Host-directed therapy for the treatment of tuberculosis: rewiring the host to recover control

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CHAPTER 1

General Introduction

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Introduction

The battle between humankind and pathogenic organisms is a never-ending one and has profoundly shaped our history. Numerous pandemics have ravaged humans and animals alike and their incidence has not decreased [18]. Contrarily, the frequency of disease outbreaks and their diversity are expected to grow steadily due to increased

population size and global travel and greater exploitation of natural resources [19]. For example, the recent pandemic of coronavirus disease 2019 (COVID-19) has claimed the lives of at least 7 million people (**Figure 1A**). Although this loss of lives is unimaginable, various pandemics have killed numbers of people several orders of magnitude larger (**Figure 1A**). Tuberculosis (TB) is estimated to have caused the death of more than a billion people throughout human history. Almost 3 persons per minute succumb to TB, roughly the same number as Measles, (non-)Typhoid fever, Malaria and Influenza combined (**Figure 1B**). Clearly, solutions for this ongoing pandemic are needed.

Mycobacteria

The causative agent of TB in humans and animals is a member of the *Mtb* complex (MTBC), of which *Mycobacterium tuberculosis* (*Mtb*), *Mycobacterium africanum* and *Mycobacterium bovis* are the most notable members. The family of Mycobacteriaceae, genus of Actinobacteria, contains close to 200 different species [20]. The second major human mycobacterial infectious disease is leprosy, which is caused by the bacterium *Mycobacterium leprae*. Some species from the non-tuberculous mycobacteria (NTM) such as *Mycobacterium avium* (*Mav*) and *Mycobacterium abscessus* (*Mab*) additionally cause disease and can be a major cause of death in the immunocompromised host, e.g. untreated AIDS patients [21]. The prevalence of these opportunistic pathogens is currently increasing and they are a major morbidity factor in patients with pre-existing lung conditions [22].

Mycobacteria are aerobic, generally rod-shaped bacteria and vary in size between 1 and 10 µm. *Mtb* is a nonmotile bacterium, although for the species *Mycobacterium marinum*, a natural fish pathogen which can cause opportunistic infections in humans such as aquarium granuloma, intracellular motility has been observed [23]. Interestingly, they are not classified as gram-positive or gram-negative since they are

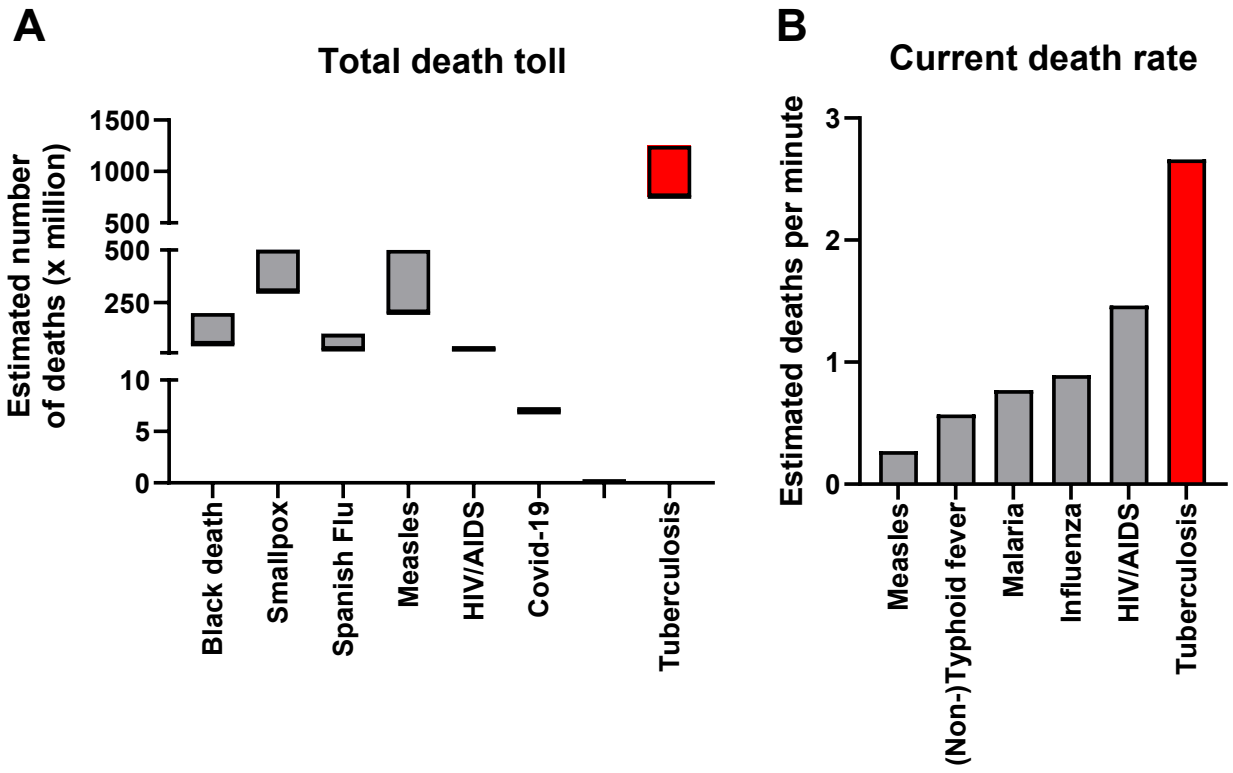

Figure 1. Deadly pandemics and their causative pathogens

A. Estimated total death toll of several pandemics, past and ongoing. Floating bars depict the spread of estimations [1-9]. **B.** Estimated current death rate of the deadliest infectious pathogens. Bars depict the estimated number of deaths per minute [10-16].

poorly stained due to the arrangement of their cell wall which is much thicker than in most other bacteria [24]. The *Mtb* cell wall starts from the inside with a cross-linked network of peptidoglycan bound to the cytoplasmic membrane, which is followed by highly branched arabinogalactan polysaccharide and ends with long-chain mycolic acids. This all is enveloped by a membrane segment containing lipids, lipoglycans, and proteins. The intricate design of the mycobacterial cell wall creates a barrier almost fully impermeable to exogenous sources. One of the

most surprising characteristics of *Mtb* is its long division time (between 16 and 20 hours), which is much slower than for other pathogenic bacteria. For example, *Salmonella enterica* serovar Typhimurium (*Stm*) and *Salmonella enterica* serovar Typhi, pathogenic bacteria causing non-typhoid fever and typhoid fever, respectively (**Figure 1B**), can have a doubling time as short as 20 minutes [25].

Pathogenesis Of Tuberculosis

Transmission of TB occurs mostly when the bacterium is spread by particles coughed up by infected individuals suffering from active TB. The bacterium-containing droplets, mostly aerosols, are then inhaled and internalized into the alveoli of a new host [26]. Active TB can be caused by either primary infection or due to reactivation of latent TB (LTBI). The former happens when the immune system fails to defend itself against *Mtb* which occurs in only a small minority (5-10%) of the cases, while reactivation of LTBI is defined as the asymptomatic continuous containment of mycobacteria [27, 28]. Current estimates are that around 25% of the world's population is infected with *Mtb* [29]. Although reinfection can be a large source of active TB in highly endemic areas, in low endemic areas the majority is thought to be due to progression of LTBI to active TB [30, 31]. It is not difficult to imagine that the enormous reservoir of LTBI represents a major obstacle for the eradication of TB.

