Electron microscopy reveals treatment options for pathogenic microbes

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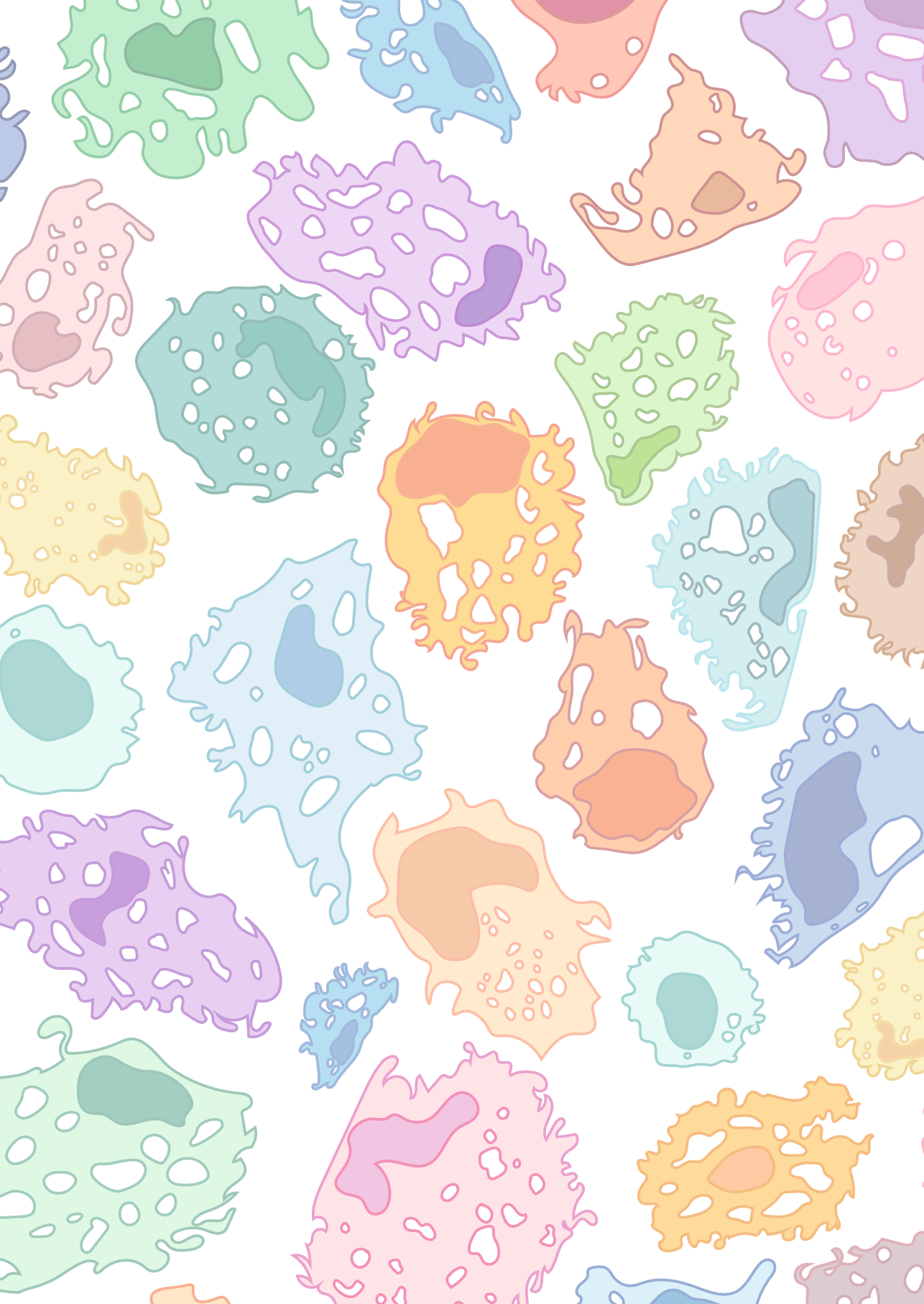

