Florence Seibert (1897-1991) standardised production and isolation of proteins used for the test, and called it purified protein derivative (PPD). The suspension he created is very similar to the formulation that is in use today.

During the time this pioneering work was ongoing, mortality rates caused by tuberculosis in the western world was gradually decreasing (16). Although the reason for this is not entirely clear, hypotheses put forward are improved social and living conditions, better nutrition, and the natural selection of a population more resistant to developing disease (17). The treatment regimens of the early days were to rest and eat well. Sanatoriums offered this, and they also served the purpose of isolating infected individuals and preventing them from spreading the bacteria to their families and other close contacts. Studies conducted at the time formally proved the benefit of this form of treatment; the cure rate was up to 20% higher for patients admitted to sanatoriums than for patients treated at home (18). That a simple treatment based on rest and a healthy and nutritious diet increased the chances of a positive outcome, underlines the fine balance in the tug of war between the bacilli and the human host during tuberculosis disease. A more drastic treatment introduced at the time was pulmonary collapse, primarily for the closure of cavities (10). This treatment was also recorded to have some degree of success in converting sputum positive patients to sputum negative.

The colleagues Albert Calmette (1863-1933) and Camille Guérin (1872-1961) set out to develop a vaccine against tuberculosis in 1908, and their chosen method was to attenuate the causative agent of bovine tuberculosis, *M. bovis*, by subculturing this pathogen in a medium consisting of bile, glycerine and potato (19). After 11 years and 230 passages, they were confident that they had an attenuated organism that failed to give progressive tuberculosis when injected into guinea pigs, rabbits, cattle and horses. The first test on humans in 1921 was a success, and after 3 years the Pasteur institute in Lille began a mass production of the vaccine. By 1928, 114,000 infants had been vaccinated without any serious complications recorded. Calmette and Guérin observed a fall in tuberculosis amongst the individuals that had been vaccinated with BCG, and within years the use of this vaccine spread to countries outside France. However, it was not until the World War II, which resulted in a large epidemic of tuberculosis, that BCG became administered on a massive scale worldwide.

The history of tuberculosis in man changed dramatically with the arrival of chemotherapies. In 1944 streptomycin was discovered, and a few months later it had been used to successfully treat a young woman with tuberculosis (10). Although the initial treatments were successful, it took between 2 and 3 months before improvements in clinical health and sputum bacteriology was observed. This prolonged use of a single antibiotic inevitably led to the development of antibiotic resistance. In the following years, isoniazid (1951) and rifamycins (1964) also became available, and it was the combined therapy using three antibiotics, all with a different mechanism of action, that eventually became the recommended therapeutic strategy. It reduced the course of treatment and largely overcame the problem with the development of drug-resistant bacteria.

The BCG vaccine, diagnostic tools, and treatments described above are still in use today, and together with a much improved socioeconomic situation, they have contributed to almost complete eradication of tuberculosis from the majority of the populations of the Western world. However, in the developing world, where the

disease is truly endemic, it is necessary to radically improve the way we fight tuberculosis. In recent years, this absolute requirement has been recognised, and interest in research in the field has been generated alongside improved funding. An initiative lead by WHO, the Stop tuberculosis Partnership (http://www.stoptb.org/), has outlined a plan to halve tuberculosis prevalence and mortality by 2015, and to eliminate the disease as a public health problem by 2050. Because of such initiatives, the incidences of tuberculosis have slowly but gradually been falling. However, the development of resistant strains and the increase in mortality caused by co-infection with HIV, have highlighted the need for additional funding to refine and develop new tools to continue the fight against tuberculosis (20).

2. The causative agent of tuberculosis

2.1 Mycobacterial taxonomy

Mycobacteria was first classified in 1896 when Lehmann and Neumann proposed the genus for the causative agents of tuberculosis and leprosy on the basis of their morphology and acid-alcohol fastness (21). It was placed in *Mycobacteriaceae* family (*Mycobacterium* is the only genus in this family), *Actinomycetales* order, and *Actinomycetes* class. Today, there are three main criteria for being included in the genus *Mycobacterium*: 1) acid-alcohol fastness (a feature that is exploited in acid-fast staining methods for diagnosis), 2) the presence of mycolic acids, and 3) a G+C content of the DNA of 61 to 71 mol% (22). The genus *Mycobacterium* is divided into two divisions: rapidly growing and slowly growing. This categorisation was initially based on their properties during *in vivo* culturing, but years later, 16S rRNA sequencing proved this division had a foundation in phylogenetic segregation (23). The slowly growing strains are generally associated with disease in human and animals, while rapidly growing strains are regarded as non-pathogenic. However, there are important exceptions to this correlation: for an immunocompromised individual almost all members of *Mycobacterium* can be pathogenic. To date, there are 120 recognised and proposed species that meet the standards for inclusion in the genus, and the vast majority is considered non-pathogenic.

Members of the *Mycobacterium tuberculosis* complex are the causative agents of tuberculosis, and form a tight cluster in taxonomical studies: *Mycobacterium tuberculosis*, *Mycobacterium bovis* (including the BCG vaccine strains, that was attenuated from a clinical isolate of *M. bovis* called “lait Nocard”), *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium caprae*, *Mycobacterium canetti* and *Mycobacterium pinnipedii*, which are all pathogenic for human and/or animals. The properties described in the next chapter are specific to the members of this *Mycobacterium tuberculosis* complex, primarily *Mycobacterium tuberculosis*.

2.2 Mycobacterial characteristics

2.2.1 Historic observations

In 1880, after Robert Koch accepted the position as director of the bacteriological laboratory at the Imperial Health Office in Berlin, he immediately started to develop the tools required for bacteriological work, including staining methods, solid culture media, incubation boxes, experimental animal studies, and microscopy and microphotography (12). These methods allowed him to both cultivate the agent of tuberculosis, observe the bacterium inside and outside cells in animal specimens, and to document his findings. For *M. tuberculosis*, what he described was a slightly bent, rod-shaped cell, approximately 2-4 μm long and 0.2-0.5 μm wide. These bacteria are slow-growing, with a replication time of around 21-23 hours in human macrophages. Most mycobacterial species can grow on simple substrates, and optimal growth temperature can vary greatly. The exceptions are *M. leprae* and the closely related *Mycobacterium lepromatosis*, which causes a special form of diffuse lepromatous leprosy. *M. leprae* has till this day not been successfully cultivated in culture medium, and is therefore grown in armadillos when cultivated for scientific purposes. Mycobacteria are aerobic, and considered to be non-motile and non-sporulating. They are described as acid-fast, a feature that stems from its unique cell envelope. These bacilli have for a long time erroneously been thought to lack a true outer membrane, and they have therefore traditionally been considered Gram-positive, even if they do not retain the crystal violet stain well like other Gram-positive bacteria.

2.2.2 The unique cell envelope

The complex wax-rich cell envelope, which consists of a cytoplasmic membrane and a cell wall, is one of the distinguishing features of this organism. It makes up a very rigid structure, and function as a permeability barrier that plays a crucial role in survival under tough conditions and gives the bacteria intrinsic drug resistance (24). Mycolic acids account for 30% to 40% of the envelope. These are long fatty acids that are linked to peptidoglycan by an arabinogalactan polymer, which is a

polysaccharide composed of arabinose and galactose subunits. This acid-arabinogalactan-peptidoglycan polymer is arranged to form a hydrophobic layer together with other lipids (25). The outer membrane of mycobacteria has been the subject of some controversy, where some have been sceptical to the existence of a 'true' membrane and have instead argued that the structure was similar to that of Gram-positive bacteria. However, recent cryo-electron tomography and electron microscopy has revealed a bilayer structure of a true outer membrane. It is approximately 8 nm thick and morphologically symmetrical, which suggests that free lipids with heterogeneous head groups are distributed over both leaflets in the outer membrane (26,27).

Components of the cell envelope play an important role in mycobacterial virulence ranging from granuloma formation to inhibiting killing mechanisms of infected macrophages. A more detailed description will be given in chapter 5, which deals with the interaction between the pathogen and its host. The cell envelope differs between mycobacterial species, and it gives some of these species properties that are not found in others. An example of this is the ability to aggregate into structures known as cords, which has been linked to the lipid composition of the *M. tuberculosis* cell wall (28-32). The cord-formation has been linked to virulence, and underlines importance of the mycobacterial cell envelope for bacterial perseverance and virulence in the host.

2.2.3 The genome sequence

The complete genome of the laboratory reference strain *M. tuberculosis* H37Rv was published in 1998 (33), and since then, genome sequences from several other *M. tuberculosis* strains and species of mycobacteria have been published (34-39).

The genome of *M. tuberculosis* consists of approximately 4.5 million base pairs, making up a predicted 3995 genes (40). The G+C content is higher than most known bacterial species and averages at 65.6%. The areas of the genome that contain the two novel PE- and PPE gene families were found to have an even higher G+C ratio, and

an amino acid composition that differs from other mycobacterial proteins. Despite making up to 10% of the coding potential of the genome, the function of these genes is still unknown, although a role in pathogenesis and antigenic variation has been suggested (41). The basis for the lipid rich cell envelope can be traced back to the genome, where every known lipid and polyketide biosynthetic system has been identified, including enzymes that are typically found in mammals and plants. In fact, more potential lipid biosynthetic activities were identified than there are known metabolites, which suggests the existence of novel lipid and polyketide species that are yet to be found. Many genes seems to encode enzymes that are involved in fatty acid degradation, which could be linked to the degradation of lipids that are present in host membranes, and thus contribute to the energy metabolism of this intracellular pathogen (33).

The genome of *M. tuberculosis* has three types of repetitive sequences: duplicated genes and gene families, insertion sequence (IS) elements, and dispersed non-coding sequences. Gene duplication is a common feature of many organisms, but in *M. tuberculosis* the duplication of certain genes has lead to the presence of copies that are identical in sequence. Typically, extensive sequence divergence is more common after duplication events. The PE and PPE gene families are the dominant examples of duplication events producing near identical genes. There are 56 copies of IS elements that belong to 9 different families of genes, and they often appear in clusters. These elements are thought to be the targets of transposons, since insertions into these sequences will not disrupt essential genes and alter the function of the organism (42). However, with the exception of IS6110, most elements found in *M. tuberculosis* H37Rv appear to be stable and maintain a conserved location in the genome. Seven copies of repetitive sequences exist that are collectively known as the 13E12-family. They contain an open reading frame, that in some cases appear to require frame shift to produce a protein. In addition, there exists smaller repetitive sequences called mycobacterial interspersed repetitive unit (MIRU), variable number tandem repeats

(VNTR), and exact tandem repeats. Many MIRUs are situated in between genes in operons, and therefore have the potential to encode peptides (43).

2.2.4 From genome to proteome

The output of genome-wide sequencing is a raw sequence of nucleic acids, and from this sequence the researchers try to accurately predict, or annotate, every gene this genome might contain. The value of a genome sequence, and the biological information it provides, is therefore only as good as its annotation. They will normally start by mapping the genome for known genes, repetitive elements, duplications, and so forth to get an overview over landmark features. To identify the genes, it is necessary to identify long open reading frames that starts with a start codon and terminate with a stop codon. The stop codon is unambiguous, but the alternative start codons, ATG, TTG, and GTG, also codes for the amino acids methionine, leucine, and valine, respectively. These might therefore appear at any place within the final protein, in which case a different start codon further upstream will be the actual start of the protein. Finding the true starting point is therefore essential for a correct gene annotation. Furthermore, open reading frames exist on both strands of the genome, and in some cases genes overlap on the opposite strand. Gene annotation is therefore a complex task, and luckily several software algorithms exist that can aid the prediction by detecting features based on motifs or other properties of the DNA. Alongside being analysed by prediction algorithms, the genome is compared to gene sequences from other species that already have been characterised. This comparison is particularly valuable for elucidating the function of each of the predicted proteins. The result is a division of proteins into the following 3 broad categories: 1) proteins with great similarities to known proteins and that can be attributed precise functions, 2) proteins with similarities to known proteins, and 3) proteins that do not resemble any known proteins, often referred to as hypothetical proteins. The hardest task is to annotate genes that belong to the latter category. However, mistakes can be made even if a sequence shows great similarity to a previously annotated gene. For example, a pseudogene has lost its protein-coding

ability and has no function in the bacilli, yet it is highly similar to the original functional gene from which it is derived. When the genome of *M. tuberculosis* was first sequenced in 1998, around 40% of the genes were attributed precise functions, 44% was found to have great similarity to known genes, while 16% did not resemble any known protein (44).

The annotated genome can further be subjected to *in silico* analyses. Software algorithms have been developed that use sequence information from proteins that have already been characterised, and which have been found to contain a specific property. The algorithms are "trained" on sequence information from several proteins that share the same feature, and they can subsequently predict the probability of this trait being present in new and less well-characterised proteins. Good examples of such prediction algorithms are computer programs used for identifying N-terminal signal peptides. These amino acid sequences are responsible for transporting proteins to the membrane, where they are processed by the general secretory system, and released on the exterior of the cell as the signal peptide is cleaved off (this is described in more detail in chapter 3.2.1). The algorithm can therefore both disclose the final destination of a protein, and reveal the mature protein sequence after removal of the signal sequence. The best of these algorithms can accurately identify signal sequences in many different eukaryotic and prokaryotic species (45), including *M. tuberculosis* (46). However, since such computational methods are developed from benchmark datasets consisting of pre-existing experimental data, there is no guarantee that the prediction is correct. Only when experimental research verify such predictions can we be certain the organism actually expresses these proteins.

The study of proteins, often referred to as proteomics, predates genomic analyses, but traditionally the methods used were laborious and generated a relatively low-output relative to the effort. However, several important mycobacterial proteins were identified and described many years before the gene sequence was made available, making it possible to isolate and characterise several important antigens and to identify features that distinguish pathogenic from non-pathogenic mycobacterial

strains. These days the new and refined proteomic tools are used to both verify and correct gene annotations that were published following the sequencing of the *M. tuberculosis* genome.