Clinical Aspects Of Tuberculosis

The most common symptom of active TB is a persistent cough, but symptoms such as fever, night sweats and weight loss are also frequent. Most patients suffer from pulmonary disease, however, TB can manifest in every organ [32]. By now various techniques are used for the diagnosis of TB, including chest X-ray, sputum smear examination by microscopy and molecular confirmation using quantitative polymerase chain reaction (qPCR) [33]. Additional genotyping or functional testing is applied for the identification of drug resistance. Currently, still an estimated 30% of worldwide cases remain undetected due to underdiagnosis and underreporting [16].

Although everyone can develop TB, there are certain risk factors increasing the risk for developing active TB significantly. The five most prominent of these factors are undernourishment, smoking, alcohol abuse, HIV infection, and diabetes mellitus (DM) [16]. The latter represents an increasingly growing problem since currently close to half a billion people have DM and this number is expected to keep rising [34], while TB and DM worsen each other's clinical outcome [35]. Also, the coinfection of HIV and *Mtb* is very problematic. HIV-infected patients have a higher risk of progressing from latent TB to active TB, which is the greatest cause of mortality in these patients [16].

Vaccines For Tuberculosis

The attenuated strain of *Mycobacterium bovis*, Bacille Calmette-Guérin (BCG), is so far the only licenced vaccine since its introduction in 1921. It is given early in life (neonates, young children) by a single intradermal injection and while being the most widely used vaccine, it only partially protects against disease. Nevertheless, it is still recommended for all countries with a high burden of TB or leprosy [16]. Because of the

lack of protection against pulmonary TB in adults when given early in life, numerous endeavours are being undertaken to find either new vaccines or improved versions of the current one [36]. A recent analysis of a trial with the recombinant protein vaccine M72/AS01$_E$ showed around 50% protection against progression to active TB in people with LTBI, lasting for a period of at least 3 years [37]. This encouraging approach of vaccine design is worthy of exploring further. The search for an effective vaccine is however, significantly hampered by the absence of known immune correlates or biomarkers of protective immunity, thus necessitating long and resource-demanding follow-up trials [38].

Experimental Models For The Study Of Tuberculosis

Models that display some or all the pathological processes observed in humans are needed to help understand TB pathogenesis and development and, in addition, to facilitate the testing of new drugs and vaccines in a translatable setting. Three main model categories can be distinguished, *in silico* (computer simulation), *in vivo* (in whole living organisms) and *in vitro* (outside biological context). Although most of the work in this thesis has been performed *in vitro*, *in silico* and *in vivo* studies have been employed additionally in chapter 1 and chapters 3, 5 and 6, respectively.

In Silico Models

Most of the *in silico* models are used for computer aided drug design (CADD). Programs are designed to either discover new ligands for a structurally known target protein or combine the knowledge on known ligands of an uncharacterized target to predict a new ligand [39]. Some models combine both approaches [40]. With the current advances in data availability, structural resolution and computational power, these methods are expected to yield new candidate drugs in a swift manner. A recent example of this is the prediction of the so-called molecule Halicin, which shows high antibacterial activity *in vitro* [41]. One other use of *in silico* models in the TB field is the simulation of disease kinetics for example in virtual lungs [42], in virtual granulomas [43] and between multiple physical compartments, such as the lung, lung draining lymph node and blood [44]. Importantly, results from these models can explain experimental observations *in vivo* which demonstrates their usefulness [45].

In Vivo Animal Models

Most animals used for TB research are mice [46, 47]. They are versatile, cost-effective and numerous strains with specific genetic modifications exist. A major drawback, which is that they are not a natural host for *Mtb*, is compensated by the availability of diverse mouse strains that allow elements of TB pathogenesis to be studied individually and the availability of a wide range of research tools [48]. Contrarily, guinea

pigs are more susceptible to TB, can be infected by a low aerosol dose and are considered a more relevant model for human TB [49]. Although already used in the 19th century, rabbit models are since recently also implemented more often because they were found to carry mycobacteria naturally [50]. Although infrequently used, rat TB models exist as well [47].

Furthermore, cattle and goats are the natural host of the pathogen *Mycobacterium bovis* (*Mb*) that is the source of our current only vaccine BCG. Bovine TB is still a large problem in developing countries and is a challenge to animal health and major cause of economic loss [51]. Because transmission from cattle to humans has been reduced enormously since the pasteurization of milk and BCG vaccination effectively prevents disseminated *Mb* infection, cow or milk transmitted *Mb* infection in humans is nowadays rarely diagnosed [52]. As a model, cattle and goats are well suited for the validation of vaccine efficacy [47, 53]. Recently, the use of minipigs as experimental model for TB has gained attention due to their high resemblance to humans in terms of physiology, immune system and the spectrum of TB that they develop [54, 55].

There exist also two invertebrate models using fruit flies and zebrafish. Although innate immunity is well conserved in fruit flies, adaptive immunity is lacking and mycobacteria are not their natural pathogens [47]. Zebrafish *(Danio rerio*) on the other hand have as natural pathogen *Mycobacterium marinum* (*Mm*) and show quite similar disease progression and pathology compared to TB in humans [47, 56]. They form granulomas and are transparent in their embryonic and larval stage of development allowing highly detailed microscopy studies on complete organisms [57].

The model genetically, physiologically and immunologically most close to humans is the non-human primate (NHP) model. NHPs suffer from mycobacteria in nature and are typically used for validating vaccine and treatment induced responses [58]. Rhesus macaques and cynomolgus macaques are the most commonly used species and display slightly different TB pathology due to their different susceptibility to TB [59]. Ethical concerns, high costs, low availability and the significant investment in facilities necessary, however, restrict the use of these models.

In Vitro Models

Compared to *in vivo* models, *in vitro* models are significantly cheaper, easier to use, short-cycled and they allow the screening of large chemical and genetic libraries. The simplest models use cell lines, derived from either human or murine origin, which are easy to obtain and are a good option for exploratory work. Experimental work using cell lines is generally highly reproducible, although cell lines tend to be genetically unstable resulting in subclones and hybrid phenotypes.

The RAW264.7 and J774 macrophage cell lines, both derived from BALB/c mice, are frequently used, though they come with some limitations. First of all, BALB/c mice are traditionally deemed fairly resistant to TB and how this relates to the Mϕ response is unclear [60]. Secondly, the transcriptional response of J774 upon *Mtb* infection clearly differs from primary murine Mϕs [61]. Human cell lines, such as THP-1, U937 and A549, represent an alternative option. The human monocytic leukaemia cell line THP-1 and lymphoma patient derived monocyte cell line U937, can, using phorbol 12-myristate 13-acetate (PMA), be differentiated into a macrophage like cell that shows a similar phenotype upon *Mtb* infection compared to primary human Mϕs [62, 63]. However, the necessary stimulation with PMA perturbs major intracellular signalling pathways and could be a major confounder in the interpretation of both fundamental and drug efficacy studies. Additionally, THP-1 cells miss certain surface receptors, including the mannose receptor important in *Mtb* phagocytosis, which also represents a caveat [64]. Furthermore, phagocytosis and control of mycobacterial growth of U937 cells differ significantly from primary Mϕs [65]. Other more infrequently used options include human lung epithelial cell lines like A549 cells [66].