Chapter 1

General introduction

Tuberculosis

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (Mtb), where pulmonary infection is the most common manifestation of disease. Infection with Mtb can cause active or latent infection. Common symptoms of acute pulmonary tuberculosis are cough, sputum with blood, chest pain, weakness, weight loss, fever and night sweats. Mtb infection can occur in other organs; for example, lymph node infections are the most common extra-pulmonary infections (Prakasha *et al.*, 2013). Mtb has infected humans for at least 70,000 years and in this period Mtb has adapted to the changing human population (Comas *et al.*, 2014). Despite tremendous progress in the medical field, the TB problem is not solved, as in 2021, Mtb is estimated to have caused 1.6 million deaths (WHO, 2022b). A complicating factor is the global prevalence of latent Mtb, which is recently estimated to be around 21-25% of the total world population (Houben and Dodd, 2016; Cohen *et al.*, 2019). During latent infection, a slow growth rate but continuous mutation rate of Mtb was observed (Colangeli *et al.*, 2014). The risk of reactivation of latent tuberculosis was the highest until 5 years after primary infection and was higher in patients that did not complete antibiotic treatment (Erkens *et al.*, 2022). Human immunodeficiency virus (HIV) infection is the strongest risk factor for reactivation of latent tuberculosis (Horsburgh *et al.*, 2001). The current widely used vaccine to prevent tuberculosis is *Mycobacterium bovis* bacille Calmette Guèrin (BCG), which is a non-pathogenic mycobacterium. This vaccine strain was developed more than 100 years ago and is still used to protect children in areas with high tuberculosis burden despite its variable and likely very low efficiency (0-80%) in adults (Ahmed *et al.*, 2021). Currently, there is no universally effective drug treatment and current regimes still require months-long therapy with multiple agents. Current antibiotic strategy to cure TB consists of 2 months treatment with Ethambutol, Isoniazid, Pyrazinamide and Rifampicin, followed by 4 months of Rifampicin and Isoniazid (Peloquin and Davies, 2021). Despite this elaborate treatment regime, the treatment success rate is only 60% in drug resistant patients, who still have stubbornly high death rates from TB disease (WHO, 2022b). In addition to a low treatment success rate, the COVID-19 pandemic reversed years of progress in treating tuberculosis patients due to limited access to TB care (WHO, 2022b). Between 2020 and 2021 the TB incidence rate increased by 3.6% instead of a yearly 2% decline in the past 2 decades (WHO, 2022b).

Mycobacterium abscessus

Another pathogenic mycobacterium studied in **chapter 2** of the thesis is *Mycobacterium abscessus*. This is a rapidly growing non-tuberculous mycobacterial species, that it is able to form visible colonies in less than 7 days (reviewed in Medjahed, Gaillard and Reyrat, 2010). The species *M. abscessus* consists of 3 subspecies: *abscessus*, *bolletii* and *massiliense*. Despite its rapid growing phenotype, *M. abscessus* shares features with the slow growing Mtb like latency and granuloma formation, unlike other rapid growing mycobacteria (reviewed in Medjahed, Gaillard and Reyrat, 2010). *M. abscessus* can cause a wide range of different infections in skin, soft tissue and lungs (Brown-Elliott and Wallace, 2002). Infection with *M. abscessus* is an emerging thread for cystic fibrosis patients (Martiniano, Nick and Daley, 2019). In contrast to Mtb, infection can potentially occur through exposure to *M. abscessus* in domestic and

hospital water sources (Thomson *et al.*, 2013), and it was shown for cystic fibrosis patients that infection can occur from environmental sources and through human transmission (Bryant *et al.*, 2016). Like Mtb, *M. abscessus* is extremely resistant to antibiotics (Nessar *et al.*, 2012). For example, in cystic fibrosis patients, treatment showed a success rate of only around 33% (Kwak *et al.*, 2019). A treatment regime with more than 2 antibiotics based on susceptibility of the *M. abscessus* strain, is recommended to treat infections (Daley *et al.*, 2020). A major factor in designing the treatment regime is the expression of *erm(41)*, this gene causes resistance to macrolides that are widely used for *M. abscessus* treatment (Nash, Brown-Elliott and Wallace, 2009). *M. abscessus* can grow in a smooth or rough colony variant, and the colony morphology is dependent on the expression of surface lipids (Howard *et al.*, 2006; Pawlik *et al.*, 2013). The rough colony forming variant is more virulent than the smooth colony variant, as it has the ability to cause persistent and more invasive infections (Howard *et al.*, 2006). Further, the smooth and rough colony forming *M. abscessus* are targeted differently by the immune system in adult zebrafish (Kam *et al.*, 2022).

Antibiotic resistance

Mycobacteria have a whole repertoire of mechanisms to increase their antibiotic resistance in order to survive within the host. These can be classified as intrinsic resistance and acquired resistance. Intrinsic resistance mechanisms reside in the multilayer cell envelope, consisting of the cell wall and capsular layer of Mtb (Nasiri *et al.*, 2017). Other pathogenic mycobacteria such as *M. abscessus* also form hydrophobic non-permeable first defense mechanism that prevent antibiotics to reach the cytosol (Nessar *et al.*, 2012; Nasiri *et al.*, 2017). In addition, mycobacteria have inducible or acquired resistance mechanisms. When antibiotics reach the cytosol, efflux pumps are able to pump antibiotics out of the bacterium (Nessar *et al.*, 2012; Nasiri *et al.*, 2017; Mudde *et al.*, 2022). In addition, Mtb is able to go in a dormant state with a reduced metabolism, which causes decreased killing of drugs that act on Mtb metabolism genes (Gengenbacher and Kaufmann, 2012). Very little is known about factors that control the dormancy of *M. abscessus*, but it does contain the DosR regulon, that is required for the transition to a dormant state in response to stress (Gerasimova *et al.*, 2011). In addition, this regulon is required for growth of *M. abscessus* under hypoxia (Simcox *et al.*, 2023). Another strategy to achieve antibiotic resistance is to modify or degrade the antibiotic that reaches the cytosol. *M. abscessus* produces enzymes that can modify of degrade antibiotics, which has been shown for Rifampicin and macrolide resistance (Nessar *et al.*, 2012). The presence of antibiotic modifying enzymes is also observed for Mtb (Nasiri *et al.*, 2017). Acquired resistance is also achieved through genetic mutations in the targets of antibiotics. In Mtb, genetic mutations that result in antibiotic resistance for the first line drugs Isoniazid, Rifampicin, Ethambutol and Pyrazinamide are known and even some mutations to gain resistance to second line drugs are observed (Nasiri *et al.*, 2017). Genetic mutations that lead to antibiotic resistance are also found in *M. abscessus* for aminoglycosides, macrolides and fluoroquinolone (Nessar *et al.*, 2012). These data demonstrate that it is extremely difficult to design an effective antibiotic to treat mycobacterial infections.