As mentioned above, genome annotation relies heavily on computational methods, which in turn are based on the fairly limited information gained from prior experimental research. Sequence comparisons are often made to unrelated organisms, and mistakes are commonly found in the available gene and protein databases. The vulnerability of such methods are exemplified by the sequencing and annotation of the genome of the lab strain *M. tuberculosis* H37Rv. The work was carried out in parallel by two separate institutions, the Pasteur Institute and the J. Craig Venter Institute, and the number of genes annotated by the two groups turned out to differ by 12% (35,44). After the *M. tuberculosis*-genome sequence was made available, several proteomic studies sought to either verify, or correct, the annotations and predictions, and to identify proteins expressed from genes that has not been annotated at all (47-55). These studies were performed using mass spectrometry based methods that are both sensitive and allows large scale screenings (56). In 2008, de Souza and co-workers performed a proteomic characterisation of *M. tuberculosis* H37Rv, and were able to conclude that the annotation from the Pasteur Institute was the most accurate annotation of the two (52).

This is not the only achievement of modern proteomics with regard to mycobacterial species. Mycobacterial proteins located within or attached to the membrane, or secreted into the environment, have important roles in pathogen-host interactions. They are therefore particularly interesting for the development of new vaccines, drugs, and diagnostic tools. Proteomic analysis of membrane fractions have extensively documented its protein content (53,57,58), and a thorough screening of culture filtrates have characterised a large amount of the proteins that are thought to be secreted (51,52,59). Other types of analyses have revealed several examples of post-translational modifications of mycobacterial proteins. These types of findings are important to better understand how the bacteria can regulate key pathways and

respond to signals from its environment. Prisic and co-workers recently identified 301 proteins that were found to be phosphorylated under one or more external conditions such as nitric oxide stress, hypoxia, and in response to variable carbon sources (60). Phosphorylation and dephosphorylation activate or deactivate many proteins, and are important steps in many signal transduction pathways. Another type of modification, ubiquitination, is used to target proteins to proteasomal degradation. In 2008, the ubiquitin-like protein Rv2111c was described in *M. tuberculosis*, and this was the first time an ubiquitin-like system was described for a bacterial species (61). Subsequently, proteomic approaches have identified a total of 48 sites in 41 proteins as targets for this type of modification (62). Lipoproteins exported by the general secretory pathway become acetylated at the N-terminal cysteine, and several are also O-glycosylated close to the N-terminal end. Both modifications have been suggested to protect these lipoproteins from proteolytic cleavage before they reach their final destination. In *M. tuberculosis*, such modifications have been found for various lipoproteins including MPT83 (63) and SodC (64). The non-lipoprotein MPT32 (Apa) (65) is known to be glycosylated, and is currently the only mycobacterial non-lipoprotein where this type of modification has been described (66). There is evidence to suggest that post-translational modifications directly affect how the immune system recognises mycobacterial antigens. The heparin-binding hemaglutinin is exposed on the surface of the bacilli, and has been found to be methylated on several lysine residues (67). These methyl lysines are now believed to be part of the epitopes recognised by T-cells, and may be important for generating a specific immune response (68).

Another relatively new field within proteomics are quantitative studies that allow the description of the relative abundance of identified proteins. So far these methods have confirmed that many of the already well-established immunogens of mycobacteria are indeed highly expressed. The hope and promise of quantitative proteomics is to identify proteins with significant expression, but which so far have largely been overlooked. These proteins can be evaluated as candidates for vaccines, target

antigens in diagnostic tests, and drug targets. Furthermore, they may be involved in important biological activities of the bacteria, and are therefore interesting in their own right. A study that identified several proteins with relatively high expression was recently performed for *M. leprae* (69).

3. Protein expression and localisation

3.1 Transcription and translation

Mycobacteria, like all prokaryotes, do not have a separate compartment, a nucleus, where DNA is located. Both transcription of DNA and translation into proteins therefore takes place in the bacterial cytoplasm. Since the bacterial genome is relatively streamlined, with no exons or introns that needs to be edited out before the mRNA can encode a functional protein, translation of mRNA can start before transcription has been completed. Transcription is carried out by a four-subunit RNA polymerase, and a transcription factor (in prokaryote often referred to as sigma factor) that enables this complex to recognise the promoter region that consist of a TATAAT and TTGACA sequence that are 10 bp and 35 bp upstream the start codon, respectively. Transcription factors bind directly to the promoter region of the genes they regulate, and function to either promote or block the binding of RNA polymerase. Their role is therefore to increase or decrease gene transcription. The transcription factor can be aided by other cofactors, but these do not have the capacity to bind DNA directly. When the polymerase binds to the promoter region, it starts transcribing the gene, and it generates an mRNA sequence that will be translated into a protein. An operon consists of several genes located close together, which are transcribed from the same promoter region, and thus controlled by the same transcription factors. Each gene in this region can have its own start- and stop-codons, and a separate mRNA string for each gene is made. Sometimes the mRNA encodes more than one protein, in which case it is called polycistronic (as opposed to monocistronic) and an intercistronic region separates each gene on the single mRNA string. The chromosome can be transcribed on both strands of DNA, both 5’ to 3’ and 3’ to 5’, and genes can therefore overlap by utilising the same genome sequence albeit in opposite directions. Genes can also overlap on the same strand, in which case there is an offset in the reading frame by 1 or 2 codons.

Since expression of proteins can be controlled at the level of transcription, the activities of transcription factors are important for adapting to changes in the environment. The genome of *M. tuberculosis* have been found to contain a large number of putative transcription factors, including 12 sigma factors (33), and furthermore, microarray data has revealed shifts in transcription profiles when the bacteria encounter the hostile environment of the macrophage (70). This underlines the important roles of transcription factors and gene regulation for bacterial adaptation, survival and pathogenicity.

The polypeptide chain that emerges from the ribosome during translation consists of amino acids that differ in the chemical nature of their side chains. These side chains can react with various molecules already present in the cytoplasm, which can prevent formation of a stable and functional mature structure (71). To prevent this, polypeptides therefore often start folding before the protein has been completely translated (72). In addition, stabilising chaperons, like heat shock proteins, associate with the newly synthesised polypeptide during translation and aid the correct folding of the protein. Under normal conditions, heat shock proteins constitutes around 5% of somatic proteins, but under stressful conditions these proteins can represent up to 15% of the prokaryotic cellular protein mass. Since the stress experienced by intracellular pathogens inside host cells cause an up-regulated production of heat shock proteins, they typically become important immunogens inducing strong humoral and cellular immune responses (73).

Once synthesised, the protein can either remain intracellular or it can be transported to the membrane into which it can be inserted, or exported through and eventually released on the exterior of the cell. The latter two groups of proteins, membrane- and secreted proteins, are particularly important for host-pathogen interactions, and are frequently recognised by the host immune system. These proteins require a system for localising to sites where they will be further processed. As it turns out, many of these proteins are recognised and processed by the same system despite their

divergent destinies. It is the intrinsic properties of the individual protein that determine if it will be retained within the membrane or released on the outside.

3.2 Protein secretion mechanisms

Secretion mechanisms in *M. tuberculosis* consist of highly conserved systems, but more specialised systems also exist. Protein transport through the SecYEG-integral membrane complex, which involves the activity of Signal peptidase (SPase) I, is the core system in all domains of life. It is highly conserved and therefore called the general secretory pathway (74). The transport of lipoproteins through the twin-arginine translocation (TAT) machinery seems to be conserved across prokaryotes (75). In addition, mycobacteria have a secretion system that is more specialised and unique to a limited number of prokaryotes. The ESX-1 secretion system seems to be functionally equivalent to the Type I-V secretion systems of some pathogenic Gram-negative bacteria, which serve to transport proteins into the host cell and interfere with the host response to infection.

3.2.1 The general secretory pathway and signal peptides

The general secretory pathway secretes proteins that are “labelled” with an N-terminal amino acids sequence, called the signal peptide. These signal peptides ensure the correct transport and post-processing of the protein. The N-terminal sequence interacts with a transport system that transfers the protein to a secretion system located on the cytoplasmic side of the cell envelope. At this site, the protein adapts to a structural confirmation that exposes a specific region of the signal peptide to an enzyme that cleaves the signal peptide from the protein. After the cleavage event, the protein becomes “mature”, and it can be released from the cell or contained within the membrane fraction. Typically, proteins that contain one or more transmembrane regions are retained in the membrane and perform their functions as part of the membrane structure, be it on the surface, on the cytoplasmic side, or within the membrane layers.

The protein complex involved in translocating proteins, the translocon, consists of 3 proteins, SecY, SecE, and SecG (SecYEG) that forms a channel across the cytoplasmic membrane. Peripherally attached on the cytoplasmic side is the ATPase motor, SecA, which drives the translocation in an ATP-dependent manner. In addition, a protein complex called SecDF, and the protein YidC, associates transiently with the translocon, but their role in the translocation process in not clear. Soon after transcription the pre-protein, which still have an N-terminal signal sequence, is transported to the translocon. It first interacts with SecA that binds both to the signal peptide and the mature protein. Not all proteins require a signal peptide for this interaction, and appear to associate just as well when the signal peptide is impaired (76). The protein domain downstream of the signal peptide therefore seems to be the primary interaction partner with SecA.

Some proteins require the aid of molecules to keep them in an unfolded non-aggregative state compatible with the translocation system. Signal recognition chaperons immediately bind to the peptide emerging from the ribosomal tunnel, and delivers the protein to the translocon via a membrane bound protein. Other proteins do not require the assistance of chaperons; they remain unfolded and can associate with the translocon independently. It is not understood why some proteins require assistance while others do not. For example, the chaperon SecB found in proteobacteria cannot distinguish between secreted and non-secreted proteins by its binding specificity. Furthermore, a study utilising an *Escherichia coli secB* null mutant, have shown that a protein normally associated with SecB, remained unfolded and was secreted, albeit with a significantly reduced export efficacy (77). These chaperons might therefore have additional roles in protein processing and secretion. Mycobacteria lack *secB*, but are likely to have other cytosolic chaperons to escort proteins to SecA.

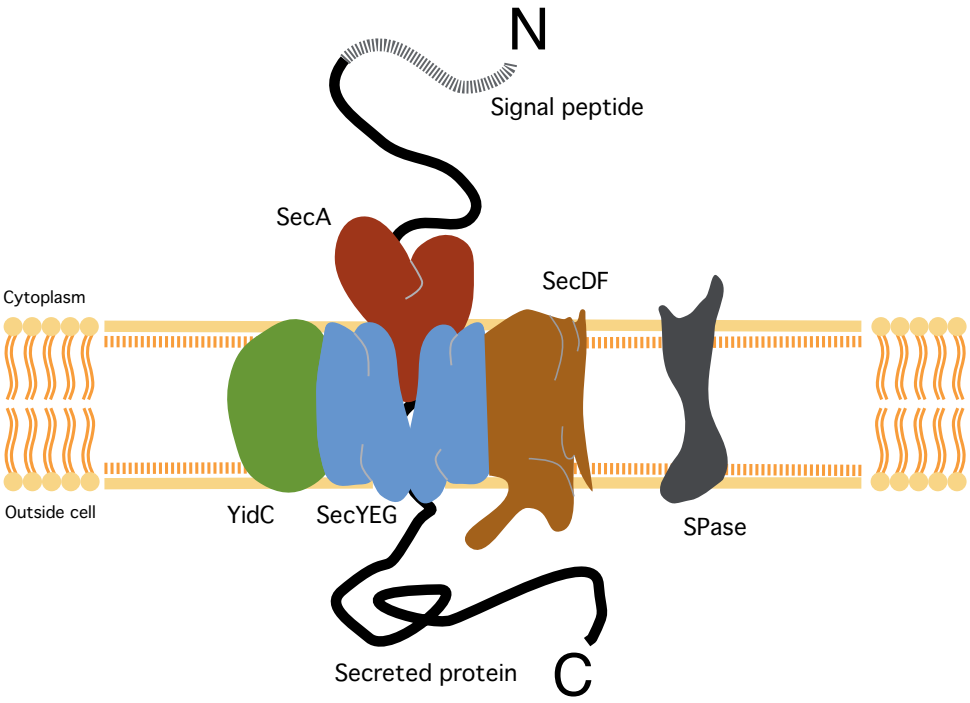

Figure 2. The SEC translocase. See text for details.

SecA binds the pre-protein in a clamp-like manner, inserts itself into the SecYEG translocon, and the pre-protein subsequently moves through the clamp to enter the translocon (78). ATP drives the translocation; it is required for the insertion of SecA into the translocon, and the subsequent hydrolysation of ATP causes the dissociation of SecA from the pre-protein and a weakening of the binding between SecA-SecYEG. Sometimes this causes SecA to dissociate completely. However, SecA will then once more partially bind to the pre-protein and, upon binding to ATP, cause further translocation of the protein (79). This cycle of stepwise translocation continues until the process is completed, after which the enzyme SPase cleaves off the signal peptide.

As noted above, the signal peptide might not be required for the localisation to, and interaction with, the translocase system. It does, however, appear to have an essential role as activator of the translocase. Although the signal peptide of the protein PhoA is

not required for association with SecA, Gouridis and colleagues (2009) showed that the signal peptide was essential for protein translocation (76). By comparing the translocation events of a wild-type protein to the same protein devoid of its signal peptide, they showed that this sequence initiated the translocation process by lowering the activation energy state of the translocase. It was further responsible for the insertion of the N-terminal segment of the protein into the translocase, and for a continued association with the translocon throughout the secretion process.

Signal peptides have common features that are similar in prokaryotic and eukaryotic cells. Although they vary in length, they typically have a three-domain structure where each domain serves a particular purpose. The amino-terminal region, 1-5 residues long, is positively charged, primarily due to the presence of lysine and/or arginine. Site-specific mutagenesis in the signal peptide of maltose-binding protein of *E. coli* has shown that a negatively charged N-terminal leads to a more slowly transport of the protein, but the transport is not inhibited per se (80). Other examples have shown that exchanging positive residues with neutral residues can completely abrogate its ability to be transported (81). The effect of amino acid-substitutions presumably depends on the amino acid composition of the rest of the signal peptide, which may have overlapping functions. The hydrophobic region that extends from the N-terminal to the cleavage site appears to be necessary for translocating the protein across the membrane. Enhanced export efficacy can be achieved by making the region more hydrophobic or by extending it, while introducing charged residues, or shortening the region, can negatively affect protein export (82). Extending this region can also compensate for the absence of positively charged amino acids in the N-terminal, and it has been suggested that both these regions can be involved in interaction with SecA (83).