Primary Mϕs, either human or murine, are mostly used to confirm results obtained from cell line models, since they are physiologically more relevant but also more difficult to access. An advantage of obtaining murine Mϕs is that numerous genetic mutant mouse strains exist, allowing the straightforward dissection of specific protein functionality in TB. Human cells on the other hand can be relatively easily obtained in large quantities from buffy coats by differentiating monocytes extracted from human peripheral blood mononuclear cells (PBMCs) in the presence of certain stimulating factors [46]. A disadvantage however is that relative long culture times and differentiation are needed, likely causing differences from tissue-resident cells *in vivo*. Additionally, the interpretation of results is often complicated by the genetic variation that underlies the use of human donors. Ideally, alveolar macrophages (AMϕ) would be used, but these are difficult to obtain owing to lengthy ethical approval, the procedure and costs. The Max Planck Institute (MPI) cells represent another interesting option [67]. Of murine embryonic origin, they are self-renewing tissue-resident Mϕs and display a response that is functionally very close to AMϕs [68].

Currently, more intricate models like *in vitro* granuloma models are being advocated for since they allow the study of host-pathogen interactions in a 3-dimensional (3D) organization, which is important since the immune response is “3D organized” in granulomas as well [69]. *In vitro* granuloma models can be generated by either placing peripheral blood mononuclear cells (PBMCs) combined with *Mtb* in an extracellular matrix with a 3D structure as result [70], by infecting existing lung tissue models [71], or by encapsulating cells in microspheres [72]. Interestingly, these models can also be

employed to study latent *Mtb* infection either by forcing a latent phenotype in the bacterium [70] or by using cells from LTBI individuals that in an *in vitro* granuloma model have been shown to control *Mtb* infection better than cells from naïve individuals [73]. Next to generating granulomas *in vitro*, a deconstructive approach also exists wherein mature murine granulomas are dissociated that allows drug testing in an elaborate model containing diverse cell populations, which is even amenable to high-throughput screening (HTS) [74]. Currently, organ-on-a-chip technology is becoming increasingly elaborate and accessible and will in the near future likely give rise to better and easier approaches to manipulate *in vitro* 3D TB models [75].

Amoeba species such as Acanthamoeba and *Dictyostelium discoideum* represent an interesting unicellular eukaryotic model of infection. Amoebae and mycobacteria were found to share environments and can have close interactions [76]. Interestingly, Amoebae can phagocytose mycobacteria for the purpose of nutrient acquisition, however, various mycobacterial species can survive this process and even proliferate [77]. For this reason amoebae could represent the mycobacterial environmental reservoir and might have significantly contributed to the evolution of mycobacterial mechanisms of host evasion, particularly those displayed intra-macrophage [77].

The Macrophage And Its Environment

When Élie Metchnikoff discovered the macrophage and its phagocytic capabilities in 1882, he introduced the concept of cell-mediated immunity. Now we know that Mϕs are among the first actors of the immune system and that they can display a huge variety of different behaviours. Additionally, organs have their own specialized Mϕs referred to a resident-tissue macrophages (RTMϕs), for example, the liver has Kupffer cells, the brain microglia, the epidermis Langerhans cells and the lung airways lumen AMϕs, mentioned earlier [78] (**Figure 2**). While most hematopoietic cells derive from hematopoietic stem cells, these RTMϕs originate mostly from embryonic precursor cells and they maintain their numbers through self-renewal with just a small contribution from blood monocytes [79].

The lung mononuclear phagocyte system

In the lung there is a complex mononuclear phagocyte system (MPS) characterized by multiple macrophages besides AMϕs (**Figure 2**). Interstitial macrophages (IMϕs), found in the lung interstitium, have a mixed origin, since they are to a significant extent derived from blood circulating monocytes [80]. They are thought to be important

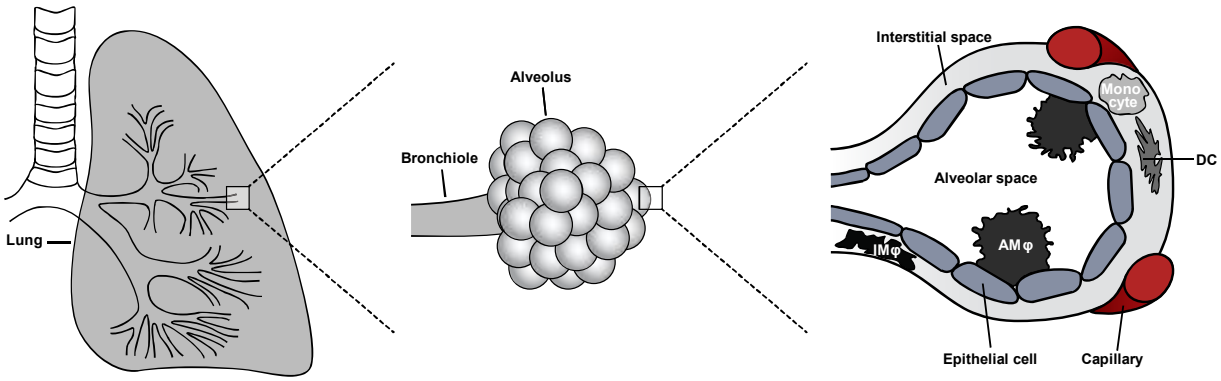

Figure 2. Structural overview of the alveolus

Overview of the lung with detailed cross-section of an alveolus. The alveolar wall consists out of epithelial cells patrolled by alveolar macrophages (AMϕs) and the interstitial space contains interstitial macrophages (IMϕs). The capillaries mitigate, besides their primary purpose of oxygen and carbon dioxide exchange, the transport of additional cells, such as monocytes, dendritic cells (DCs) and neutrophils.

mediators of lung homeostasis and are characterized by the production of the anti-inflammatory cytokine Interleukin 10 (IL-10) in steady-state, which is strongly induced upon inflammatory stimulants such as bacteria-derived Lipopolysaccharide (LPS) [81]. Within the class of IMϕs, at least two distinct populations can be observed, which are conserved between mice and humans [82, 83].

The recently proposed niche model represents our current understanding of RTMϕs [78]. Rather than cellular origin, the local environment is proposed to determine cellular polarization and function. This local environment or niche instructs cells by the release of various factors, the most important being cytokines and metabolites. These factors induce the activity of transcription factors (TFs) that institute the transcriptional and epigenetic profile underlying the core macrophage program [84, 85]. Lineage- and tissue-specific TFs lead to further Mϕ specialization, such as the AMϕ specific catabolic capability of lung surfactant [86]. Interestingly, even oxygen concentration can play a vital role in Mϕ differentiation, since AMϕs that are unable to sense oxygen fail to mature to complete functionality [87]. Furthermore, depletion of both Mϕ types also showed that while IMϕs rapidly reappeared through the swift influx of monocytes, AMϕ numbers took much longer to recover demonstrating that their population kinetics differ [82].