Nucleoid associated proteins and DNA organization

A potential target to treat mycobacterial infections is interfering with the organization of the DNA. When mycobacteria encounter stress conditions, such as starvation and antibiotic stress, the DNA condenses as a response to protect it from damage (Scutigliani *et al.*, 2018). Mycobacterial DNA is organized by nucleoid associated proteins. These proteins are involved in protection of the DNA and regulation of genes (Hołówka and Zakrzewska-Czerwińska, 2020). Mycobacteria have multiple nucleoid associated proteins that each have their own distinct roles. For example, HupB is involved in antibiotic resistance towards Rifampicin and Isoniazid, where it acts by regulating expression of efflux pumps and the permeability of the cell wall (Singh *et al.*, 2022). In **chapter 2** and **3** of the thesis, we focus on the nucleoid associated protein Lsr2. This protein binds A-T rich DNA and is involved in DNA bridging and regulation of genes (Gordon *et al.*, 2010; Qu *et al.*, 2013; Kołodziej *et al.*, 2021). By influencing gene expression, Lsr2 plays a role in antibiotic resistance, synthesis of cell wall components, adaptation to unfavorable conditions and virulence of Mtb (Colangeli *et al.*, 2007, 2009; Gordon *et al.*, 2010; Bartek *et al.*, 2014). In *M. abscessus*, Lsr2 is upregulated in the more virulent rough colony forming variant and it is required for virulence in both cells and animal infections (Le Moigne *et al.*, 2019). Interestingly, inhibition of Lsr2 by the asthma drug Zafirlukast results in killing of Mtb and *M. smegmatis* (Pinault *et al.*, 2013). Thus, Lsr2 is a promising target as a new therapeutic for mycobacterial infections.

***Mycobacterium tuberculosis* virulence**

Mtb is a pathogen with various strategies to survive in the host macrophages. An important strategy is the subcellular behavior of mycobacteria, which is studied in **chapters 4 and 5**. Intracellular adaptation of Mtb to the host results in multiple mechanisms to enhance Mtb survival. More than 20 years ago the difference between non-pathogenic vaccine strain *M. bovis* BCG and Mtb was determined by a genomic screen and a major difference between the two mycobacteria is the presence of region of differentiation 1 (RD1) in Mtb (Behr *et al.*, 1999). This and other genetic regions of deletion (RD) contribute to Mtb virulence (Lewis *et al.*, 2003). Mutations in the RD1 region result in loss of export of virulence factors ESAT-6 and CFP-10 (Guinn *et al.*, 2004). Mtb with mutations in a part of the RD1 region is able to grow within macrophages but is not able to spread to other macrophages, which causes attenuated infection in mice (Guinn *et al.*, 2004). The ESX-1 secretion system plays an important role in multiple defense mechanisms. Mtb prevents fusion of phagosomes with lysosomes, resulting in a less acidic environment (Armstrong and D'Arcy Hart, 1971; Sturgill-Koszycki *et al.*, 1994), this is dependent on the presence of the ESX-1 secretion system (MacGurn and Cox, 2007; Brodin *et al.*, 2010). Interestingly, direct contact of the bacterium and phagosomal membrane is required to prevent acidification of the phagosome (de Chastellier *et al.*, 2009). In addition, virulent Mtb impairs the autophagic flux by preventing fusion of auto-phagosomes with lysosomes through ESX-1 in dendritic cells (Romagnoli *et al.*, 2012). Induction of autophagy results in more acidic phagosomes and promotes maturation of mycobacteria containing phagosomes, this results in decreased mycobacterial survival (Gutierrez *et al.*, 2004). ESAT-6 reduces autophagic

degradation in macrophages (Dong *et al.*, 2016). Another virulence factor of Mtb is its complex multilayer cell wall and capsular layer.