The cleavage site itself seems to adhere quite strictly to amino acid specificity in the -3 and -1 position upstream relative to the cleavage site. An alanine in the -1 position

Figure 3. Illustration of a signal peptide of the mycobacterial protein MYCP3 (in light green) and the cleavage site (marked by red arrowheads).

appear to be particularly conserved, and only substitutions with Glycine or Serine appear to be acceptable (84). Alanine is also predominant in the -3 position, but here Serine, Glycine, Valine, Threonine, Leucine, and Isoleucine also seem to be permitted. All these amino acids have small neutral side chains, which seems to be an important feature for allowing the cleavage site to dock into the catalytic site of the SPase. A study revealed that extending the cleavage region with Glutamine up to 9 amino acids was tolerated for processing of a signal peptide in *E. coli* (85). Certain deviations from a preferred signal peptide structure therefore seem to be acceptable, but this is likely dependent on structural properties of the rest of the peptide (86). Nevertheless, the AXA-motif is found in the majority of experimentally verified signal peptide cleavage sites, suggesting a preference for this sequence for most of the proteins that are secreted by the general secretory pathway.

3.2.2 The twin-arginine transporter (Tat) pathway

The Tat secretion system allows translocation of folded proteins across the plasma membrane. These proteins carry a signal sequence with a structure similar to the sequence used by the general secretory pathway, but it also includes a double arginine motif followed by two uncharged residues near the N-terminus (87). In *M. tuberculosis*, the *tat* genes appear to be essential for growth in culture (88), and substrates of the Tat-pathway have also been implicated in interaction with the host and bacterial virulence (88,89).

3.2.3 The ESX-1 secretion system

In mycobacteria, a unique secretion system, termed the ESX-1 secretion system, has been described. Early secretory antigenic target 6 kDa (ESAT-6) and Culture filtrate

protein, 10 kDa (CFP-10) are the two prototypic proteins secreted through this system. These proteins are immunodominant antigens of *M. tuberculosis*, and are important for its virulence (90). They are found in the region of difference 1 (RD1), the loss of which represented an important attenuation event when the BCG vaccine was developed from a pathogenic *M. bovis* strain. It has been shown that several genes in the RD1 locus are required for ESX-1 secretion (91). However, the gene locus Rv3616c-Rv3614c, which lies outside the RD1 region, is also involved. Rv3616c and Rv3615c are secreted in an ESX-1 dependent manner, and Rv3614c is required for secretion of ESAT-6 and CFP-10. Other proteins involved are ATPases that provides the energy for translocation, a protease with extracytoplasmic activity, and transmembrane proteins whose functions are still not known (91). ESAT-6 and CFP-10 form a dimer and are interdependent on each other for stability within the mycobacterial cytoplasm. This dimer is escorted to the membrane and delivered in an ATPase dependent manner to Rv3870 and Rv3877 that together make up the pore spanning the cytosolic membrane (92,93). Interestingly, it is the C-terminal of CFP-10 that carries the signal sequence; this 15 amino acid-long peptide is unstructured and function as a handle for the protein Rv3871, which target the complex for secretion (93).

3.3 Membrane protein integration

Membrane proteins are attached to, or associated with, the membrane of the cell. They can be permanently bound to the membrane or temporarily associated with the lipid bilayer or with integral membrane proteins. Some proteins are permanently associated with the membrane because of their hydrophobic nature, while other proteins can exist in more than one state, both water soluble and lipid bilayer-bound. The membrane proteins have various functions, including mediating selective transport, energy conversion, cell division, extracellular signal sensing, and membrane and cell wall biogenesis (reviewed in (94)).

Many proteins rely on the protein secretion systems for localisation and insertion into the membrane, and have intrinsic properties that cause them to be retained in the membrane rather than being secreted into the exterior of the cell. This is usually due to one or more transmembrane stretches of approximately 20 hydrophobic amino acid residues that form an α-helix and are long enough to span the hydrophobic core of the lipid bilayer. These transmembrane domains are also passed through SecY during translocation, but leave SecYEG laterally into the lipid bilayer. Proteins that are inserted into the outer membrane bypass the lateral integration into the plasma membrane, and most of them possess an all-β-barrel that crosses the outer membrane structure. These proteins require a machinery consisting of the Omp85 outer membrane protein and several chaperones for inserting the barrel into the outer membrane (94).

4. Immunology of tuberculosis

This chapter will first outline how the infection is typically established and how it develops. It then introduces the components of the immune system, and describe their role in combatting the invading tuberculosis pathogen. It will largely be presented as the “ideal situation”, where the immune system function adequately to contain the infection, and it will not address how the bacterium resists and modulates the immune response to its own favour. This will instead be covered in chapter 5.

4.1 The course of infection

Tuberculosis is primarily a disease of the lungs. The infection starts when droplet nuclei carrying the bacilli are exhaled by a patient with active disease, and subsequently inhaled by another individual, which then becomes infected. The droplet is so small that it can remain airborne for several hours. Key factors that determine the likelihood of transmission include the number of bacilli expelled into the air, the total concentration of organisms in the air in a defined room or area, and the duration of exposure to the contaminated air (3). The bacteria reach the bronchi, and are phagocytosed by alveolar macrophages, which subsequently invade the epithelial layer. The signals secreted by the infected cell attract neutrophils, NK-cells, CD4+ T-cells, CD8+ T-cells, which in turn adds to the cytokine- and chemokine milieu, recruiting even more cells and model a stable cellular structure at the infection site (95). A functional microvascular network develops within this granuloma, and an extensive fibrotic capsule forms that eventually separates macrophages, granulocytes, foamy macrophages and giant cells, from the lymphocytic infiltrate. Eventually the centre becomes necrotic, which in turn leads to formation of caseous material as the cells in the granuloma start to die (96). The solid caseous material is depleted in oxygen, the pH is low, and it contains enzymes released from dead cells. All these factors are hostile to the bacterium, and caseating necrosis is linked to a drastic reduction in the number of viable bacilli. In rare

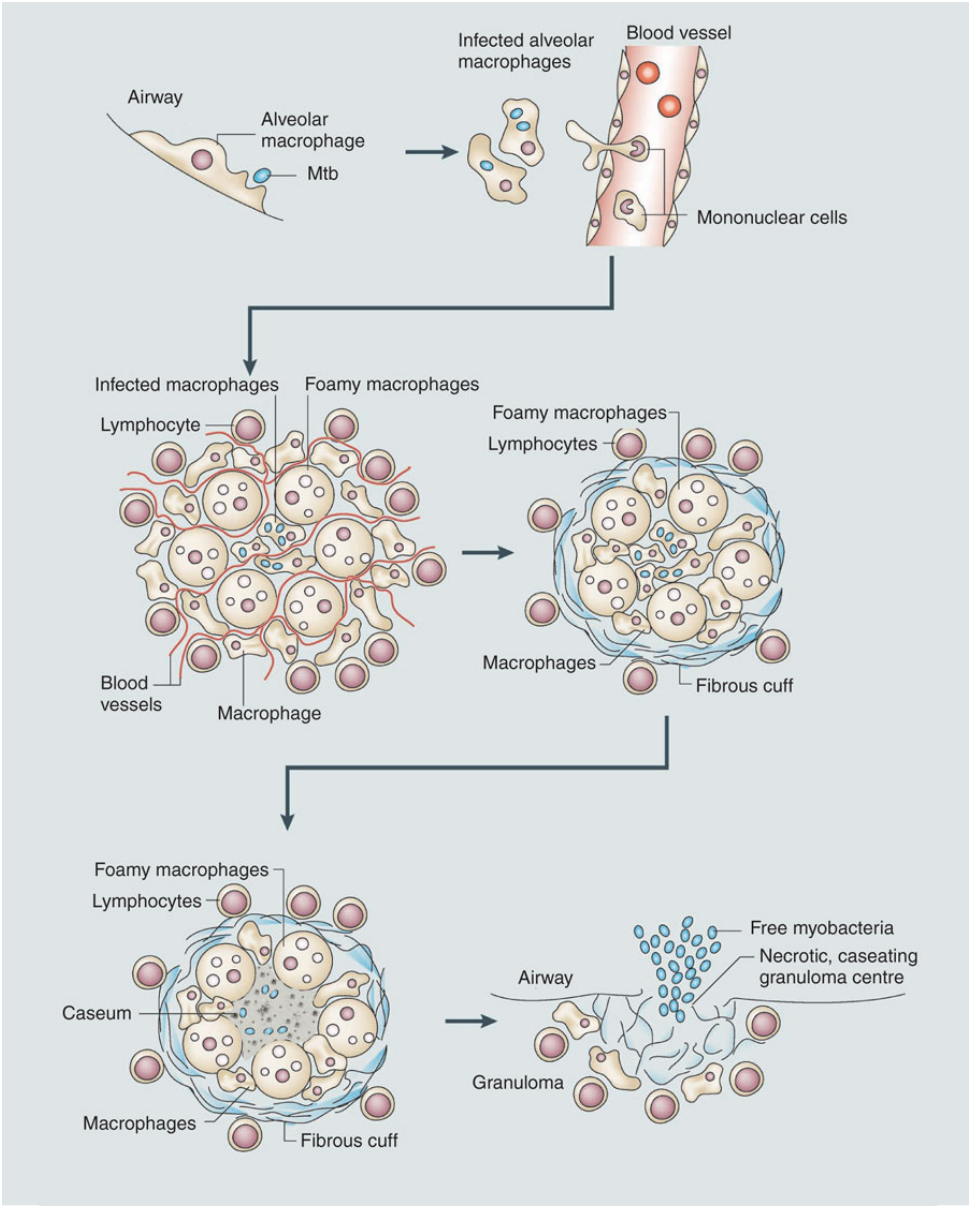

Figure 4. Stages of pathology during tuberculosis infection. See text for details (Russell, 2009)

instances events occur that lead to softening, or liquefaction, of the caseum, and a subsequent destruction of tissue provides direct contact with the bronchi. The resulting oxygen rich cavity significantly improves the living conditions for the bacilli, and promotes extensive extracellular growth. The establishment of an opening

to the bronchial tree also permits the release of bacteria into the airways (96). The ability to contain these organisms is therefore dependent upon a properly structured framework within the barriers of the granuloma, and is presumably a prerequisite for controlling the disease (97).

M. tuberculosis is not necessarily confined to the centre of the pulmonary granulomas, and can sometimes be found at sites recognised as primary areas for lymphocyte proliferation (98). Furthermore, mycobacterial DNA have been detected in lung tissue without histological evidence of tuberculous lesions (99), which suggests the bacteria can persist outside the cellular infiltrates. Lastly, *M. tuberculosis* can also establish infection in sites outside the lung. This is regarded as a failure by the host to retain the bacteria at the primary site of infection. Lymph nodes are the most common extra-pulmonary site of infection, but *M. tuberculosis* can also establish infection in the pleural space, genitourinary system, bones and joints, central nervous system, abdomen, and the pericardium (100).

4.2 Innate immunity

The innate immunity allows the host to combat pathogens before adaptive immunity is activated. The system is often referred to as a non-specific immune system, since it recognises conserved structures shared by a whole range of pathogens. The cells of the innate immune system can immediately defend against the infection, and simultaneously activate the adaptive immune system.

Alveolar macrophages are likely the first cells to respond to the mycobacterial invasion, and are considered to be primary host cells for the intracellular infection. The interaction with several types of surface receptors results in phagocytosis of the bacterium, and the resulting phagosome matures and fuses with lysosomes to create a phagolysosome (101). The lysosome contains hydrolytic enzymes that can efficiently degrade microbes, and since these enzymes function optimally at acidic pH, the lysosome employs ATP-dependent proton pumps to maintain an acidic milieu at pH

4.5-5.0, which in itself is stressful to the pathogen (102). The host cell also starts producing reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) (103), and at least the latter appears to be essential for control of the bacterial infection (104-106). Killing and degradation of the bacilli within the phagolysosome is eventually followed by processing and presentation of its peptides on MHC Class II molecules on the surface of the host cell. Infected and activated macrophages also stimulate immune responses by producing and secreting signal molecules like chemokines and inflammatory cytokines. The induction of a strong IL-12 response is particularly important, as it stimulates the secretion of INF-γ by several cells of the immune system (107,108). If the invasion-event itself does not activate the macrophage, this cytokine can stimulate and enhance its killing mechanisms. As they become activated, some macrophages become rich in lipid bodies, and are referred to as foamy macrophages, while others differentiate into epitheliod cells or merge with other macrophages to form multinucleated giant cells (109).

Dendritic cells are also important antigen presenting cells during tuberculosis infection, although their functions are more specialised. After ingestion of the bacterium, these cells migrate to the draining lymphoid organs where they present the mycobacterial antigens to naive T-lymphocytes. This is a key event in the initiation of the adaptive immune response (110). Dendritic cells also seem to present antigens to, and stimulate, newly arrived lymphocytes in focal lymphocyte-dense infiltrates in infected organs (111).

Neutrophils are among the first innate immune cells to migrate to a site of inflammation, and influx of these cells are found in acute pulmonary tuberculosis (112). They have receptors that can recognise pathogens, and facilitate both phagocytosis and stimulate the production of chemokines and inflammatory cytokines (113,114). Neutrophils contain high amounts of granules with microbiocidal activity, and in response to inflammatory stimulation, they can undergo apoptosis and the resulting apoptotic bodies contain a high concentration of these

granules. It has been shown that *M. tuberculosis*-infected macrophages can phagocytose these bodies, and thereby increase their own antimicrobial arsenal (115).