Host-Pathogen Interactions – A Macro Level

The First Phase Of Infection

As mentioned before, infection starts with the phagocytosis of *Mtb* by AMϕs. Contrary to their behaviour in steady-state, the *Mtb*-infected AMϕs then travel from the airway to the lung interstitium thereby spreading the infection, also known as the 'Trojan Horse' phenomenon [88, 89] (**Figure 3**). This process depends on both bacterial and host components, since Interleukin 1 beta (IL-1β) release through the inflammasome pathway, which is activated by the mycobacterial ESX-1 secretion system, was shown to be crucial in this behaviour [88, 90]. Additionally, endothelial cells might play a role in bacterial dissemination by allowing replication and migration of *Mtb* across the barrier [91] (**Figure 3**).

Following these events, a high influx of monocytes into the lung tissue increases IMϕ and DC numbers concomitant with massive neutrophil invasion [92, 93] (**Figure 3**). From the host perspective, this seems sensible because IMϕs display better capabilities in restricting *Mtb* growth, while AMϕs on the other hand are more permissive to *Mtb* replication [92]. Selective depletion experiments confirmed these observations because AMϕ ablation showed decreased bacterial burden, while IMϕ depletion exacerbated infection [92, 94]. The role of neutrophils, however, is more ambiguous since they are known to be permissive to *Mtb* growth, but their absence is detrimental to acute host control of infection [95, 96]. Their highly tissue-destructive nature is likely beneficial in the onset of infection, but becomes harmful in later stages leading to excessive tissue damage [97].

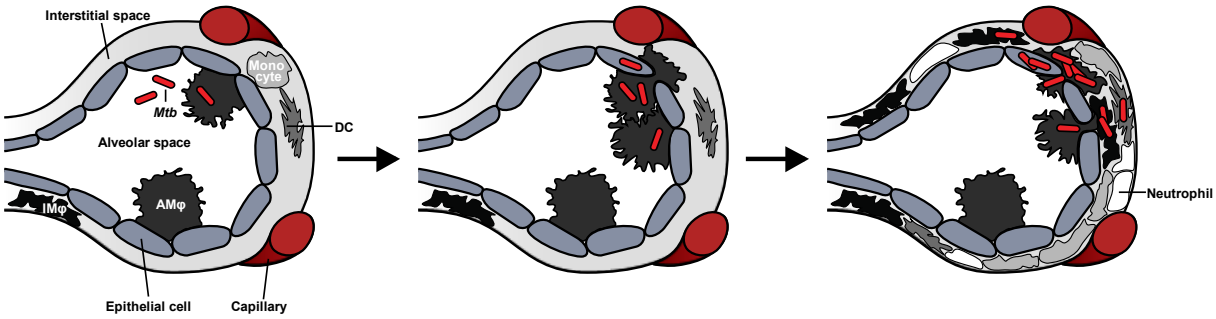

Figure 3. The onset of *Mtb* infection

Mycobacterium tuberculosis (*Mtb*) bacteria enter the alveoli through inhalation and are phagocytosed by alveolar macrophages (AMϕs). Infected AMϕs migrate to the interstitial space and secrete cytokines and chemokines to recruit additional immune cells. A large influx of neutrophils and monocytes from the blood follows, of which the latter differentiate into (among others) interstitial macrophages (IMϕs) and dendritic cells (DCs). *Mtb* proliferates in permissive cells and disseminates [17].

The Granuloma

The next stage of the immune response, adaptive immunity, starts when DCs prime the T-cell response in the lung-draining lymph nodes [98, 99]. The infection now usually transitions into a containment phase and the bacterial burden remains quite static. This phase is characterized by the formation of granulomas, a hallmark of TB. Granuloma formation is a very complex phenomenon and despite decades of research still poorly understood. Although traditionally considered as a host-driven response that works in favour of the host, recent works suggest that this underestimates their possible benefit to the mycobacterium [100]. Indeed, it has been shown that pathogenic bacteria drive granuloma formation as protection against the host response and the cell-to-cell spread of infection [101].

Both the innate and the adaptive immune response, as well as specific mycobacterial components, have been proven to be necessary for the process of granuloma formation [102]. Different models of granuloma formation currently exist but the main consensus is that immune cells of both the innate and adaptive immune response are recruited to the site of infection which results in the organization of a multicellular structure. When this structure is fully formed, it classically develops a necrotic core, rich in lipids and caseous in nature, surrounded by a layer of macrophages (Mϕs) that often contain *Mtb* [103]. Typically, this is enclosed by a layer of matrix proteins and collagen with lymphocytes in the outer periphery [103]. Interestingly, each granuloma develops as its own entity and can display different fates. In some, infection is controlled leading to a so-called sterile granuloma, while in others, bacteria prosper, potentially leading to progression of active disease. What exactly determines this differential outcome in the non-immuno-compromised host is unfortunately still unclear. However, several host factors are implicated such as TNF-α, IL-12 and IFN-γ, since TNF inhibition therapies and genetic defects negatively affecting these factors, called Mendelian susceptibility to mycobacterial disease (MSMD), predispose to TB [104-106].

Host-Pathogen Interactions – In Detail

Phagocytosis

Phagocytosis starts with the recognition of bacterial PAMPs by non-opsonic receptors, including C-type Lectins, Toll-like receptors (TLRs), scavenger receptors and opsonic receptors, such as immunoglobulin G (IgG)-Fc receptors and complement receptor type 3 (CR3) [107, 108]. After pathogen recognition, actin filaments start to polymerize and form a pseudopod engulfing the bacterium. The bacterium is then surrounded by the host cell membrane, which closes at the distal end to form the bacterium containing phagosome. Of note, the type of receptor engaged decides the activation

that follows based on the intracellular signalling domain of the receptor. *Mtb* interferes with host cell activation by modification of its PAMPs or by producing PPR antagonists, for example sulfoglycolipids that inhibit TLR2 activation [109].

To acquire degradative capacity, the phagosome requires maturation by fusion with endosomes and then lysosomes (**Figure 4**). Early phagosomes are characterized by the presence of Rab5 protein, early endosomal antigen 1 (EEA1), soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) and phosphoinositide 3-kinases (PI3Ks) [110]. Rab GTPase are the central regulators of intracellular compartments and determine the fusion partners, i.e. vesicle identity, membrane composition but also vesicle transport [111]. In detail, Rab5 starts the process of maturation by the fusion of the phagosome with endosomal compartments. Through this process, the active form of Rab7 is acquired on the phagosomal membrane that binds Rab7-interacting lysosomal protein (RILP) [112]. This complex promotes the dissociation of Rab5 and further maturation follows via the fusion with lysosomes, thereby creating the phagolysosome [113]. This phagolysosome contains hydrolases, proteases, vacuolar-type ATPases (V-ATPase) that acidify the intravesical environment to a pH to 4.5 - 5.5, and NADPH oxidase complexes (NOXs) that generate ROS [114], all necessary to control and eliminate intracellular pathogens.