Since the cell envelope is in contact with the host, it plays an important role in host-pathogen interactions (reviewed in Jackson, 2014). The cell envelope of Mtb has a specific structure containing multiple layers, the unique composition results in a highly impermeable, asymmetric membrane that contributes to the antibiotic resistance of Mtb (reviewed in Jackson, 2014). The outermost part of the mycobacterial cell envelope is the capsular layer with a thickness of around 30nm, this layer is shown to contain the host activating factor like α-glucan, phthiocerol dimycocerosates and ESX secreted proteins (Sani *et al.*, 2010). When the capsular layer is absent, an increase in pro-inflammatory cytokines was observed compared to intact mycobacteria (Sani *et al.*, 2010). This demonstrate that the capsular layer can be considered a candidate virulence factor of mycobacteria.

Mycobacterial escape from the phagosome

To survive in the hostile environment of the host, pathogenic mycobacteria rupture the lysosome to reach the nutrient rich cytosol (Figure 1). This process was visualized in early studies using transmission electron microscopy by Leake *et al.*, 1984; Myrvik, Leake and Wright, 1984; Mcdonough, Kress and Bloom, 1993 and later by van der Wel *et al.*, 2007. This phagosomal escape is dependent on the ESX-1 secretion system (van der Wel *et al.*, 2007; Houben *et al.*, 2012) that is absent in vaccine strain BCG. When the ESX-1 secretion system was reintroduced in BCG, the mycobacterium was again able to rupture the phagosome and access the cytosol in THP-1 cells (Houben *et al.*, 2012; Simeone *et al.*, 2012). This also works the other way around, Mtb was not able to reach the cytosol when the ESX-1 secretion system was deleted from the genome (Simeone *et al.*, 2012) and even deletion of 1 secreted component (CFP10) can abolished escape (van der Wel *et al.*, 2007). Phthiocerol dimycocerosates (PDIM) and EsxA (ESAT-6) together are required to induce phagosomal damage and rupture, since PDIM mediates ESAT-6 ability to permeabilize the phagosomal membrane (Augenstreich *et al.*, 2017). When PDIM is knocked out in Mtb, less bacteria are able to reach the cytosol (Lerner *et al.*, 2018). As a response to a damaged phagosomal membrane, galectins are recruited to the phagosome to target the phagosomes for autophagy (Thurston *et al.*, 2012; Bell *et al.*, 2021). However, Mtb is able to block the autophagic flux, causing reduced autophagy (Figure 1.2) (Romagnoli *et al.*, 2012; Dong *et al.*, 2016). When Mtb is able to reach the cytosol, increased replication was observed compared to that seen within phagosomes (van der Wel *et al.*, 2007). The subcellular localization of Mtb was not only important for replication, as the intracellular localization of Mtb is important for the efficacy of the first line drug pyrazinamide (Santucci *et al.*, 2021). If the ESX-1 secretion system is knocked out the drug was more efficient in killing Mtb (Santucci *et al.*, 2021). These data indicate that ESX-1 mediated escape of pathogenic mycobacteria is an important virulence factor even though the balance between escape and immune responses are not completely understood.