Natural killer (NK) cells are large granular lymphocytes that can lyse infected cells without prior sensitisation (116). The majority of NK cells have a cytotoxic function, and form synapses with infected or abnormal cells, through which they transfer cytolytic granules that kill the target cell, including cells infected with *M. tuberculosis* (117). Interestingly, NK cells have also been shown to bind and lyse activated regulatory T-cells, whose primary activity is to reduce inflammatory responses (118). A small group of NK cells do not lyse cells directly, but instead produce INF-γ that adds to the pro-inflammatory environment (119). NK cells can also produce IL-22, which promotes phagolysosome fusion within the infected macrophages (120). Finally, NK-cells appear to possess antigen capture and presentation capabilities, and have therefore been suggested to enhance pro-inflammatory T-cell responses alongside other antigen presenting cells (121).

Epithelial cells are non-phagocytic and regarded as fairly resistant to invasion by *M. tuberculosis*, and the bacilli are therefore assumed to cross the epithelium via alveolar macrophages and dendritic cells. However, both *in vitro* culture studies (122-124), and observations in human autopsies (99), suggest that these cells can also be invaded by mycobacteria. Epithelial cells can respond to cytokine stimulation, and have been shown to exhibit antimicrobial activities, including the production of nitric oxide (NO) (125) and Lipocalin 2, which seize iron and therefore appear to limit mycobacterial growth (126). However, the killing mechanisms exerted by these cells is regarded as insufficient for eradicating the bacilli (127).

4.3 Adaptive immunity

The adaptive immune system comprises cells that are highly specialised against a particular pathogen, and it is activated by antigen-presentation and stimulation from cells of the innate immune system. The first time a host encounter a new pathogen,

specific adaptive immune responses will develop around three to four weeks after infection. However, upon re-exposure, cells with antigen-specificities developed during the initial exposure, rapidly triggers an efficient response. Adaptive immunity is traditionally divided into two branches: cell-mediated immunity and humoral immunity, and it is primarily the former that is associated with protection against tuberculosis. Humoral immunity is mediated by antibodies, which long have been considered by many to be of little help in battling this disease. In cell-mediated immunity, the protection is mediated by T-cells that specifically recognise antigens presented by antigen presenting cells. They can modulate functions of the innate immune system by secretion of signal molecules like cytokines, or they can directly kill infected cells. In tuberculosis, the distinction between two T helper cell subsets, Th1 and Th2, is believed to be important, and it is Th1 responses that correlates with protective immunity (128). This subset consists of CD4+ T-cells that secrete proinflammatory cytokines, which enhances the ability of the infected cells to kill the intracellular pathogen. Th2-subsets counter-regulates Th1-responses by secretion of anti-inflammatory cytokines like IL-4, IL-10 and TGF-β.

After the bacteria have been phagocytosed by the antigen-presenting cell, they primarily reside within vacuoles. The presentation of mycobacterial peptides on MHC class II molecules to T helper (Th) cells (CD4+ T-cells), is therefore believed to be the most important protective mechanisms against *M. tuberculosis* (129-132). The primary anti-mycobacterial effector function of CD4+ T-cells is the secretion of INF-γ, which can activate macrophages and enable them to control and eliminate the intracellular pathogen, but they also produce other proinflammatory cytokines, including IL-2 and TNF-α. TNF-α seems to have at least two roles: it contributes to the activation of macrophages (133,134), and it affects immune cell migration and localisation within tissues and contributes to the organisation of granulomas. Exactly how this cytokine contributes to granuloma formation is not known, but it is known to invoke expression of adhesion molecules, along with chemokines and their receptors (135,136).

Proteins present in the cytosol of antigen presenting cells are presented to cytotoxic (CD8+) T-cells by MHC class I molecules. Since mycobacteria are primarily thought to reside within phagosomes, the role of CD8+ T-cells in tuberculosis infections has been thought to be limited. However, mycobacteria have been observed outside the phagosome in infected cells (137), and there is evidence of proteins being translocated from the mycobacterium-containing phagosome into the host cell cytosol(138). CD1 molecules on dendritic cells can also present antigens to CD8+ T-cells. These molecules are structurally similar to MHC class I, but they have an antigen binding groove that is deeper and more hydrophobic, which allows them to present both lipids and glycolipids (139). CD8+ T-cells can kill tuberculosis-infected cells by secreting perforins and granulysin (140), or by Fas-L-interaction with Fas-receptors that leads to apoptosis. They also secrete INF-γ during mycobacterial infections (141), and this is regarded as a positive contribution of CD8+ T-cells towards fighting the infection (142).

Th17 cells distinguish themselves from the Th1 and Th2 subtypes by the secretion of IL-17, and are believed to aid in the elimination of pathogens that have resisted the efforts of Th1 and Th2 cells. Several cell types express the IL-17-receptor and can respond to its stimuli, including dendritic cells, macrophages, lymphocytes, epithelial cells, keratinocytes and fibroblasts (143). The responses induced are primarily pro-inflammatory and lead to accumulation of other cells of the immune system (144,145). Th17 cells might also have role in developing long-term protection against *M. tuberculosis*; Th1 memory cells that produce IL-17 appear to respond more quickly and populate the lungs faster than Th1 memory cells that do not secrete this cytokine (145).

The name of γ/δ T-cells are derived from their unique T-cell receptor composed of a gamma and a delta chain, which appear to recognise small phosphorylated molecules (146). It is not yet understood how these cells become activated, but they are thought to be involved in immune responses during early stages of infection, where they facilitate crosstalk between the innate and adaptive immune system (147). During *M.*

tuberculosis-infection, they have been found to produce INF-γ and IL-17 (148,149), and to express Fas-FasL and perforin (150). Studies in mice also suggests that γ/δ T-cells have anti-inflammatory properties, as depletion of these cells or replacing them with non-functional cells, was shown to accelerate inflammatory damage during *M. tuberculosis*-infection (151). Lastly, these cells can function as antigen presenting cells, and provide sufficient co-stimulatory signals to other cells for induction of proliferation and differentiation (152).

Regulatory T-cells are characterised by expression of the transcription factor forkhead box p3 (Foxp3), and since many express CD25 on their surface, their phenotype is often denominated as CD4+CD25+ cells. Regulatory T-cell expression has been associated with bacterial persistence in tuberculosis infection models in mice (153), and a rise in cell numbers have been found in human patients with active tuberculosis (154,155). Regulatory T-cells produce the anti-inflammatory cytokines TGF-β and IL-10, which inhibits the pro-inflammatory activity of all other CD4+ T-cells (156). The primary role for regulatory T-cells is probably to limit pathology once a pathogen has been eliminated. However, with a persistent organism such as *M. tuberculosis*, these cells might prematurely down-regulate the pro-inflammatory immune response. Indeed, increased levels of regulatory T-cells have been found in patients with extrapulmonary tuberculosis, which in turn is linked to a failure of the Th1-response to kill the bacilli, or at least to limit dissemination (157).

Antibodies have long been thought to offer little protection against tuberculosis. This notion is based on the premise that cell-mediated immunity protects against intracellular organisms, and B-cells and antibodies protect against extracellular infections. Furthermore, early attempts to treat tuberculosis by serum therapy gave quite variable results (158). For this reason, the two primary T helper-cell responses, Th1 and Th2, have been regarded to be on the opposite ends of the spectrum with regard to controlling and eliminating mycobacterial infections. In recent years, the rigid distinction between Th1 and Th2 responses have been questioned, and the role of B-cells against mycobacterial infections are now considered more favourably.

Naive CD4+ T-cells are not committed to a particular cytokine-secretion profile, but are influenced by the local environment when exposed to their cognate antigen; if the antigen presenting cells secretes IL-12, the T-cells are likely to develop into a Th1 cell, while IL-4 seems to drive the cell into a Th2 phenotype. Furthermore, both nutrients and hormones will influence the outcome of the Th1 and Th2 balance (159). The classification is based on the differential set of secreted cytokines, the most important being INF-γ secretion by Th1 cells, and IL-4 by Th2 cells. The strict division between the two profiles are primarily derived from studies in mice, but in humans, the Th1 and Th2 subsets do not adhere so strictly to a certain set of cytokines. Furthermore, one subset of activated B cells, the effector (Be) 1 cells, secrete INF-γ and IL-12, while the subset Be 2 cells produce IL-4. B-cells also produce IL-6, which is an important co-stimulator of T-cells responses, and IL-10, which inhibits activation of both macrophages and dendritic cells (160).

There is also evidence to suggest that antibodies actually do play a role during *M. tuberculosis*-infections. Monoclonal antibodies against arabinomannan on the bacterial surface have been shown to prolong survival of *M. tuberculosis*-infected mice (161). Also, passive transfer of monoclonal IgA that targets the major membrane protein α-crystallin, have been shown to reduce bacterial load and pathology in mice infected with *M. tuberculosis*, whereas monoclonal antibodies directed against a different protein, PstS-1, did not confer protection at all (162,163). The mechanism of antibody-protection in tuberculosis is not completely understood. Antibodies can presumably bind to the bacilli during brief extracellular exposures, which would allow uptake of the pathogen by a wider variety of cells. Furthermore, an Fc-receptor-mediated endocytosis might lead to more efficient fusion with lysosomes (164). Recently, a research group has identified an intracellular IgG-receptor, tripartite motif-containing 21 (TRIM21), which binds to the constant region of antibodies (165). The intracellular effector mechanism was shown to be protective in cells infected with adenovirus, but TRIM21 and similar receptors, as well as their

downstream pathways, might also be involved in the protection against a whole range of intracellular pathogens, including *M. tuberculosis*.

4.4 The mouse model for mycobacterial infections

Inbred mice are widely used to study *in vivo* properties of mycobacteria and host immune responses, and since there are extensive similarities between mice and men in terms of the basic immune responses, the use of this animal model has provided researchers with an invaluable understanding of human tuberculosis. It is frequently used to test new vaccine candidates, and to evaluate the effect of newly developed drugs. Experiments in mice are relatively inexpensive, and the genetic standardisation and minimal variation between animals reduce the number of animals needed in experimental groups.

In both humans and mice, the bacteria primarily target the lungs, and efficient host responses rely heavily on the actions of T-cells, INF-γ, and TNF-α (166). Furthermore, immune responses can be readily investigated using the vast array of reagents that are available against immunological markers in mice. There are also some differences that are important to keep in mind. The central necrosis described for human granulomas are not typically found in the most commonly used mouse strains (167). The organisation of macrophages and lymphocytes is also described to be different, as the macrophage- and lymphocyte aggregates found in mice are more loosely organised (168). Perhaps as a consequence, there does not appear to be any zones of hypoxia in the lungs of these animals during infection, which are comparable to the caseous centres in human granulomas (169). However, there are genetic differences between mouse strains, just as there are significant differences between infected individuals, and certain phenomena can be investigated by selecting the appropriate model (170). For example, well-organised granulomas with necrotic centres have been described in the lungs of the mouse strain C3HeB/FeJ after challenge with *M. tuberculosis* (171). In conclusion, a complex disease such as tuberculosis cannot be fully understood by the use of a single animal model, but it

can provide information on key aspects of disease progression and immune responses, and by doing so this animal have, and will continue to provide, very important pieces of the puzzle.

5. Mycobacterial modulation of the host response

5.1 First contact and entry

Specific interactions between the mycobacteria and its host start moments after the bacilli have gained entry in the lung. Cells of the immune system recognise distinct surface features of the bacterium by various classes of phagocytic receptors: Toll-like receptors (TLRs), C-lectin type receptors (CLRs), immunoglobulin Fc receptors (FcRs) and scavenger receptors (SRs) (101). Some of these receptors will immediately prime the immune cells for activation by cytokines, and trigger the production of antimicrobial molecules, chemokines, and cytokines. Other receptors do not trigger such responses, and may therefore allow a "silent" entry of the bacterium into the cell. The outcome of this initial interaction can therefore be influenced by the type of receptors expressed by the cells that first encounter the bacilli.

The TLRs are perhaps the best described receptors in tuberculosis. TLR-2 recognises several mycobacterial proteins and structures, including Hsp65, Hsp70, 19 kDa lipoprotein, lipoarabinomannan, and phosphatidylinositol mannoside (PIM) (172,173). This interaction has been suggested to induce synthesis of an antimicrobial peptide, cathelicidin, which has been shown to be effective against *M. tuberculosis* (174). However, it appears that TLR-2-signalling and responses are dampened when the receptor interacts with the secreted mycobacterial protein ESAT-6 (175). Furthermore, activation through this receptor can also limit the up-regulation of MHC class II by macrophages in response to INF-γ (176).

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which is a member of the NOD-like receptor-family, recognises mycolylarabinogalactan, and has been reported to be important for cell activation during tuberculosis-infections (177). Soluble and membrane-bound CLRs are involved in sensing *M. tuberculosis*, and collectins or integrins that carry the lectin domain, CR3, can facilitate

phagocytosis of the bacilli (178). The CLR mannose receptor recognise ManLAM, arabinomannan, and mannosylated proteins (179), and has also been shown to be important for internalization of bacteria. Both CR3 and mannose receptors are associated with a silent entry that does not activate the host cell (180-182). Dectin-1 is a major beta-glucan receptor that appears to sense heat-stable constituents like lipoglycans and α-glucans from the envelope of *M. tuberculosis* (183). Another CLR, Mincle, is a receptor that binds to trehalose 6,6'dimycolate (TDM), also known as the cord factor, which play a role in tissue remodelling and granuloma formation (184). TDM is the most abundant lipid to be extracted from virulent *M. tuberculosis* (185). On dendritic cells, DC-SIGN senses ManLAM, arabinomannan, PIM6, α-glucans and glycosylated proteins, and it is involved in internalization of *M. tuberculosis* (186). The receptor is also thought to initiate anti-inflammatory responses (187), and it has been found to be important in controlling immune responses during acute and chronic tuberculosis in mice (188).