Mtb can inhibit this phagosome maturation in different ways. For instance, it can induce Rab5 accumulation that prevents the acquisition of Rab7 and thereby fusion with late endosomes and lysosomes [115]. It can elicit lipoarabinomannan (LAM) mediated suppression of intracellular Ca2+ levels, which halts the accumulation of specific PI3Ks necessary for the continuation of phagosome maturation [116]. Furthermore, the *Mtb* secreted protein tyrosine phosphatase A (PtpA) interferes with the binding of V-ATPase on the phagosomal membrane. Additionally, *Mtb* can activate cytokine-inducible SH2-containing protein (CISH) via STAT5 leading to V-ATPase ubiquitination and degradation [117, 118].

In addition, mycobacterial lipids can disrupt the actin polymerization process essential to form an integral phagosome and furthermore also its migration from the cell periphery to the centre [119]. Using the 6 kDa early secretory antigenic target (ESAT6) protein family secretion (ESX) systems, mycobacteria secrete various proteins that additionally aid the bacterium in blocking the host response. ESAT6 for example can cause phagosomal membrane damage allowing the escape of *Mtb* or its secreted components to the cytosol (**Figure 4**) [120]. The ESX systems are of vital importance in

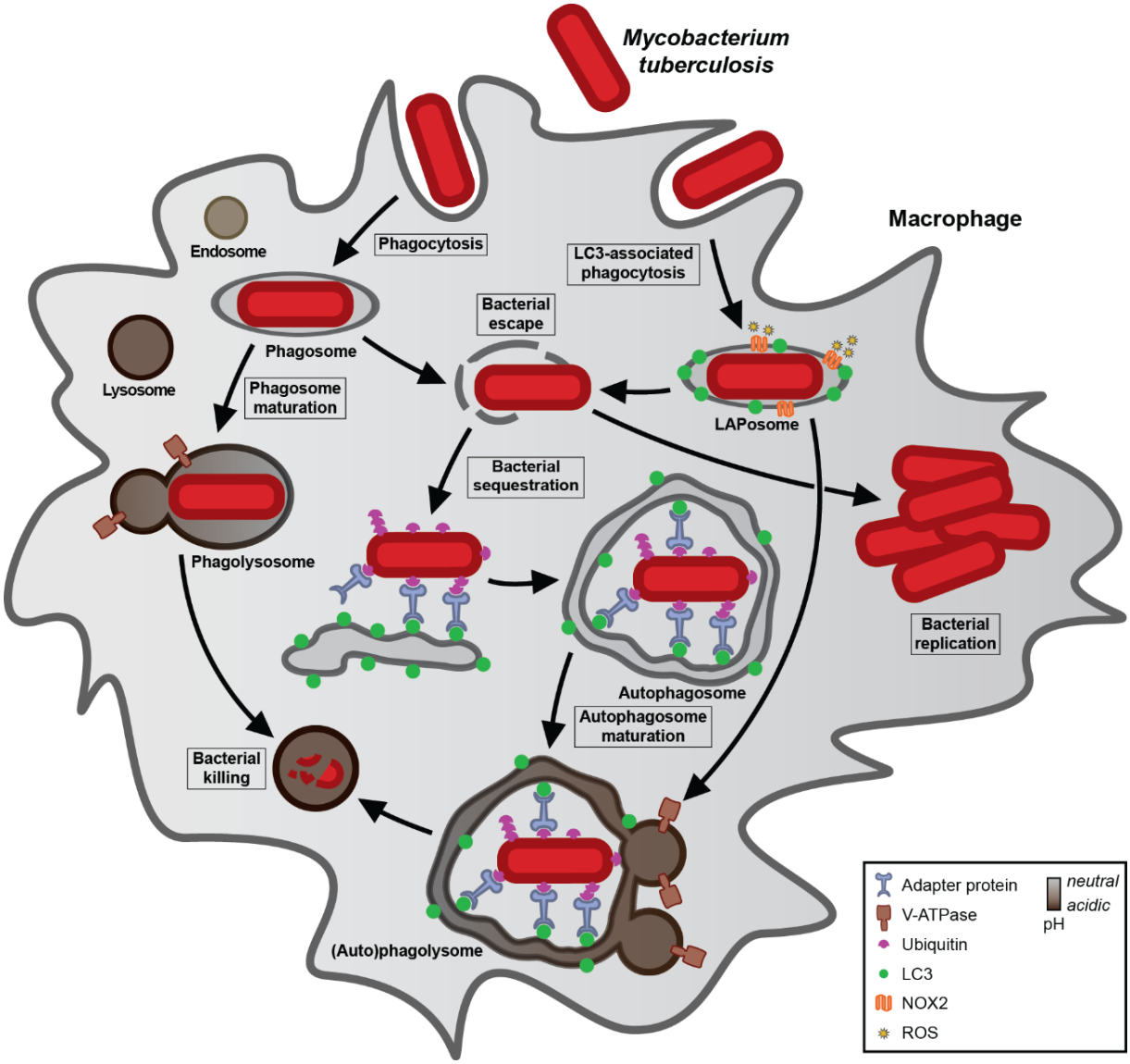

Figure 4. The intracellular life cycle of *Mtb* in the macrophage

Mtb is phagocytosed by the macrophage via either canonical phagocytosis or LC3-associated phagocytosis. The phagosome or LAPosome fuses with lysosomes to acquire microbicidal enzymes and low pH needed to kill the bacterium. In case of phagosomal rupture and bacterial escape, selective autophagy can recognize and target the bacterium for degradation. *Mtb* has evolved mechanisms to interfere with these steps in the host defence response in various ways.

phagosomal escape since mutants deficient for ESX and naturally ESX deficient BCG, are unable to rupture the phagosomal membrane [121]. Although phagosomal escape can be beneficial for the pathogen, cytosolic detection systems such as nucleotide-binding oligomerization domain (NOD)–like receptors, retinoid acid–inducible gene I (RIG-I)–like receptors and stimulator of interferon genes (STING), are capable of recognizing PAMPs and danger-associated molecular pattern (DAMPs) resulting in the activation of other antimicrobial pathways as consequence [122].