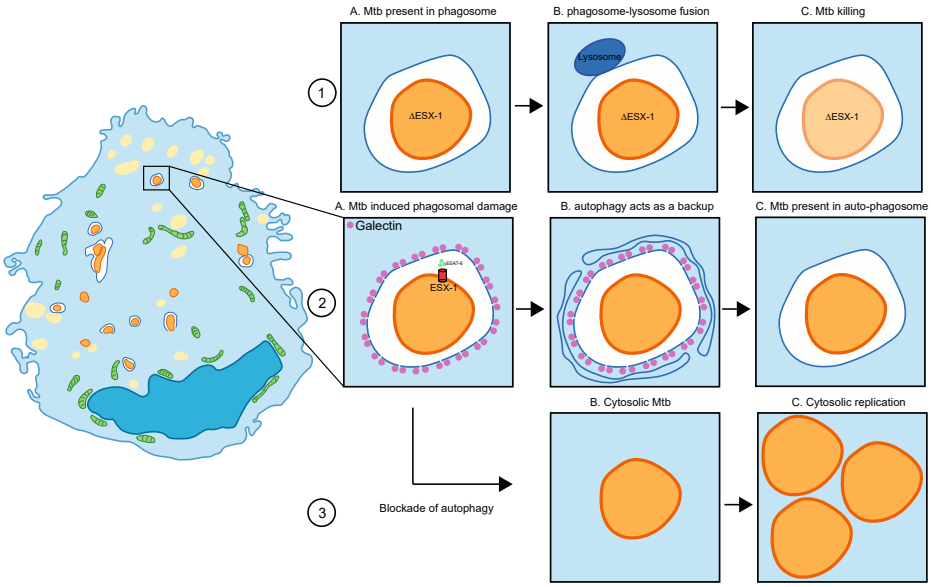

Figure 1: schematic representation of regulation of escape in a Mtb infected macrophage. When Mtb is present in a phagosome 3 options for subcellular localization are present. Option 1, when the ESX-1 secretion system is lacking, the phagosome matures and fuses with a lysosome. This promotes a lower pH and Mtb killing. Option 2, when a functional ESX-1 secretion system is present, Mtb can permeabilize the phagosomal membrane, which leads to recruitment of galectins to the damaged membrane and autophagy is initiated. These effects result in maturation of the phagosome with Mtb into an autophagosome. Option 3, when Mtb is able to block autophagy, autophagy is not initiated and Mtb is able to reach the cytosol and replicates. Lipid accumulation is indicated in yellow, mitochondria in green, Galectin in pink, lysosomes in dark blue, ESX-1 in red, ESAT-6 in light green, Mtb in orange and host membranes in blue.

The role of the immune system on Mtb control

In **chapter 4** of the thesis, the role of the host immune system to control phagosomal escape is studied. Both the innate and adaptive immune system promote defense against Mtb infection (reviewed in de Martino *et al.*, 2019). TB is the leading cause of death in people with a defective immune system, like for example human immunodeficiency virus-1 (HIV) patients (WHO, 2022b). In HIV infected patients many immune factors are impaired: $CD4^+$T cells are depleted and $CD4^+$ T cells, $CD8^+$ T cells and natural killer cells have a reduced function, which also causes reduced macrophage defense (reviewed in Beck, 2005). In addition, reactivation of latent tuberculosis can occur through treatment with immune checkpoint inhibitors through dysregulation of TNF-α (Langan *et al.*, 2020; Tezera *et al.*, 2020). The immune response to control Mtb infection is complex and not yet completely understood. When Mtb enters the human host, Mtb encounters alveolar macrophages (Cohen *et al.*, 2018) and primary airway epithelial cells (Reuschl *et al.*, 2017). Primary infection with Mtb occurs in alveolar macrophages that migrate to the lung interstitium where monocyte derived macrophages and neutrophils can be infected (Cohen *et al.*, 2018). In airway epithelial cells, Mtb is present in late endosomes and infected cells are recognized by $CD4^+$ and $CD8^+$ T cells that activate upon recognition (Harriff *et al.*, 2014). When the macrophage is not able to kill Mtb, a granuloma will form.

Granulomas are organized structures of immune cells surrounding and containing the bacteria (reviewed in Pagán and Ramakrishnan, 2018). Granulomas were observed in active, latent and reactivated tuberculosis (Flynn, Chan and Lin, 2011) and are generally protective for the host and evolve during the duration of infection (reviewed in Pagán and Ramakrishnan, 2018). However, in active tuberculosis, the granuloma is not always capable of controlling infection (Flynn, Chan and Lin, 2011).

Cytokines produced by immune cells play an important role in controlling Mtb infection and a key player is interleukin 1 (IL1), which is a pro-inflammatory cytokine produced by various immune cells. IL1 signaling is important to control Mtb infection in mice (Juffermans *et al.*, 2000), and when IL1 signaling is absent, inflammation is reduced in IL1 receptor knockout mice (Labow *et al.*, 1997). When the IL1 receptor was knocked out in mice, the mice die earlier and more mice die than wildtype mice, which indicates an important role in controlling Mtb infection (Sugawara *et al.*, 2001; Mayer-Barber *et al.*, 2014). Furthermore, the IL1 pathways is dysregulated in patients with active tuberculosis (Llibre *et al.*, 2022). IL1 also plays a role in the effectiveness of antibiotics, IL1 together with TNF is required for induced function of Isoniazid (Yamashiro *et al.*, 2016). These data indicate that proper IL1 signaling is important in Mtb control, and thus in **chapter 4** the effect of this cytokine on subcellular trafficking is studied.

Mtb has multiple mechanism to modulate the host immune response. Mtb can reduce macrophage MHC class 1 antigen presentation function by binding of ESAT-6 to beta-2-microglobulin in the endoplasmatic reticulum and thereby reduce macrophage antigen display (Sreejit *et al.*, 2014). In addition, an intact $CD4^+$ T cell response is needed to clear Mtb, these T cells are activated by dendritic cells. Mtb is able to infect dendritic cells and impair their antigen presenting function (Wolf *et al.*, 2007). Furthermore, regulatory T cells have an immunosuppressive effect by suppressing the proliferation of effector $CD4^+$ T cells. In blood and broncheo-alveolar lavage of tuberculosis patients the levels of $CD4^+$ regulatory T cells are elevated, this results in a decrease in the ability of monocyte derived macrophages to restrict Mtb growth (Semple *et al.*, 2013).