From this brief overview, it would appear that interaction with certain receptor types favour the initiation of an inflammatory response, while other receptors suppress this type of response by allowing the bacilli to enter the cell without activating it. However, mycobacteria are likely to interact with several different pattern recognition receptors simultaneously as it encounters the immune cells of the host. The initial host-pathogen interaction, and the subsequent host response, is therefore multi-factorial and complex.

5.2 Surviving the phagolysosome

After mycobacteria have entered their host cell by endocytosis, they are immediately subjected to host killing mechanisms. Activated macrophages express phagocyte NADPH oxidase (NOX2) that generates ROIs (189), and nitric oxide synthase that produce RNIs (103). Both ROIs and RNIs can kill the bacteria by interaction with molecules like nucleic acids, proteins, lipids and carbohydrates. However, this pathogen appears to have different means of resisting this type of stress. It has been

suggested that the mycobacterial proteasome can efficiently eliminate or refold proteins that have been damaged (190). The bacteria can also prevent damage by inhibiting the activities of oxidants. The mycobacterial protein SodA is an iron co-factored superoxide dismutase that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. This enzyme is secreted through the SecA2 secretion channel, and has been found to suppress macrophage activation *in vitro* and inflammatory responses *in vivo* (191,192). Studies have implicated the activity of several other mycobacteria-produced antioxidants in bacterial virulence, including catalase-peroxidase, alkylhydroperoxidase C, and Rv3303c (193-195). Expression of many antioxidants have been found to be regulated by the sigma factor, SigH, and *M. tuberculosis* mutants that lack this transcription cofactor are highly attenuated in mouse models of infection (196,197).

Efficient and complete clearance of the bacteria within the host cell requires fusion of the bacteria-containing phagosome with lysosomes containing an arsenal of antimicrobial molecules. For this fusion event to occur, the phagosome is required to expose several proteins on its surface that will interact with the lysosome. However, the bacilli have ways of interfering with the machinery involved in maturation of the phagolysosomes. The effect of this can be seen in the atypical retention of the GTPase Rab5 to the surface of the phagosome, and a subsequent deficient recruitment Rab7 (198). Rab5 is normally found on early, but not late, phagosomes, while Rab7 is required for late endosome trafficking. The mycobacterial interference therefore seems to take place around the time of Rab5 acquisition. However, one experiment showed that even with an abundance of Rab7 on the phagosome surface, the phagosome failed to acquire the lysosome-associated membrane glycoprotein 1 (LAMP1), which is required for phagolysosome fusion (199). This suggests that maturation-arrest also can occur after Rab7 has been recruited. Furthermore, mycobacterial ManLAM attenuates the activity of the phosphatidylinositol-3(OH) (PI(3)P) kinase hVPS34, and by doing so it inhibits recruitment of the early endosome autoantigen 1 (EEA1) (200). The latter protein interacts with the soluble

N-ethylmaleimide-sensitive factor-attachment protein receptors (SNAREs), which is involved in the fusion event. It has also been reported that a SNARE protein called cellubrevin exists in an unusual form on mycobacteria-containing endosomes, and a structural modification of this protein might be another way to prevent phagosome maturation (201). PI(3)P has a role in anchoring many effector proteins on the phagosome surface, but by inhibiting recruitment of EEA1 and the lipid kinase hVps34, *M. tuberculosis* prevent PI(3)P from assembling on this surface. The bacteria also produce a phosphatase, SapM, that specifically hydrolyses PI(3)P, and the combined effect is a near complete depletion of PI(3)P from early phagosomes (202).

Mycobacteria interact with the host tryptophan aspartate containing coat protein (TACO) (also called coronin-1), and it has been shown that phagosomes containing live, but not dead BCG, co-localize with this protein (203). TACO prevents phagolysosome fusion by regulating calcium-dependent signalling processes; a sustained calcium influx activate the phosphatase calcineurin, which subsequently interferes with the phagolysosome fusion by an unknown mechanism (204). Some reports suggests that retention of TACO only occurs in phagosomes where bacterial clumping is observed, but the significance of this is not understood (205).

M. tuberculosis possesses 11 proteins of the eukaryotic-like serine/threonine kinase family, which is likely to be involved in the modulation of signal transduction cascades (206). Most of these enzymes are transmembrane proteins with the active domain placed in the bacterial cytosol, which suggests they are involved in regulating the bacterial physiology. However, two of the kinases, PknG and PknK, are soluble proteins and at least PknG is secreted into the phagosome and eventually reach the macrophage cytosol. PknG-deficient bacteria grow well in *in vitro*-cultures, but in macrophages they are attenuated since they cannot prevent lysosomal transfer (207). The precise mechanism by which PknG prevents the phagolysosomal fusion is not known, but presumably it can phosphorylate one or more host molecules, and thereby interfere with signalling pathways that would normally lead to the fusion event.

Perhaps one of the most intriguing observations of *M. tuberculosis* during infection, is the ability to escape from the confinement of the phagosome altogether. This has been linked to the function of the ESX-secretion machinery, but details of this mechanism have yet to be revealed (208).

5.3 Inhibition of signalling pathways

Mitogen-activated protein kinase (MAPK) phosphorylates substrates like transcription- and nuclear factors, which in turn lead to production of pro-inflammatory mediators like TNF-α. Activation of MAPK is caused by signalling pathways that involve a cascade of kinases, including p38, extracellular signal-related kinases (ERKs) and Jun N-terminal kinases (JNKs). The pathways can be initiated upon pathogen entry, and by the influence of cytokines and growth factors. Pathogenic mycobacteria, but not non-pathogenic mycobacteria, can inhibit activation of p38 and ERK1/2, and by so doing they abrogate the standard pro-inflammatory host response (209,210). Mycobacteria can also interfere with the signalling events that are initiated when INF-γ binds to its receptor on the macrophage surface. Under normal circumstances, this receptor will recruit Janus kinases (JAKs) that in turn activate the signal transducer and activator of transcription (STAT) protein. STAT then translocate to the nucleus where it initiates a potent antibacterial response by activating transcription of INF-γ-target genes. While *M. avium* has been found to down-regulate the expression of INF-γ-receptor (211), *M. tuberculosis* has been shown to interfere with the ability of STAT-1 to bind to DNA (212). Both actions lead to a reduced transcription of INF-γ-responsive genes.

5.4 Apoptosis

Apoptosis, also called programmed cell death, is a precisely controlled event that is either triggered by an extrinsic pathway initiated by the binding of ligands to the so-called death receptors, or via the intrinsic pathway that involves translocation of

cytochrome c from mitochondria to the cytosol. Both pathways activate a cascade that leads to degradation of genomic DNA, and eventually fragmentation and packaging of cellular contents into vesicles called apoptotic bodies. These vesicles are in turn taken up by nearby cells, including macrophages. This is a common and efficient response by immune cells for resolving persistent intracellular infections. Whether apoptosis is a favourable event for mycobacteria is a matter of debate. Apoptosis of the infected cell attracts other immune cells, and the bacteria can spread to new potential hosts via the apoptotic bodies. On the other hand, if the infected cell fail to undergo apoptosis it might become a safe harbour for the bacteria that can replicate unhindered for a prolonged time. Furthermore, it has been shown that small apoptotic vesicles derived from infected cells can present mycobacterial antigens to T-cells, and thereby stimulate a range of pro-inflammatory responses (213). It has been suggested that the bacteria can prevent apoptosis in the early phase of infection, which gives them time to adapt and replicate, but that they are unable to prevent this form of cell death at later stages. There is little doubt that at least some mycobacterial species have the capacity to down-regulate host cell apoptosis, and that this property is correlated with increased virulence (214,215).

Mycobacteria have several mechanisms that can influence host apoptosis. Ca^{2+} can facilitate apoptosis by increasing the permeability of mitochondrial membranes, enabling the release of cytochrome c (216). *M. tuberculosis* has been shown to limit the Ca^{2+} influx in infected cells by an unknown action of mycobacterial ManLAM (217). Bcl-2 is an anti-apoptotic host protein that exert its function by preventing a Bax-mediated release of cytochrome c (218). It is inactivated when bound to the pro-apoptotic protein Bad, however, ManLAM stimulates phosphorylation of Bad, which prevents it from binding to Bcl-2 (219). In murine tuberculous granulomas, it has been shown that infected macrophages contain increased amount of Bcl-2 and reduced amounts of Bax (220). Infection with *M. tuberculosis* also stimulates the host to produce the immunosuppressive IL-10 (221). This interleukin blocks the apoptosis-stimulatory abilities of TNF-α by inducing the release of TNF receptor type

2 protein. This molecule binds to TNF-α to form an inactive complex, which in turn prevents the cytokine from binding to death receptors and stimulate apoptosis. Infection with *M. tuberculosis* also seems to interfere with the expression of apoptosis-inducing receptor Fas and its pro-apoptotic ligand FasL. Infected macrophages are shown to strongly express FasL, but the expression of Fas appears to be down-regulated. Furthermore, up-regulation of FasL expression may induce selective immunosuppression by inducing apoptosis of immune cells in the vicinity of the infected cell (214,222). Lastly, MPT64 is a mycobacterial protein that is implicated in the inhibition of apoptosis, but how it affects the apoptotic pathways are not yet understood. Mustafa and co-workers have described how the expression of this protein was found to be inversely correlated with apoptosis in infected cells in human granulomas (223,224), and this is supported by the recent findings of Kozak *et al* (2011) (225).

5.5 Metabolic adaptations

Besides inhibiting host killing mechanisms, *M. tuberculosis* ensures its survival in the host by adapting its metabolism to the nutrients that are available at different stages of infection. Like other heterotrophs, *M. tuberculosis* is dependent on at least one carbon source for the generation of energy, and for production of proteins, nucleic acids and cell envelope components. Specialised uptake systems and pathways are involved in the transport and metabolism of available nutrients, and genes involved in these pathways are controlled by global and specific transcriptional regulators (226). The expression of several genes related to metabolism is upregulated in bacteria residing within cells, compared to growth in a standard culture medium, including genes characteristic for C_2-based metabolism and gluconeogenesis, *fad*-genes encoding enzymes for fatty acid uptake and degradation, and genes for glycerol-3P uptake and metabolism. Indeed, fatty acids, and glycerol or glycerol-3P, seem to be the favoured source of carbon during intracellular existence.

M. tuberculosis has 4 homologous mammalian cell entry (*mce*) operons that encode membrane lipid transporters and enzymes for metabolism and modification of imported substrates (227). Deletions *mce* operons have been shown to attenuate *M. tuberculosis*-virulence in infected mice via the intratracheal route (228), and fatty acids have been shown to be required for carbon metabolism in both acute and persistent infections in mice (229,230). It is likely that lipids from the host cell are essential sources for carbon during infection, although host carbohydrates also appear to be used (231). Interestingly, it seems that lipids produced by *M. tuberculosis* can stimulate the host to make nutrients available to the bacteria. Oxygenated forms of bacterial mycolic acids induce the formation of foamy macrophages that are rich in low-density lipoprotein particles containing cholesterol, triacylglycerides and phospholipids. The bacteria can access these lipid droplets, which have been suggested to be an important nutrient source for pathogenic mycobacteria in the latent stage of infection. In fact, bacteria-containing phagosomes have even been found within such lipid bodies (232). Foamy macrophages have also been shown to be induced by the cell wall lipid trehalose dimycolate, the cord factor, of *M. bovis* BCG by way of stimulating TLR-2 of the host (233).

5.6 Caseation, liquefaction and spread

The accumulation of caseum in the granuloma appears to be linked to the formation of foamy macrophages, since the lipids of the caseum are the same as those involved in foam-cell formation (234). As mentioned previously, the caseum forms after the immune cells have been organised into the granuloma structure, and its primary purpose is to form a physical barrier from which the bacteria cannot immediately escape. In some cases, however, a build-up of tuberculin-like products seems to cause a response that eventually result in a liquefaction of the solid caseum (235). The liquefaction is probably driven by hydrolytic enzymes that are either produced by the surrounding host cells, or by enzymes that are already present within the caseum. Mycobacteria have been suggested to produce inhibitors of these enzymes to delay an

early destruction of the tissue it has invaded. This would explain why the tuberculous lung under normal circumstances do not liquefy for several weeks, if at all (236).

6. The substrains of BCG

After Albert Calmette and Camille Guérin had attenuated a pathogenic strain to develop the vaccine *M. bovis* Bacille Calmette-Guérin (BCG), and it had been proven to be both protective and safe, large-scale vaccination programs were initiated. Many countries received their own batch of the BCG Pasteur strain culture for local maintenance and distribution. However, variable sub-culturing practices at these facilities eventually lead to the rise of substrains with divergent genetic content and alterations in the regulation of gene expression. Proteomic studies and genotyping in later years have shed some more light on the evolution of these substrains, and based on this work they have been divided into 4 groups based on similarities and divergence from the originating *M. bovis* strain. This division is illustrated in Figure 5.