Autophagy

The primary defence mechanism against escaped intracellular pathogens is autophagy. Autophagy is a highly conserved process that enables the degradation of damaged organelles and cytoplasmic components to maintain cellular homeostasis. Autophagy also plays a major role against intracellular pathogens, including *Mtb* [123], and is then referred to as xenophagy. Upon phagosomal rupture and exposure to the cytosol, the bacteria or remnants of it are ubiquitinated through proteins such as Galectin, Parkin, SMAD specific E3 ubiquitin protein ligase 1 (Smurf1) and TANK binding kinase 1 (TBK1), [124], which trigger the recruitment of adapter proteins that have an ubiquitin binding site (**Figure 4**) [125]. The most well-known adapter protein is sequestosome 1 (SQSTM1/p62), but also others play a role; nuclear domain 10 protein 52 (NDP52), optineurin (OPTN), neighbor of BRCA1 gene 1 (NBR1) [126]. Atg8-like proteins that can be divided in microtubule-associated protein 1 light chain 3 (LC3) and GABARAP subfamilies, bind the formed complex using the LC3-interacting region (LIR) that the adapter molecules contain, which is then encapsulated by a lipid bilayer [127]. Numerous other host proteins are involved as well, for example DNA-damage regulated autophagy modulator 1 (DRAM1) that stimulates the formation and maturation of autophagosomes under stress conditions and protects against mycobacterial infection [128, 129]. A unique characteristic of autophagy is that the phagophore fuses with itself to form a double-membrane vesicle [124]. Similarly, to phagosome maturation, the autophagosome matures and acquires microbicidal tools by the fusion with lysosomes thereby generating the autolysosome.

In response to this auxiliary host pathway, *Mtb* is able to suppress autophagy by activating mammalian target of rapamycin (mTOR), a central negative regulator of autophagy, and by inhibiting AMP-activated protein kinase (AMPK), which positively regulates autophagy [130-132].

LC3-associated phagocytosis

Less well understood compared to normal phagocytosis, is the process of LC3-associated phagocytosis (LAP) where LC3 is recruited to single-membrane phagosomes, called LAPosomes (**Figure 4**) [133]. Although the exact mechanism is not clear, it has recently been proposed as a vital process in the host response against *Mtb* [134]. Different from canonical autophagy, LAP starts with the recognition of bacteria containing phagosomes using PPRs, including TLRs and Fcγ receptors, and depends on the proteins Rubicon and NADPH oxidase 2 (NOX2) [126]. Rubicon stabilizes NOX2 on the phagosomal membrane and the ROS that is subsequently generated by NOX2 is essential for the recruitment of LC3, necessary for the trafficking to lysosomes and their fusion [135].

Via the secretion of CpsA, a protein absent in non-virulent mycobacteria, *Mtb* interferes with the recruitment of NOX2 [133]. LAPosomes lacking NOX2 and therefore LC3, fail to mature and allow *Mtb* to evade LAP-mediated killing.

Macrophage cell death

The host cell can undergo several types of death following mycobacterial infection. The main mechanisms involved are apoptosis, pyroptosis and necrosis [122].

Apoptosis is a regulated cell death mechanism of host cells with a series of characteristic series of events that starts with the disruption of the membrane-actin interaction resulting in blebbing and release of apoptotic bodies [136]. Apoptotic bodies are flagged for recognition by other macrophages that can easily clear and degrade their contents, a process called efferocytosis. This process is vital for the antimicrobial response and is thus generally regarded as favourable to the host in the context of TB [137].

Pyroptosis is an inflammatory type of cell death started by the recognition of pathogen-associated molecular patterns (PAMPs). In pyroptosis, a multi-protein complex called the inflammasome activates caspase-1 that converts pro-IL-1 and pro-IL-18 to the mature forms IL-1β and IL-18 and additionally generates pore-forming proteins, which are responsible for host cell lysis [136]. Pyroptosis, as well as apoptosis, is generally considered favourable to the host and reduces bacterial burden, but can contribute to pathology through excessive inflammation [138]. Mycobacteria inhibit macrophage pyroptosis through the secreted protein Rv3364c that inhibits the activity of a serine protease, cathepsin G and downstream caspase-1 [139]. Another secreted factor, Zmp-1, inhibits the inflammasome, the hallmark of pyroptosis, and thereby contributes to *Mtb* survival [138].

Although initially thought as accidental or undesired, evidence accumulated during the last years strongly suggests necrosis can in fact be regulated, a process also referred to as necroptosis that depends on Receptor-interacting serine/threonine-protein kinase 3 (RIPK3). TNF-α and activation of pattern recognition receptors (PRRs) among others can start the process of necrosis wherein reactive oxygen species (ROS) play a central role [136]. Additionally, there is an iron-dependent form of necrosis called ferroptosis, which is thought to contribute to *Mtb* dissemination [140]. Necrotic cells represent a perfect niche for *Mtb* to replicate and necrosis and its associated modes of necrotic cell death are thus beneficial for the pathogen [141].

The above-mentioned modes of cell death share characteristics and specific host- as well as bacteria-derived factors impact the pathway that follows [136]. The tug of war

between processes beneficial to the host on one side and pathways beneficial to the pathogen on the other side, reflects the complex interactions during *Mtb* infection.

Comparison between *Mycobacterium tuberculosis* and *Salmonella enterica*

Not only *Mtb*, but also numerous other bacteria are highly skilled in the exploitation and manipulation of host processes. The *Salmonella enterica* species are another example of successful intracellular pathogens. The *Salmonella enterica* serovar Typhi, known for causing Typhoid fever, and serovars Typhi and Typhimurium, causative agents of gastroenteritis, contribute significantly to the global burden of infectious disease related deaths [142, 143]. *Salmonellae* and *Mtb* share some of their strategies in how to subvert the host response. Differently from *Mtb*, *Salmonellae* can, besides being phagocytosed, actively invade the host cell and persist and replicate in vesicles known as *Salmonella*-containing vacuoles (SCVs) [144]. The host response is usually only effective when the protective SCV is damaged, which prompts rapid recognition, ubiquitination and clearance by autophagy [145]. The SCV does however, mature slowly as observed by the acquisition of Rab7 and V-ATPases among others [146]. Interestingly, the stress induced by the low pH of around 5.5 in this matured SCV, results in the expression of *Stm* type three secretion systems (T3SS) and consequential secretion of virulence factors, thereby driving *Salmonellae* survival and replication [147]. Although *Mtb* and *Salmonellae* are clearly idiosyncratic in the ways they manipulate the host, their survival depends on the subversion of the same host cell pathways.

Antibiotics And The Development Of Resistance To Antibiotics

Halfway through the 20th century, humankind thought to have an answer to bacterial pathogens in the form of antibiotics. Unfortunately, improper use of antibiotics and the huge genetic flexibility of pathogens proved this wrong [148]. Scientists were not able to match the speed of new drug discovery to the emergence of bacterial drug resistance and nowadays, antibiotic resistance is recognized as one of the biggest threats to human society [149]. Most of the antibiotics currently in use are either produced by bacteria or fungi themselves as defence, or modified versions thereof. For example, numerous clinically used antibiotics are derived from the family of Streptomycetaceae which easily explains the emergence of drug resistance against these chemicals since natural selection already favoured this [150]. The production of a certain version of the β-lactamase enzyme by various bacteria that confers resistance to Penicillin among others is a prime example of this [151]. In addition, genetic mutations that alter the drug-binding site of a target on an individual level or the

exchange of genetic material encoding drug resistance on a population level are major drivers of the global emergence of drug resistance [152].