(Intra-bacterial) lipid storage

Lipid filled 'foamy' macrophages are a hallmark of Mtb infection (Peyron *et al.*, 2008; Russell *et al.*, 2009). Proteomics showed that live infection with Mtb affects protein abundance of lipid droplet associated proteins in macrophages (Menon *et al.*, 2019). Lipid droplets are lipid storage organelles that have a mono-layer membrane and are coated by perilipins. *In vitro,* it was shown that in macrophages and granulomas that Mtb but not *M. smegmatis* causes induction of lipid accumulation, this is caused by pathogenic mycobacteria specific oxygenated mycolic acids (Peyron *et al.*, 2008). Lipid droplets and Mtb containing phagosomes can interact with each other, which is driven by mycobacterial cell wall lipids and is dependent on Rab7 mechanisms (Roque *et al.*, 2020). The interaction of Mtb with lipid droplets is also observed using electron microscopy (Peyron *et al.*, 2008). Live cell imaging experiments showed that lipid droplets are in close proximity to Mtb shrink in size over time, suggesting utilization of

lipids form the lipid droplets by Mtb (Greenwood *et al.*, 2019). Lipid droplets potentially have also another role during Mtb treatment. Correlative light, electron and ion microscopy showed that the antibiotic Bedaquiline accumulates in lipid droplets of Mtb infected macrophages (Greenwood *et al.*, 2019). These data suggest a potential role of lipid droplets in Mtb treatment.

Also inside the bacteria lipids are stored as 'intra-bacterial lipids inclusions' (ILI). Multiple studies showed that Mtb imports fatty acids from foamy macrophages to synthesize triacylglycerol (TAG) (Daniel *et al.*, 2004; Nazarova *et al.*, 2017). The presence of intra-bacterial lipid inclusions is linked to non-replicating persistence both in vitro and in patient isolates (Garton *et al.*, 2008; Rodríguez *et al.*, 2014; Vijay *et al.*, 2018). However, intra-bacterial lipid inclusions can form in the absence of macrophages (Knight *et al.*, 2018). TAG synthesis is important for the following processes: energy storage during dormancy and reactivation (Low *et al.*, 2010; Galagan *et al.*, 2013), arrest of growth and gain of antibiotic resistance by reducing the carbon flux (Baek, Li and Sassetti, 2011). Mtb has a protein with weak similarity with human perilipin-1 (Daniel *et al.*, 2016). This protein is required for uptake and accumulation of host lipids, dormancy and Rifampicin resistance (Daniel *et al.*, 2016). When TAG synthesis was reduced, Mtb was more susceptible to antibiotics during mice infection (Baek, Li and Sassetti, 2011). This indicates that uptake of lipids by Mtb and the formation of ILI can be a potential target for Mtb drug development.

In **chapter 5** we study the Mtb specific lipid 1-tuberculosinyladenosine (1-TbAd). This lipid was discovered in a comparative lipidomics screen between Mtb and *Mycobacterium bovis bacille Calmette Guèrin* by Layre *et al.*, 2014. The enzyme Rv3378c is required for 1-TbAd synthesis (Layre *et al.*, 2014). Rv3377c was shown to act as a diterpene synthases and is required for the production of Tuberculosinol and (13R,S)-Isotuberculosinol (Nakano *et al.*, 2011). In a screen to detect Mtb mutants that are able to inhibit the fusion of phagosomes with lysosomes, Rv3378c was found to inhibit phago-lysosomal acidification (Pethe *et al.*, 2004). The effects of 1-TbAd were studied on macrophages and it was observed that 1-TbAd is responsible for around 1% of the total lipids production of Mtb and it is able to raise the phagosomal pH since it acts as a basic lipid in an acid environment (Buter *et al.*, 2019) (Figure 2). When the gene for Rv3378c was placed in *Mycobacterium kansasii*, this bacterium was able to raise the pH, showing clear gain of function (Buter *et al.*, 2019). Using transmission electron microscopy, we previously showed that treatment with pure 1-TbAd remodels the lysosomes dramatically in macrophages and that infection with a Rv3378c mutant did not cause the foamy phenotype in infected macrophages (Buter *et al.*, 2019) (Figure 2). These findings identify 1-TbAd as a host-activating virulence factor, which now emerges as a potential drug target.