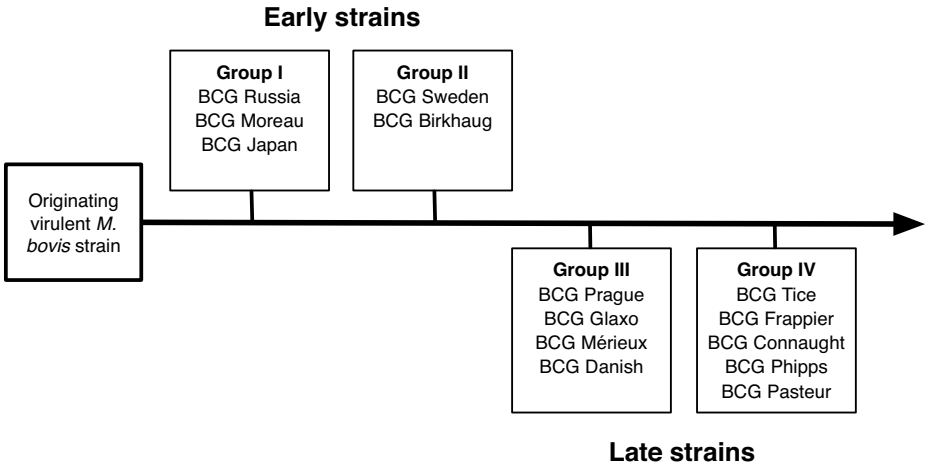

Figure 5. The division of BCG into early and late strains (Adapted from Brosch, 2007)

One of the major differences between these groups is the loss of region-of-difference 2 (RD2) from the BCG strains that often are referred to as late strains (Group III and IV in Figure 5) (237,238). Recently, the RD2 region was shown to contribute to the virulence of a wild type *M. tuberculosis* H37Rv strain, when compared to a H37Rv:ΔRD2 in both an *in vitro* and murine model (225). The authors described

reduced bacterial burden and lung pathology at early time-points for the gene knock-out-strain. The late BCG strains also lack the gene *mma3*, which is involved in synthesising methoxymycolic acids (239), and a missense mutation in the start codon of *sigK* leads to a greatly reduced expression of MPB70 and MPB83 (240-242). Gene deletions and missense mutations are not the only modifications that differentiate the BCG substrains. The alterations through cultivation have also led to duplication of genes (37,243). Two duplication units have been described, DU1 and DU2; the former appear to be unique to the substrain BCG Pasteur, while the latter is more widely distributed. Within the DU2 unit lies the gene encoding the sigma factor SigH, which has been found to increase expression of multiple antioxidants during oxidative stress (244). This region also harbours *whiB1* and *whiB3* that belongs to the WhiB family of transcription factors, which have been found to regulate virulence, cell division and stress responses. *WhiB1* have been found to be duplicated in all BCG strains, while the expression of WhiB3 is increased 9-fold in BCG Pasteur compared to BCG Japan and *M. bovis* (245). Also, expression of the oxidoreductase Rv1774 has been described to be up-regulated in BCG Pasteur when compared to BCG Russia, which is linked to the loss of a repressor that lies within RD14. BCG Pasteur is the only BCG strain that lacks this region (246). To complicate the picture further, Chen and colleagues (2007) found that BCG Japan (group I), BCG Moreau (group I), and BCG Glaxo (group III) do not produce two cell wall lipids that have been associated with virulence in *M. tuberculosis* and *M. bovis* (247,248). The lipids were found to be expressed in other BCG strains representing all the four groups. The phylogenetic distance from the original *M. bovis* strain does therefore not necessarily represent a gradual increase in attenuation and decrease in vaccine efficacy; the BCG substrains appear to differ in their properties in a less predictable manner. In fact, the efficacy of the BCG vaccine has long been a matter of controversy. After it was determined to be both safe and protective, a close look into the vaccination programs showed inconsistencies in the reported results, and a meta-review published in 1994 describe reports of BCG efficacy ranging from 0% to 80% (249). It has been speculated that an ‘over’-attenuation of certain BCG substrains might be the cause for

these highly variable results (250). However, other meta-reviews that have evaluated vaccine programs in humans and animal trials, conclude that the administered BCG vaccine strain could not be used as a determinant of overall efficacy (251,252). Another suggested factor is variable handling of the vaccine leading to differential numbers of viable bacilli per vaccine dose. Situations where these differences might come into play include vaccine production (both cultivation and the process of freeze drying), storage, reconstitution and preparation. In 1948, Heimbeck showed that live but not dead BCG bacilli protected vaccinated nurses against tuberculosis (253), indicating that the metabolic activity, and secretion of proteins, is important for inducing a protective immune response. It has also been suggested that differences between the vaccinated individuals, like the presence or absence of resistance and susceptibility genes, and nutritional status, have influenced the outcome of these trials. Another confounding factor is possibly the exposure and interference of non-pathogenic environmental mycobacteria like *M. avium* (254,255). However, while dead environmental bacteria might theoretically interfere with the efficacy of BCG vaccination, live environmental bacteria actually seem to offer some protection by secreting antigens that are cross-reactive. Non-pathogenic mycobacteria may also influence results of the BCG trials in less obvious ways. The negative control group, consisting of non-vaccinated individuals, will be exposed to environmental mycobacteria to the same extent as the BCG vaccinated group during the trial, and the efficacy of the BCG vaccine might be occluded by any protection that these species might offer. This could explain the poor results noted for some of these trials. Today, the BCG vaccine is generally viewed as insufficient for protecting adults from pulmonary tuberculosis. However, it is still regarded to be efficient against tuberculous meningitis and miliary disease in children, and is therefore still recommended in countries with high prevalence of tuberculosis.

7. Aims of the study

7.1 Overall aim

The overall aim of the current thesis was to increase the understanding of bacterial factors for mycobacterial pathogenicity with a particular focus on secreted proteins.

7.2 Specific aims

- To determine the best signal peptide prediction algorithm for mycobacterial proteins, using data sets verified by proteomic methods.
- To evaluate that nature of proteins with a predicted signal peptide, but that remain uncleaved and enriched in the membrane fraction.
- To investigate the pathology in mice infected with either of two BCG strains, BCG Russia and BCG Pasteur, that differ in their genetic content due to alterations arisen during *in vitro* culturing.
- To study the *in vivo* pattern of expression of mycobacterial proteins within murine lungs by using immunohistochemistry.
- To study the *in vivo* pattern of expression of mycobacterial proteins within human tuberculous lesions obtained from lungs and lymph node biopsies.

8. Materials and methods

8.1 Ethical considerations

The animal experiments in paper III were performed according to protocols approved by the Norwegian Animal Research Authority. Ethical clearance for the use of human biopsies was obtained from the regional ethical committee in Norway.

8.2 Paper I

Construction of validation sets

To test the signal peptide prediction algorithms we created two validation sets: a positive validation set that contained proteins known to be cleaved by signal peptidase and where the exact cleavage site was known, and a negative validation set consisting of proteins that we knew were not cleaved. We included only proteins where the mature sequence had been verified experimentally. Some were found in previously published literature, but we also used novel data generated by our group. In total, we included 57 proteins in the positive validation set, and 61 proteins in the negative set.

Prediction algorithms

We tested the performance of 9 different signal peptide prediction algorithms: SIGCLEAVE, PrediSi, SPEPLip, Signal-CF, Signal-3L, and the hidden Markov model and neural network method from versions 2 and 3 of SignalP. The selected algorithms were free of charge and publicly accessible from their respective web interface. Since these programs have been developed to separately analyse eukaryotes, Gram-negative bacteria and Gram-positive bacteria, we chose the Gram-positive option since mycobacteria are regarded to belong to this group. The exception was SIGCLEAVE, which only had options for eukaryotes and prokaryotes.

Protein sequence data

The prediction algorithm that performed best on our positive and negative validation sets was used to analyse proteins from all mycobacterial proteome annotations that were available at the time we conducted our study. These included two separate annotations of *M. tuberculosis* isolate H37Rv, *M. tuberculosis* isolate CDC1551, *M. tuberculosi*s isolate H37Ra, *M. bovis*, *M. bovis* BCG Pasteur, *M. leprae*, *M. avium*, *M. avium* subsp. paratuberculosis, and *M. smegmatis*.

8.3 Paper II

Preparation of mycobacterial proteins for proteomic analyses

We harvested colonies of *M. tuberculosis* H37Rv grown on agar plates for around 4 weeks. The colonies were mixed with an extraction buffer that contained protease inhibitors, and transferred to tubes with glass beads. The bacilli were subsequently lysed by bead beating. For preparation of whole cell lysate, the solution was centrifuged and the supernatant containing the proteins was collected. To isolate membrane proteins, the supernatant was treated with the detergent Triton X-114 to obtain lipophilic proteins from the lipid phase.

Proteins from each preparation were further separated by size using an SDS-PAGE gel electrophoresis system, and each lane was divided into smaller segments for digestion with trypsin. The enzyme trypsin cleaves peptide chains at the carboxyl side of lysine and arginine, except when these amino acids are followed by a proline. The mass spectrometry technique requires the proteins to be broken down into smaller fragments for analysis. By using trypsin digestion, the proteins are broken into smaller peptides with a mass that can be matched to theoretical peptides acquired by *in silico* trypsin cleavage of annotated protein sequences. The mass of a peptide, and the masses of its fragments following fragmentation, gives the peptide a unique peptide sequence tag that is used to identify the peptide in a protein database.

Identification of proteins by mass spectrometry

Peptides were detected using an Orbitrap mass spectrometer, which generated a list of peptide sequence tags representing the most abundant peptides in our samples. These were identified by searching through databases of theoretical peptides generated from the Tuberculist database made available by the Pasteur Institute, and the CMR-TIGR database. Proteins were validated statistically based on the score of the identified sequence tags. If a particular protein was represented by only a single peptide, this peptide was required to have a higher score to be accepted as a positive identification, than if a protein was identified by two or more peptides.

Label free quantification of proteins based on MS data is a relatively new development. We calculated the abundance of all identified proteins by calculating an emPAI value for each protein, and by further dividing this value by the sum of the emPAI values for all identified proteins in the sample. The emPAI value of a protein is calculated by dividing the parent ions of observed peptides by the number theoretically observable peptides for a particular protein.

8.4 Paper III

Infection of mice

We grew two BCG strains, BCG Russia and BCG Pasteur, in parallel in 7H9 culture medium. When the bacilli had reached mid-log phase, we broke up any large bacterial aggregates that might be present in the medium, and froze the suspension at -80°C. Female mice of the C57BL/6 strain were infected intranasally with either of the two BCG strains. The infection inoculates were verified to contain the same number of CFU, and to be similar with regard to clumping. After infection, we sacrificed groups of mice at various intervals for 17 months. The right lung was removed and homogenised for enumerating bacterial colony forming units (CFU), and the left lung was perfused with formalin and later embedded in paraffin for histological examination.

Table 1: Antibodies used for protein-specific staining by immunohistochemistry.

Antibody	Target antigen	BCG equivalent	Protein function	Paper	Ref.
Secreted proteins					
Anti-MPT32	**MPT32 (Apa, Rv1860)**	BCG1896	Unknown (Could mediate bacterial attachment to host cells)	III, IV	(65,256)
Anti-MPT44	**MPT44 (Ag85A, fbpA, Rv3804c)**	BCG3866c	Involved in cell wall mycoloylation. Responsible for the high affinity of mycobacteria to fibronectin. Possesses a mycolyltransferase activity required for the biogenesis of trehalose dimycolate (cord factor), a dominant structure necessary for maintaining cell wall integrity	IV	(65)
Anti-MPT46	**MPT46 (Thioredoxin TrxC, Rv3914)**	BCG3972	Participates in various redox reactions through the reversible oxidation of its active center dithiol, to a disulfide, & catalyzes dithiol-disulfide exchange reactions.	III, IV	(65,257)
Anti-MPT51	**MPT51 (Ag85D, fbpD, Rv3803c)/TB22.2**	MPB51 (BCG3865c)	Same as MPT44	III, IV	(65)
Anti-MPT53	**MPT53 (DsbE, Rv2878c)**	MPB53 (BCG1715)	Unknown	IV	(65,258)
Anti-MPT59	**MPT59 (Ag85B, fbpB, Rv1886c)**	BCG1923c	Same as MPT44	IV	(65)
Anti-MPT63	**MPT63 (Rv1926c)**	MPB63 (BCG1965c)	Unknown	III, IV	(65,259)
Anti-MPT64	**MPT64 (Rv1980c)**	MPB64	Unknown (Suggested to inhibit apoptosis of infected cells)	IV	(264)
Anti-ESAT6 (HyYB76-8)	**ESAT6 (Rv3875)**	n/a	Unknown (Proposed to bind to TLR2 receptor and inhibit signal transduction)	IV	(260,261)
Somatic proteins					
Anti-MPT57	**MPT57 (GroES, Rv3418c)**	MPB57 (BCG3488c)	Binds to Cpn60 in the presence of Mg-ATP and suppresses the ATPase activity of the latter.	III, IV	(65)
Anti-Hsp65	**Hsp65 (Ag82, GroEL2, Rv0440)**	BCG0479 (Ag82)	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.	III, IV	(263)
Anti-Mce1A	**Mce1A (Rv0169)**	BCG0206	Thought to be involved in cell invasion (entry and survival)	IV	(262)
Polyvalent antibodies					
Anti-BCG	**BCG whole cell sonicate**		Various	III	(265)

Histology and immunohistochemistry

The paraffin embedded lungs were cut in serial sections, and one section from each lung was stained with hematoxylin and eosin to investigate lung pathology. We defined 4 types of regions that were measured separately: macrophage-dominant areas, lymphocyte-dominant areas, bronchus-associated lymphoid tissue (BALT), and areas with no observable pathology. We then measured the total size of each of these areas, which was compared to the total size of the lung section. The remaining paraffin sections were used for immunohistochemistry. These were stained using a set of rabbit polyclonal antibodies that were specific to somatic (cytoplasmic and membrane) and secreted mycobacterial proteins, and we also used antibodies developed against a BCG whole cell lysate (see Table 1). Visualisation of staining was achieved with biotinylated secondary goat anti-rabbit antibodies, streptavidin-coupled horseradish peroxidase, and finally a 3-amino-9-ethylcarbazole (AEC) substrate chromagen. We determined the ratio of stained cells versus unstained cells in the 4 types of regions outlined above, and we made a semi-quantitative measurement of the staining intensity.

Statistical analyses

All statistical analyses were performed using the SPSS Statistics software. Values were expressed as the median. A two-tailed non-parametric Mann-Whitney U-test was used to compare data from the two BCG strains, and for correlation between parameters we used Spearman's rank correlation for non-parametric data.

8.5 Paper IV

Collection of samples

We obtained biopsies from patients with pathologic findings confirmed to be caused by *M. tuberculosis* complex organisms. In total we studied 17 cases of tuberculous lymphadenitis and 4 cases of pulmonary tuberculosis. Twelve cases of non-

tuberculous inflammation were used as controls. In addition, lung tissues from 11 mice infected with *M. tuberculosis*, and 4 uninfected control mice were included.

Histology and immunohistochemistry

Staining with hematoxylin and eosin, as well as immunohistochemistry, were performed as described for paper III.