Since the first whole genome sequencing study on *Mtb* was published in 1998, our understanding of drug resistance in TB has greatly increased. Unlike other pathogens like *Stm*, *Mtb* does not contain plasmids and generally does not share genetic information horizontally, although evidence suggests that this did play a major role in the establishment of the *Mtb* genome itself [153, 154]. Instead, mutations are passed along to daughter cells. Despite a slow evolutionary rate, estimated at 0.3 - 0.6 single nucleotide polymorphisms (SNPs) per genome per year [153], even resistance against recently approved drugs, such as Bedaquiline, has been found [155]. Furthermore, suboptimal concentrations of antibiotics at the site of infection caused by the low accessibility of the granuloma structure and the poor antibiotic pharmacokinetics require multiple drugs to be administered simultaneously [156]. Of bacteriologically confirmed pulmonary TB cases, 71% were caused by Rifampicin-resistant *Mtb* of which a significant portion were multi drug-resistant (MDR) *Mtb* strains [16]. Treatment of drug-sensitive (DS)-*Mtb* consists of a 6-month regimen containing Isoniazid, Rifampicin, Ethambutol, and Pyrazinamide. The regimen is even longer for the treatment of MDR-*Mtb*, which requires 9 to 20 months of second-line drugs, i.e., a Fluoroquinolone and injectables such as Levofloxacin, Moxifloxacin, Bedaquiline, Delamanid or Linezolid [16]. The complexity and duration of these treatment plans, which are aggravated by drug toxicity and high costs, cause lack of treatment adherence that facilitate and perpetuate the emergence of drug resistance [16]. Importantly, a small proportion of the MDR-*Mtb* strains (6.2% in 2018) is already resistant to at least one of the Fluoroquinolones and one of the injectable agents. These strains, which are defined as extensively drug-resistant (XDR) *Mtb* [16], pose a new emerging threat that the current TB drugs cannot face.

The TB drug pipeline has in the last decade delivered three new antibiotics for the treatment of TB, Bedaquiline, Delamanid and Pretomanid, all approved for the treatment of MDR-TB [157, 158]. Although efficacious, they are only administered as a last resort owing to significant toxicity. It is clear that better options, that are safer, more potent, cheaper and with better drug-drug interactions, are urgently needed. Currently, approximately another 20 drugs are in clinical trials as candidate anti-TB agents [159]. Most of these drugs were developed for different purposes and are categorised as repurposed or repositioned drugs. Some of these drugs do not have a direct effect on the pathogen but act on the host cells, opening to new exciting treatment possibilities, which are discussed in the following paragraph.

Host-Directed Therapy

Host-Directed Therapy (HDT) represents an attractive complement to current classical antibacterial therapies consisting of antibiotics. Since the survival of intracellular pathogens relies heavily on their ability to manipulate host cell signalling, stopping infection via targeting of the host should prove effective. There are numerous advantages of HDT (**Figure 5**). First, HDT targets the host and is therefore unaffected by the presence of mutations that confer resistance to antibiotics, allowing the treatment of DS-*Mtb*, as well as MDR- and XDR-*Mtb*. Although there is selection pressure, acquisition of drug resistance should prove very difficult because HDT targets host pathways. Additionally, metabolically low or inactive bacteria that are hard to treat with antibiotics, could be targeted by HDT irrespectively. Fourthly, since the targets of HDT and antibiotics are species apart, synergy between the two treatments is expected. Lastly, due to a higher treatment efficacy and success rate when combined with antibiotics, the duration of therapy could be shortened with less toxicity and higher treatment compliance as result.

Theoretically, all processes modified by *Mtb* to its advantage represent candidate targets for HDT. Practically, this is not necessarily the case since these processes could be vital to the host and targeting them might lead to severe toxicity. It is therefore crucial to find targets that are important enough to a host process so that the consequence of their modulation is not easily debilitated and simultaneously results in minimal damage to the host.

The maturation of the *Mtb*-containing phagosome and autophagy are obvious targets that are modified by numerous approved chemical and biological drugs, such as Imatinib, Metformin, Gefitinib, GM-CSF, Vitamin D, Clionamines, and statins among others [160-167]. Additionally, altering the production of ROS/RNS is another successful strategy. Restriction of *in vitro Mtb* survival was demonstrated by stimulating ROS/RNS using Bazedoxifene and soluble CD157 [168, 169], but conversely also through the dampening of excessive amounts of ROS that can lead to host cell death, i.e. necroptosis, using antioxidants, such as N-acetyl-cysteine (NAC) and Nicotinamide [170]. Corticosteroids, such as Dexamethasone and Doramapimod, are also capable of halting host cell death through necrosis that is advantageous to *Mtb*, but their immune suppressive effects could also cause reactivation in LTBI patients [171, 172]. Modifying host cell metabolism is a strategy gaining a lot of interest in the last years since accumulating evidence has shown that *Mtb* profoundly alters the overall bioenergetic state of the host cell [173-176].