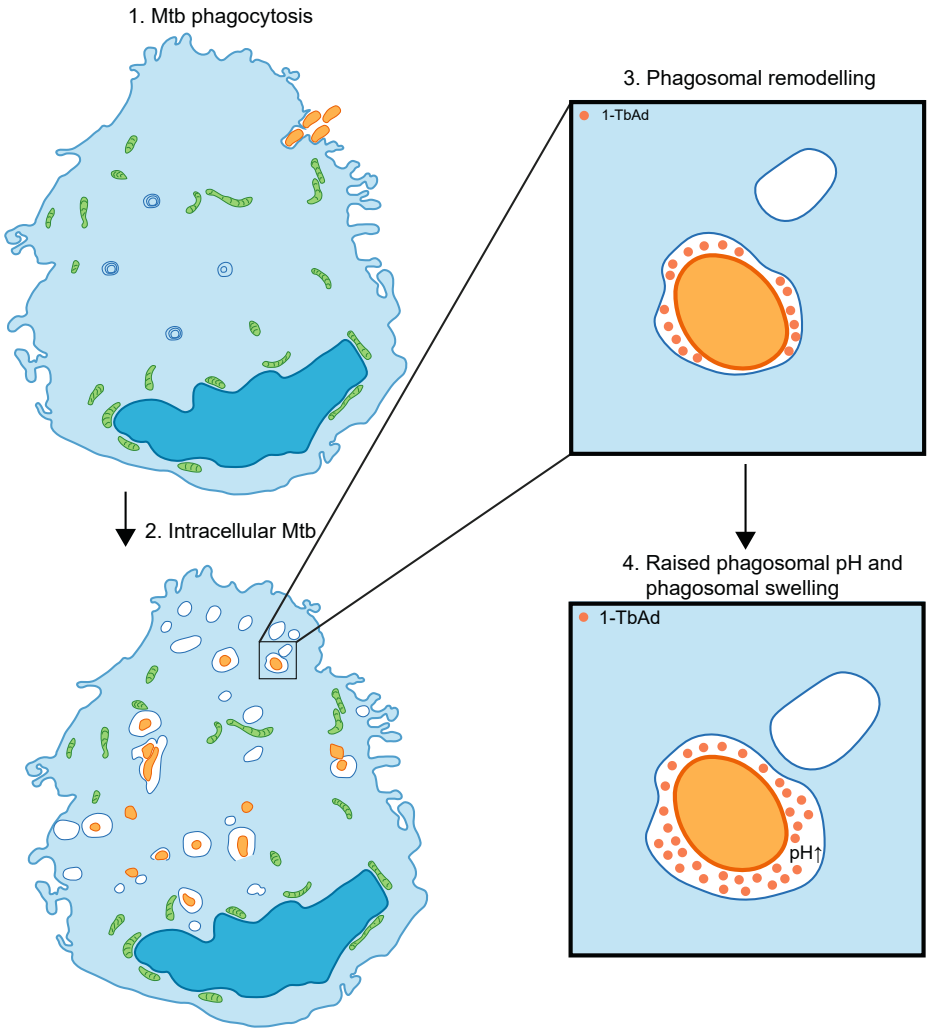

Figure 2: Schematic representation of 1-TbAd induced phagosomal remodeling. 1. Mtb is taken up by macrophages. **2.** During infection, Mtb releases 1-TbAd in the surrounding phagosome and it also affects the surrounding phagosomes. **3-4.** Upon exposure to 1-TbAd, the phagosomal pH rise and phagosomes increase in size. Mtb in orange, mitochondria in green, host membranes in blue, swollen phagosomes in white and 1-TbAd in light pink.

COVID-19

In addition to mycobacteria we also studied the effect of SARS-CoV-2 on the host in **chapter 6** and **chapter 7**. We started this research since the pathology department in our hospital analyzed material from postmortem and post-COVID-19 patients and our techniques used to study mycobacteria are ideal to apply to COVID-19 and visualize the viral proteins in high detail and resolution. Infection with SARS-CoV-2 was first discovered in December of 2019 in Wuhan and identified using polymerase chain reaction, sequencing and electron microscopy

(Zhu *et al.*, 2020). The genome of SARS-CoV-2 has around 79% homology with SARS-CoV-1 and 50% with MERS-CoV, and this homology was considered low enough to classify it a new virus (Lu *et al.*, 2020). It was demonstrated that SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor to enter the human cells (Zhou *et al.*, 2020). The ACE2 receptor is present on the surface of lung epithelial cells, enterocytes of the small intestine, arterial and venous endothelial cells and arterial smooth muscle cells in various organs (Hamming *et al.*, 2004). The presence of ACE2 in lung epithelia and small intestine explains the routes of entry of SARS corona viruses. Patients with lung disease, heart disease, obesity, diabetes and elderly people have an increased risk of severe COVID-19 symptoms (Gao *et al.*, 2020). In postmortem samples from COVID-19 patients, virus was found in the lungs, heart, upper respiratory tract, kidney and gastrointestinal tract (Schurink *et al.*, 2020). In addition, inflammation was found in lung, heart, liver, kidney and brain (Schurink *et al.*, 2020). SARS-CoV-2 consists of membrane protein, envelope protein and spike protein in the membrane and the RNA in the core is packed in a helical structure by the nucleocapsid protein (de Haan and Rottier, 2005). The membrane protein, envelope protein and nucleocapsid protein are required for assembly, trafficking and release of virus particles (Siu *et al.*, 2008). Upon a few passages in Vero cells, SARS-CoV-2 is able to acquire mutations in the spike protein (Ogando *et al.*, 2020). Interestingly, an increase in lipids was observed in cells infected with SARS-CoV-2 and in lung tissue of COVID-19 patients (Nardacci *et al.*, 2021). This is also observed in Mtb infected macrophages (Peyron *et al.*, 2008, **chapter 5**). Therefore we studied in **chapter 6** the presence of viral proteins and lipids in lungs of fatal COVID-19 patients.