9. Summery of results

9.1 Paper I

In paper I, we investigated the accuracy of 9 prediction algorithms for predicting the presence of a signal peptide, and the respective signal peptide cleavage site, in mycobacterial proteins. They were tested on two validation sets; a positive set where all the proteins had been experimentally confirmed to harbour a signal peptide, and a negative set that contained proteins without an N-terminal signal peptide.

- The hidden Markov model of SignalP v 3.0 was the best performing algorithm. It correctly detected the presence of a signal peptide in all proteins in the positive set, and it did not produce any false positive results in the negative data set. This algorithm also was the best performer for detecting the correct cleavage site; 45 of the 57 cleavage sites were correctly predicted.

- Using the hidden Markov model of SignalP v 3.0, we predicted secreted proteins from several mycobacterial proteomes. From the list, we excluded proteins predicted to have transmembrane helices in the mature protein sequence, with the presumption that these are membrane proteins. The ratio of secreted proteins ranged from 7.8% in *M. smegmatis*, to 10.5% in *M. leprae*.

- Signal peptide prediction of two different protein annotations of the *M. tuberculosis* H37Rv genome gave variable results; in one annotation, 13% of the proteins were predicted to have a signal peptide, but only 11% in the second annotation.

9.2 Paper II

In paper II, we investigated the nature of membrane proteins with a predicted signal peptide.

- We identified 30 proteins enriched in the membrane fraction, with positive identification of peptides that either completely or partially overlapped with the predicted signal peptide sequence.
- Structurally, these proteins were similar to proteins known to be cleaved by signal peptidase, but we found more variation in the -3 position relative to the cleavage site.
- Some of the proteins had a transmembrane domain that spanned the predicted cleavage site, which might make it unavailable to cleavage by signal peptidase. However, this does not explain the lack of processing for the majority of these proteins.

9.3 Paper III

In paper III, we studied the course of infection with two BCG strains in mice, and investigated the *in vivo* expression of secreted and somatic mycobacterial proteins.

- BCG Pasteur was more persistent than BCG Russia in our mouse model; while BCG Russia was cleared from the lungs after 4 months of infection, BCG Pasteur persisted until the experiment ended 17 months after infection.
- There was greater pathology in lungs of mice infected with BCG Pasteur, consistent with the higher bacterial load.
- Immunohistochemistry revealed the *in vivo* protein expression to be similar between the two BCG strains.
- For both BCG strains, immunohistological staining revealed host cells located in the normal lung parenchyma with a high load of bacterial antigens.

9.4 Paper IV

In paper IV, we investigated the staining patterns of polyclonal antibodies specific for secreted and somatic *M. tuberculosis* proteins within human lungs and lymph node tissues.

- Secreted proteins were detected more frequently in pulmonary granulomas, than in lymph node granulomas.
- In certain cases, major secreted proteins were only detected in necrotic centres, and not in the cells of the granuloma.
- The staining pattern varied between the secreted proteins. Only MPT64 was expressed in all granulomas in both lung and lymph node lesion.
- Staining of Mce1A was detected more frequently in organised granulomas, compared to the regions without proper granuloma formation.

10. Discussion

From the moment *M. tuberculosis* enters its host, there is a two-way interaction between the two organisms. The host recognises structural features of the bacilli, and mounts responses to contain and kill it. The bacterium, on the other hand, does whatever it can to resist and interfere with the killing mechanisms of the host, something it can do successfully for many years. It manages this by way of secreted- and membrane molecules that specifically interact with host proteins and disrupts and modulates host responses. However, the exposure of such molecules also generates specific immune responses by the adaptive immune system, and might become important immunogens during the course of infection. A full comprehension of the identity, location and expression pattern of such proteins is therefore crucial to understand the biology of this pathogen, and it paves the way for making enlightened decision when developing new vaccines, diagnostic tools, and drugs.

We sought to tackle different aspects of research into this field.

10.1 Prediction of signal peptides

The use of prediction algorithms in molecular biology research has greatly pushed the biology field forward. It has made it possible to screen and interpret large amounts of data, and it can provide information about properties of proteins based solely on the gene sequence information. However, their inherent nature is to extrapolate the information from a limited set of experimentally acquired data to new sequences, which in many cases encode proteins with unknown properties and destinies. The quality of these prediction programs is therefore not only determined by the architecture of the algorithm, but also the data sets that are used to train them.

The prediction of N-terminal signal peptides from proteins that are processed through the general secretory pathway seems to be one of the success stories in the field of prediction algorithms. Several algorithms have been developed for this type of

prediction, and they vary both in design, and the size and quality of the training sets. Perhaps signal peptides lend themselves particularly well for this type of prediction. They contain three distinct domains, from the positively charged N-terminal, via the hydrophobic region, to the C-terminal part that contains two quite conserved amino acids in the -3 and -1 position relative to the cleavage site. Some variations can been found between the domains of life, and the existing prediction programs have therefore created separate algorithms for eukaryotes and prokaryotes, and many also differentiate between Gram-negative and Gram-positive bacteria. The prediction algorithms are trained on protein sequences from a large number of species within these groups.

Because of their unique cell wall, mycobacteria do not comfortably fall into either of the Gram stain-based categories. They have traditionally been grouped together with Gram-positive bacteria, and mycobacterial proteins have been part of the training sets of prediction algorithms created for this group. Over the last 20-30 years, experimental work by several laboratories has established the mature sequence of several secreted and somatic proteins in a variety of mycobacterial strains. Many of these were found to be cleaved at the N-terminal, and were therefore truncated compared to the annotated protein sequence. These findings provided us with an excellent opportunity to create validation sets to test the performance of available signal peptide algorithms on mycobacterial proteins. In total, we found 57 proteins with the experimentally verified signal peptides and cleavage sites, and we also created a set of 61 proteins that we knew were not cleaved. The amino acid sequences from these proteins were submitted to 9 different signal peptide prediction algorithms, which were subsequently ranked according to performance on the positive and negative set.

The best performer was SignalP v3.0 hidden Markov model. This algorithm correctly identified the proteins that harbour a signal peptide, and the proteins that do not, and it also correctly predicted the cleavage site for 45 of the 57 secreted proteins. In fact, the second best performing algorithm was the previous version, v2.0, of the same

algorithm. This version also correctly predicted the presence or absence of signal peptides, but it only found the correct site of cleavage for 39 of the proteins. As already stated, an essential part of creating well performing prediction algorithms is to train them on a carefully chosen training set. The evolvement of the SignalP hidden Markov model from version 2.0 to 3.0 is a good example of this. The only improvement made between the two versions was to remove dubious sequences from the original training set to create a more solid set with verified sequences and cleavage sites. The result, as measured using our validation sets, was an improvement in cleavage site prediction from 68.4% to 78.9%. The training set of this algorithm is made up of protein sequences from a broad range of Gram-positive organisms, where each species is represented by only a handful of protein sequences. As mentioned above, we were uncertain if it was correct to group mycobacteria alongside Gram-positive organisms due to their unique cell wall, and if the signal peptide prediction consequently would suffer due to differences in signal peptide structures. As it turns out, the detection of signal peptides in our validation set was faultless, but with an accuracy of 78.9%, there seems to be room for improvement for the cleavage site prediction. However, this inaccuracy does not appear to be unique to mycobacterial protein sequences. When the developers of the SignalP v3.0 hidden Markov model cross validated their algorithm using sequences from Gram-positive bacteria in their training set, they found the cleavage site prediction accuracy to be 81.6%, which is quite close to our result. This cross validation also revealed that the Neural network algorithm actually performed better than the hidden Markov model, as this predicted 85% of the cleavage sites correctly. In comparison, the prediction accuracy of this algorithm was only 50.9% in our validation set. In conclusion, both the algorithm architecture and the choice of sequences used to train it, is important for the performance on protein sequences from a given organism. By using validation sets consisting of mycobacterial protein sequences, we identified the best available algorithm for finding signal peptides and their cleavage sites in this organism. However, for other species, a different algorithm might perform better, and it is only

through work such as ours the optimal algorithm can be found for any given organism.

We decided to use SignalP v3.0 hidden Markov model to analyse all the available protein annotations from different mycobacterial species that were available at the time, including two annotations of the same *M. tuberculosis* H37Rv strain. Between 10.8% and 13.3% of the proteins were predicted to harbour a signal peptide. Interestingly, the number of predicted signal peptides were higher in one of the *M. tuberculosis* H37Rv proteome annotations, at 13% in the Sanger annotation, compared to the TIGR annotation where 11% of proteins were found to have a signal peptide. Finding more of these conserved features, which the N-terminal signal peptide represents, suggests the Sanger annotation have performed better with regard to correctly predicting the N-terminal starting point of proteins. Indeed, a study by de Souza *et al* (2008) have confirmed that the Sanger annotation is the better annotation of the two (52).

As mentioned in chapter 3.2.1, the general secretory pathway is also used to integrate proteins with signal peptide into the membrane. Characteristic features of most membrane proteins are hydrophobic regions that can span the cell membrane, often referred to as transmembrane regions. To make an estimate of the number of proteins that were secreted rather than integrated in the membrane, we used a second algorithm for predicting transmembrane regions downstream of the signal peptide cleavage site. As a consequence, between 25% and 30% of proteins with a predicted signal peptide were removed from the list of potentially secreted proteins.

To conclude, we showed there was quite a variation in the performance between programs for identifying protein processed through the general secretory pathway and their respective cleavage sites. Our results showed that the SignalP v3.0 hidden Markov model scored top marks for predicting the presence or absence of signal peptides in our positive and negative data sets, and it was the best algorithm for predicting the exact peptide cleavage site.

10.2 Proteins with predicted signal peptides enriched in the membrane fraction

In paper I, we predicted the number of secreted proteins from several mycobacterial proteomes based on the following assumption: Proteins with signal peptides that did not contain a transmembrane region in the mature amino acid sequence were regarded as secreted. The premise was thus: all proteins that meet these criteria are processed through the general secretory pathway, the signal peptide is cleaved off and removed from the mature protein, and that the protein is subsequently released to the exterior of the cell. This was a deliberate simplification; we knew that the prediction for some of these proteins might be incorrect, but furthermore, we were aware of two studies that had characterised the membrane fraction of *Bacillus subtilis* and found that it contained 31 proteins with a characteristic N-terminal signal peptide sequence (266,267). Unfortunately, the authors of these articles had not convincingly shown that these proteins were not contaminants from the cytoplasm, neither did they determine if the predicted signal peptides had been removed. In our group, we had previously characterised proteins in the membrane fraction of *M. tuberculosis* H37Rv, where we found 37 proteins with predicted signal peptides that had not been removed (54). We now wanted to further characterise these proteins to identify any characteristic features that might explain why these proteins are enriched in the membrane rather than secreted.

To address the issue of possible contamination from cytosolic proteins, we also did a proteomic analysis of a whole cell lysate of *M. tuberculosis* H37Rv and determined the relative concentration of these proteins in the respective samples. This way, a protein identified in the membrane fraction, but with a higher relative concentration in the whole cell lysate, would be regarded as a soluble protein and a contaminant of the membrane sample. This turned out to be the case for 7 of the 37 proteins. The other 30 proteins, however, were found to be truly enriched in the membrane fraction. For proteins such as these, the hydrophobic property of the signal peptide itself might be required for retention in the membrane, in which case the inability of the signal

peptidase to access and process the cleavage site is important for the correct localisation. The question is why these cleavage sites are not processed by the peptidase. Structurally, they are similar to signal peptides in proteins that are known to be cleaved, but we did notice that an alanine in the -3 position relative to the predicted cleavage site was less conserved for the uncleaved proteins, where more variation was observed. We also noticed that some of these proteins had a predicted transmembrane region that encompassed the cleavage site, which might therefore become unavailable to the signal peptidase located on the cytosolic side of the membrane. However, neither of these factors explains why all 30 proteins were retained in this fraction.

10.3 BCG strain variation and its influence on the course of infection in mice

Bioinformatic tools and proteomic analysis of *in vitro* cultures will by themselves tell us little about the *in vivo* expression of proteins, and how their expression might contribute to virulence throughout infection. Experimental infection in mice is a well-established tool to investigate *in vivo* behaviour of mycobacteria and host responses. As mentioned in chapter 6, BCG strains have been divided in groups based on their phylogenetic distance to the originating BCG strain that were created at the Pasteur Institute by Calmette and Guerin in 1921. It has been argued that strains phylogenetically closer to the original BCG strain are more virulent and therefore also more potent as vaccines, compared to the strains that have diverged more. However, studies comparing vaccine efficacy of different strains have not been conclusive with regard to this aspect. We therefore decided to infect mice with BCG strains at either end of this spectrum, the “early” strain, BCG Russia, and the “late” strain, BCG Pasteur, to describe if the relatively minor differences in genetic make-up would influence the course of infection in mice.

We selected a commonly used mouse strain as our animal model of choice. The C57BL/6 strain is considered relatively resistant to mycobacterial infections. This is

at least partially attributed to the gene called natural resistance-associated macrophage protein 1 (*nramp1*), which appears to be absent from highly susceptible murine strains (268,269). The C57BL/6 strain is also considered to initiate pro-inflammatory Th1-type of responses following mycobacterial infections. The mice were infected intranasally to deliver the bacteria directly to the lungs, and we followed the course of infection for almost one and a half year. Compared to the dose of infection, the number of bacilli dropped during the first months of infection for both strains. This was expected, as the fall in bacillary load has been described previously for BCG strains (270,271). However, while the number of BCG Pasteur stabilised at a low number, BCG Russia got cleared from the lungs by 7 months infection. Furthermore, we found more macrophage- and lymphocyte infiltrates in the lungs of mice infected with BCG Pasteur, compared to mice infected with BCG Russia. Since BCG Pasteur was more persistent and induced more pathology in mice than BCG Russia, it can be considered as the more virulent strain of the two in our infection model. There is no doubt that BCG Pasteur has undergone more genetic changes than BCG Russia, compared to the original BCG strain. Since these changes have arisen through cultivation in artificial growth medium, it would be a reasonable assertion that BCG Pasteur has become more attenuated than BCG Russia. Analyses of genomes from several BCG strains have indeed revealed the loss of gene regions that are associated with virulence in mycobacterial species (37). However, other alterations have lead to duplication of genes, and at least in one case, the loss of a gene repressor caused increased expression of the affected proteins (37,272). Among the proteins found to be up-regulated in late strains are antioxidants, possibly to detoxify by-products of aerobic metabolism during *in vitro*-cultivation (273). However, increased expression of antioxidants also improves bacterial survival inside host cells by suppressing oxidant-dependent immune responses. In line with this, Jain and colleagues found that a recombinant BCG Denmark overexpressing iron-cofactored superoxide dismutase resulted in reduction of apoptosis and microbiocidal potential of macrophages, and reduced production of IL-10 and INF-γ, compared to

the wild type strain. Furthermore, the recombinant BCG strain conferred less protection against *M. tuberculosis* in guinea pigs and mice (274).