Around 10-20% of the patients that had acute SARS-CoV-2 infection develop post-COVID-19 syndrome (WHO, 2022a). Symptoms of post-COVID-19 syndrome are cognitive dysfunction, sleep disorder, altered smell and taste, cough, shortness of breath, chest pain, fatigue and muscle pain (WHO, 2022a). Another emerging symptom after COVID-19 infection is acute kidney injury (Batlle *et al.*, 2020). SARS-CoV-2 is able to infect the human kidney (Farkash, Wilson and Jentzen, 2020) and infection induces fibrosis in kidney organoids and acute tubular injury in lethal COVID-19 (Su *et al.*, 2020; Jansen *et al.*, 2022). The incidence of kidney dysfunction was high in COVID-19 patients and acute kidney injury was associated with the severity of the COVID-19 infection (Yang *et al.*, 2020). Interestingly, it was shown that urinary levels of nucleocapsid is associated with the risk of developing acute kidney injury and the severity of COVID-19 infection (Tampe *et al.*, 2021). The nucleocapsid protein of SARS-CoV-2 can regulate innate immune responses, a low dose suppresses interferon signaling and inflammatory cytokines, whereas a high dose induces these cytokines (Zhao *et al.*, 2021). These data indicate a potential role of nucleocapsid in the severity of COVID-19 infection and the development of acute kidney injury. In **chapter 7** the presence of viral proteins is studied in kidney biopsies of post-COVID-19 patients.

Aims and layout of the thesis

The aim of the thesis is to use electron microscopy to carry out detailed morphological studies of microbes and microbially infected cells to identify physiological events or vulnerabilities

in microbes that can be exploited therapeutically. To achieve this goal, we looked at different strategies of mycobacteria to survive in the host. The thesis gives an insight in the importance of host immune response on subcellular localization of mycobacteria and we studied repurposing of an anti-asthma drug to treat mycobacterial infections. Furthermore, the interplay between Mtb and host lipid metabolism was studied to identify the mechanisms of lipid accumulation leading to classical foamy macrophage formation. In addition to mycobacterial infections, we studied COVID-19 during the worldwide pandemic emergency, since the techniques used in our lab can study the effects of COVID-19 on patients in high detail.

Chapter 1 gives a general overview of the topics addressed in the thesis. In **Chapter 2** we study interfering with nucleoid associated protein Lsr2 using the anti-asthma drug Zafirlukast on *M. abscessus* affects DNA organization and the ability to condense its DNA. In addition, we monitored survival of Zafirlukast treated *M. abscessus* over time. With transmission electron microscopy we studied if Zafirlukast has an effect on morphology of the bacterium at multiple time points. After studying the effect of Zafirlukast on *M. abscessus* we focused in **Chapter 3** on inhibition of Lsr2 by Zafirlukast as a synergetic drug with first line antibiotics to treat Mtb. First we monitored the effect of Zafirlukast as a synergistic drug on *Mycobacterium smegmatis*. We studied the ability to remodel the DNA upon antibiotic stress and the survival over time. Thereafter, we studied whether Zafirlukast had an additional effect on Rifampicin in killing Mtb and if the subcellular localization of Mtb was altered. After determining the effects of Zafirlukast on multiple mycobacterial species we focus in the next 4 chapters on the interaction of host and pathogen for both mycobacteria and SARS-CoV-2. In **Chapter 4** we observe the subcellular localization of mycobacteria *in vivo* using multiple animal models to determine whether the bacteria were able to escape to the cytosol using transmission electron microscopy. The role of the immune system on controlling phagosomal escape was determined by using IL1 receptor knockout mice and we compared the subcellular localization in zebrafish embryo to adult zebrafish. In **Chapter 5** we study the effect of Mtb specific 1-TbAd on macrophage morphology and determined its role in infection. Using fluorescent microscopy, electron microscopy and correlative light and electron microscopy we monitored the lysosomal morphology and lipid accumulation over time in both 1-TbAd treated and Mtb infected macrophages. In addition, the lysosomal and lipid droplet morphology was determined to understand the role of 1-TbAd in foamy macrophage formation. Last, we show a potential inhibitor for the effects of 1-TbAd on macrophages. In **Chapter 6** we search for components of the SARS-CoV-2 virus both in infected Vero cells and lung tissue of patients who died from COVID-19. Using both fluorescent microscopy and transmission electron microscopy we examined structural and non-structural proteins of the virus in both samples. In addition, we searched for the accumulation of lipids. After testing various anti-corona-virus antibodies and developing a strategy to image COVID-19 patient samples, we searched for virus and viral proteins in kidney in **Chapter 7**. We compared samples from post-COVID-19 patients that developed acute kidney injury months after infection with patients that did not develop kidney problems. **Chapter 8** discusses the findings of the thesis and **Chapter 9** and **Chapter 10** give a summary and highlights the main conclusions of the thesis.

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