As BCG Russia disappeared from the mouse lungs, so did the macrophage- and lymphocytes aggregates. However, the bronchus-associated lymphoid tissue that was formed following the initial infection persisted until the end of the experiment for both BCG Russia and BCG Pasteur. These lymphoid follicles are believed to be important for T-cell priming and B-cell education in the host defence against respiratory pathogens. However, the cytokine environment in this tissue is thought to shape immune responses that limit immunopathology, perhaps to stimulate prolonged and persistent priming of lymphocytes, rather than pro-inflammatory responses that can stimulate bactericidal activities of lymphocytes and antigen presenting cells. This might be why this tissue has been found to be a reservoir of latent infection with latent herpes virus MHV-68 (275). Regulatory T-cells is a lymphocyte population with immunosuppressive properties, and might contribute to the anti-inflammatory BALT environment. Following infections with *M. tuberculosis* in mice, these cells have been found in all lymphocyte aggregates, including BALT (153). By staining regulatory T-cells using antibodies against the transcription factor FoxP3, we found that this was also the case for mice infected with BCG strains. A persistent BALT that continuously prime lymphocytes with antigens from pathogens it has been exposed to, might facilitate a rapid and protective response upon re-infection. This can be particularly advantageous in epidemic situations where the risk of re-exposure is high. On the other hand, suppression of immunopathological responses are considered harmful for many infectious diseases, including tuberculosis, and mice that are depleted of regulatory T cells have been shown to better control *M. tuberculosis*-infection than their wild type counterparts (153). We found no evidence for prolonged and persistent presence of bacilli in BALT in our mice, and BALT was present in all mice even if no bacilli could be cultivated from their lungs. However, after 3 months of infection, there was a significant correlation between the percentage of BALT and detectable CFU from lungs of mice infected with BCG Russia. The

situation was different from mice infected with BCG Pasteur, where the CFU correlated with both macrophage- and lymphocyte dominant areas. Perhaps this indicates that the bronchi-associated lymphoid tissue might be a last refuge for the bacteria before they are completely cleared from the lungs.

10.4 *In vivo* mycobacterial protein expression in BCG-infected mice

Despite a significant difference in the ability to persist in the mouse lungs, there was a high degree of similarity between the two BCG strains with regard to staining patterns of polyclonal antibodies specific to selected mycobacterial proteins. For both strains, the ratio of stained cells was as expected much higher in macrophage-dominant lesions than the other areas investigated. Even if the infiltrates induced by mycobacterial infections in mice are less structurally defined than those found in humans, the aggregation of macrophages represents the primary site for bacillary presence during infection. In conclusion, we were able to show that the bacilli produced both somatic and secreted proteins during *in vivo* infection, and that the level of expression was similar between the two strains. Proteins expressed by a metabolically active BCG after vaccination, and during its relatively short-lived intradermal infection, represent the primary source of immunogens that will provide protection against subsequent exposure to a pathogenic mycobacterium (253).

We made an unexpected and interesting observation in the normal lung parenchyma. Immunohistochemistry revealed cells that carried a heavy bacterial antigenic burden. This not only suggest an inability of the host cells to eliminate the infection, but also that they seem incapable of alerting other immune cells of the on-going infection. These observations illustrate the ability of mycobacteria to survive the hostile environment of the cell, and to turn off, or not trigger, signalling from the infected cell to its surroundings. There are many who argue that *M. tuberculosis* can enter a dormant state during infection, a feature that has been linked to the establishment and maintenance of latent tuberculosis. In this scenario, the bacilli are thought to almost

cease all metabolic activity, and it has also been suggested they take on a form where they are devoid of many of its antigens, and thus become almost invisible to the host. Our observations are different. The bacteria in these particular cells seemed to both replicate and to be metabolically active as evidenced by strong staining of both somatic and secreted proteins. Without the aid of other immune cells, the infected cells are deprived of the stimuli they require to fight the bacteria, which in turn might provide the bacilli a safe harbour where they can replicate and persist for a prolonged time. These cells might have been invaded by the bacilli at the time of initial infection, and have remained unnoticed by the immune system since then. Alternatively, bacteria from established foci might have escaped their confinement and infected more distantly located cells. Finally, it is possible that infected cells have migrated from infiltrates to healthy regions of the lung. To our knowledge, observations of host cells with a heavy bacterial burden in normal areas of the lung during mycobacterial infections have not been previously described.

10.5 *In vivo* expression of mycobacterial proteins in human lungs and lymph nodes

The investigation of protein-specific staining patterns in the lungs of mice infected with either BCG Pasteur or BCG Russia revealed only minor differences between the two strains. However, when we investigated expression of somatic and secreted proteins in human lung and lymph nodes infected with *M. tuberculosis*, we made several interesting observations. The secreted proteins were present at high levels in all granulomas in lungs, but the staining was markedly less frequent in granulomas in the lymph nodes. Here, these proteins were predominantly found in the necrotic centres, and less so in the granuloma cells. A well-structured granuloma is a correlate of effective immune responses that control the infection and limit bacterial growth. In the lungs, the granulomas had the appearance of disorganised infiltrates, they did not contain necrotic centres, and the bacillary load was high. Granulomas in the lymph nodes, however, had a well-organised granuloma structure with necrotic centres, and

seemed to more efficiently restrict bacillary multiplication. The latter explains why only low levels of secreted proteins were stained within these structures. The discrete stained spots in the necrotic centres might represent cells that contain multiplying bacilli that express higher amounts of secreted proteins, but that are gradually subjected to cell lysis and necrosis. Finding low levels of secreted proteins in well-structured granulomas calls into question their importance as antigens during infection, and, by extension, if they should remain a primary focus in the development of diagnostic tools and vaccines. Proteins expressed in abundance during *in vivo*-infection are preferential targets of the cellular immune response, but if the expression is drastically reduced then so is the specific T-cell activation (276). Furthermore, evaluations of antibodies in sera from patients with tuberculosis suggest that mycobacterial surface proteins are more frequently recognised than secreted proteins (277,278). Studies in both humans and guinea pigs have revealed a lack of immune responses towards the secreted mycobacterial protein MPT53 (258), which also suggests that expression of secreted proteins is reduced during stable disease.

The situation may vary between secreted mycobacterial antigens. MPT64 was detected in all granulomas, and therefore seem to be retained within the granuloma cells, rather than being gradually cleared away. In our group, we previously showed that MPT64 was found more frequently in granulomas compared to non-granulomatous inflammation, even if mycobacterial DNA was detected more frequently in the latter areas (279). In mice infected with *M. tuberculosis*, the bacillary load stabilises after an initial phase of multiplication. When the mice eventually become moribund, this is correlated to increased pathology rather than an increase in the bacterial burden (280). The change in pathology might be caused by the gradual accumulation of proteins such as MPT64, which at some point reach a threshold where they trigger exaggerated immune responses that in turn causes tissue damage.

The mycobacterial *mce1* operon is associated with granuloma formation. Strains that are disrupted in this operon do not induce formation of organised granulomas, and

have been found to be more virulent than their wild type counterparts, in murine lungs (281). This is attributed to increase in pathology rather than bacillary load, which was similar between the strains. Our findings on one of the proteins in this operon, Mce1A, are in line with such studies. In the disorganised infiltrates in pulmonary lesions, Mce1A was detected less frequently compared to secreted proteins, and it was primarily cells surrounding the inflammatory infiltrates that stained positive for this protein. In contrast, Mce1A was found in all cases within organised granulomas in the lymph nodes, and the stain was predominantly found in cells of the granuloma, rather than necrotic centres. Both pathogen and host likely favour the formation of the granulomas during tuberculosis disease. For the host it constitutes a protective structure of bacillary containment. For the bacteria, the granuloma represents a stable structure in which it can survive for many years without causing fatal pathology to the host. The expression of Mce1A might therefore be a way for mycobacteria to induce formation of granulomas in the early stages of infection. However, for a long-term survival by way of infecting new individuals, the bacteria are eventually required to escape this enclosure. As mentioned above, it is perhaps the build-up of proteins like MPT64 that eventually facilitates the exaggerated immune response that cause destruction of tissue and release of bacilli into the bronchial tree.

It is a challenging task to study the *in vivo* expression of mycobacterial proteins, yet it is important and relevant for understanding how this organism can adapt to and influence the host responses during infection. What we know so far concerning the mycobacterial proteome is largely derived from studies of bacilli grown in culture. Some studies have attempted to alter the growth conditions to reflect the stimuli during infection in the host, for example by removing oxygen from the medium and thereby simulating conditions found in the caseum in the centre of the granuloma. However, such alterations only reflect certain aspects of the environment, and the conditions affecting the intracellular existence of the bacilli are far more complex in real life. The primary challenge for characterising mycobacterial proteins expressed

in vivo, is the influence of host proteins that by sheer number and quantity far outweighs proteins expressed by the bacilli. The modern technologies are not yet optimised and sensitive enough for unbiased analyses of such complex and target-rich samples. This holds true for both microarray-based and proteomic studies, although some recent attempts within both fields have been published. For example, transcriptome analyses have been performed on bacilli that have been isolated from the lungs of mice (282) and human tuberculosis patients (283), but the complexity of such models and the need to first isolate heterogeneous groups of bacilli from the source material, do not give us information about the specific context of protein expression. This also holds true for attempts to generate an *in vivo* proteomic profile. Kruh and colleagues performed proteomic analyses of lungs from infected guinea pigs, and were able to identify a little more than 500 mycobacterial proteins (284). Although promising, this is only one-fifth of what can be identified from bacteria grown in cultures (59). Identification of proteins by immunohistochemistry is limited by the antibodies available, expression can only be measured using semi-quantification evaluation, and the method is quite laborious. However, it provides us with the opportunity to relate the specific protein expression to a particular context by correlating the staining pattern and relative staining intensity to features of the host pathology.

11. Conclusions

The characterisation of the proteome and the dynamics of protein expression is key to understand how the mycobacterium succeeds so well in inhabiting its host. It has different means to enter host cells, and have several ways to evade their killing mechanisms. It seems to influence the formation of the granuloma to limit the extent of damage to the host, but it can also disrupt this structure to escape the boundaries of its host and infect new individuals. Secreted proteins, and proteins exposed on the surface of the bacilli, are the primary mycobacterial structures to interact with the host, and they become the most important immunogens during infection. It is therefore of utmost importance to understand when and where these proteins are expressed, and to identify their correct localisation. These are also key aspects when selecting candidates for vaccine- and drug development, and to find appropriate markers for diagnosing tuberculosis.

This work has investigated various aspects of mycobacterial protein expression and localisation, and the following conclusions can be drawn:

- The SignalP v3.0 hidden Markov model was found to the best performing algorithm for identifying signal peptide sequences and the respective cleavage site in mycobacterial proteins. This algorithm correctly identified the presence, or absence, of a signal peptide in the sequences of positive and negative validation sets, however, the cleavage site prediction can be improved. For characterisation of proteins secreted by the general secretory system in mycobacteria, this algorithm is currently the best option.

- Label-free quantitation identified proteins that were predicted to harbour a signal peptide, but which were found to be uncleaved and enriched in the membrane fraction. Components of the general secretory system are likely to target and integrate these proteins into the membrane, but for unknown reasons they are not processed by the signal peptidase. An inaccessible cleavage site,

and differences in signal peptide structure, can explain why some, but not all, cleavage sites were not processed. Another possible explanation is that amino acid substitutions in the -3 position in the AXA motif have a bigger penalty for mycobacterial proteins. The retained signal peptide might in itself function to retain the protein within the membrane fraction, and might influence both their function and structure.

- The late BCG strain, BCG Pasteur, was more persistent and caused more pathology than the early strain, BCG Russia, in the lungs of C57BL/6 mice. This may be linked to an up-regulated expression of protective proteins such as antioxidants in BCG Pasteur. We confirmed the expression of both somatic and secreted proteins by immunohistochemistry, and the staining pattern was very similar between the two strains. Single cells with a high mycobacterial antigen load were detected in the normal lung parenchyma, which may reflect the potential of mycobacteria to modulate host killing responses and to limit their ability to signal other immune cells of the ongoing infection.

- The protein expression pattern in human lungs and lymphoid tissue infected with *M. tuberculosis* revealed differences seemingly related to the structural integrity of the granuloma. In the lungs, the granuloma was loosely formed and contained a high amount of secreted mycobacterial antigens. In contrast, the lymphoid tissue contained granulomas with a well-organised structure and necrotic centres, and few secreted proteins were found within host cells. The exception was MPT64, which were identified in all granulomas. Well-structured granulomas are a correlate of protective immune responses, which could lead to a down-regulated expression of mycobacterial protein. In these regions, secreted proteins seemed to be rapidly degraded and/or transported away. In contrast, MPT64 appeared to accumulate, which might imply a role for this protein in persistence of infection. The mycobacterial protein Mce1A was found in all well-structured granulomas, and to a lesser extent in disorganised infiltrates. This is in line with other reports linking the *mce*-

operon to granuloma formation, and it iterates a shared interest of the host and bacterium to limit the extent of pathology.

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