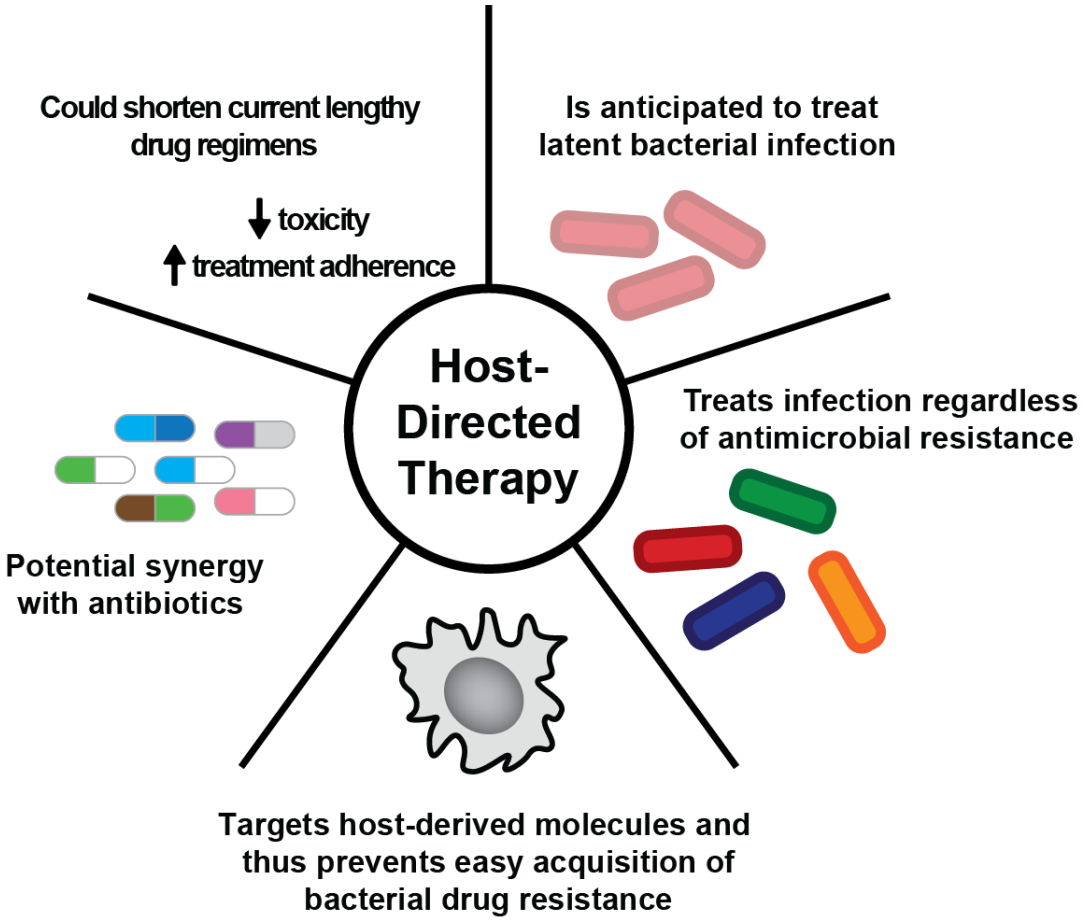

Figure 5. Advantages of host-directed therapy over bacteria-directed therapy

Host-directed therapy has several advantages over bacteria-directed therapy, such as its potential regardless of the pathogen's state of antibiotic resistance and metabolic activity. Additionally, HDT targets the host preventing bacterial resistance while simultaneously synergizing with bacteria-directed therapy. Lastly, HDT could decrease toxicity of current treatment regimens either directly through lowering tissue damage or indirectly via enhancing bacterial clearance thus allowing shorter treatment regimens.

Not only therapies aimed at the cellular level, but also therapies directed at the immune response as a whole show promising results. Enhancing the T helper 1 (Th1) response by IFN-γ, IL-2 and IL-12 [177-179], and conversely diminishing the T helper 2 (Th2) response by abrogating the IL-4 pathway [180], showed beneficial effects on bacterial burden. Similarly, enhancing antigen presentation in innate immune cells that is essential to an effective adaptive response shows great potential [181, 182]. Lastly, the targeting of the eicosanoid pathway involved in the inflammatory response by drugs including Aspirin [183], Ibuprofen [184], Zileuton and Prostaglandin E2 (PGE2) [185] is successful as well.

The granuloma is also an interesting target for HDT. The extracellular matrix is vital for the alveoli to function and the lungs aim to protect this matrix by maintaining its integrity [186]. *Mtb*, on the other hand, secretes proteases and induces host cell secretion of proteases, in particular matrix metalloproteinases (MMPs), to break down the matrix and effectively disseminate [186]. Interestingly, while inhibiting MMPS alone could worsen TB infection [187], combinational therapy of MMP inhibitors with standard antibiotic therapy improves TB infection [188]. Another feature of the *Mtb* granuloma is its vasculature, since immune-controlled granulomas have low but active granulomas have extensive vasculature [189]. Infection induced angiogenesis thus represents a target for HDT as hypoxia may slow bacterial growth and indeed the neutralization of vascular endothelial growth factor (VEGF) has in several patients been successfully used as adjunctive HDT [190, 191].

Lastly, recent studies focusing on host epigenetics, either from the perspective of the *Mtb*-infected cell or the immune response as a whole, show encouraging results. Modulation of the histone deacetylase activity of histone deacetylase 3 (HDAC3) [192], histone deacetylase 6 (HDAC6) [193], Sirtuin 1 (SIRT1) [194] and Sirtuin 7 (SIRT7) [195], showed promising outcomes in *Mtb* infection. Additionally, the use of pan-HDAC inhibitors Valproic acid (VPA) and Suberoylanilide hydroxamic acid (SAHA) inhibited intracellular *Mtb* survival as well [196].

Several trials on adjunctive HDT for TB have been performed or are currently ongoing (see Table 1 for an overview). For example, the addition of Aspirin but not Cyclooxygenase-2 (COX2) inhibitor to standard TB therapy demonstrated accelerated sputum smear conversion, a key indicator of treatment response [197-199]. Patients treated with the Phosphodiesterase-4 (PDE4) inhibitor CC-11050 and mTOR inhibitor Everolimus adjunctive to standard treatment showed superior recovery of forced expiratory volume (FEV_1), a measure of lung function, but auranofin and ergocalciferol (vitamin D) did not, although, unfortunately none of the study arms showed enhanced eradication of infection [200]. The addition of Vitamin D, Vitamin A and Zinc lacked efficacy in several other trials as well, although Vitamin D was shown to enhance sputum smear conversion rate in a meta-analysis [201-207]. Implementation of the anti-oxidant NAC, that restores Glutathione (GSH) levels that is limited by cysteine availability, improved several treatment outcome parameters including sputum negativity in one trial, while it reduced hepatotoxicity of standard antibiotic therapy in in two others, demonstrating that HDT could also serve by reducing adverse treatment effects directly [208-210]. Inclusion of IL-2 or GM-CSF in the treatment regimen lacked clear beneficial effects on treatment outcome [211, 212]. Prednisolone has been found to shorten sputum culture time, but the dose needed to achieve this is anticipated to cause unacceptable toxicity [213]. 4-Phenyl butyrate (PBA) adjunctive to standard

therapy significantly improved resolution of TB symptoms [214], and L-arginine, substrate for nitric oxide synthase (NOS), additionally enhanced sputum conversion and reduced symptoms in one trial, although it lacked efficacy in another [207, 215]. Two weeks of Doxycycline reduced pulmonary cavity volume and suppressed MMP levels that lasted for another 6 weeks [216]. Importantly, this demonstrates that an adjunctive HDT for merely a short period can change the course of TB. Lastly, patients with concomitant TB and Diabetes mellitus (DM) that are at higher risk of morbidity and mortality have been included in trials investigating the anti-diabetic drug Metformin. Metformin improved sputum culture conversion in patients with cavitary pulmonary TB, decreased mortality during TB treatment and reduced the risk of latent TB reactivation [217-219].

Advances in HDT for TB have clearly been made, however, the current number of drugs showing efficacy and safety is insufficient. Additional drugs are needed and the repurposing of already approved drugs as HDT for TB, will allow rapid translation of preclinical findings into clinical application. Additionally, a deeper understanding of the host-pathogen interactions is needed to find new targets to expand the therapeutic options. Chemical genetic screening can provide these targets and therapeutics.

Table 1. Non-exhaustive table of clinical trials of host-directed therapeutics for Tuberculosis

Substance	Mean effects	Main findings	References
Aspirin	↓ Inflammation	↓ Mortality	[197]
Etoricoxib	↓ Prostaglandins through COX2	No improvement	[198, 199]
CC-11050	↓ PDE4 activity	↑ FEV_1	[200]
Everolimus	↓ mTOR	↑ FEV_1	[200]
Auranofin	↓ Selenoprotein thioredoxin reductase ↓ Proteasome activity	No improvement	[200]
Vitamin D	↓ Inflammation ↑ Autophagy	↑ Sputum conversion based on a meta-analysis	[206]
Vitamin A	↑ Immune response	No improvement	[201-203]
Zinc	↑ Immune response	No improvement	[202, 203]
NAC	↓ ROS through restoring GSH levels	↑ Sputum conversion ↓ Hepatotoxicity	[208-210]
IL-2	↑ Th1 immunity	No improvement	[211]
GM-CSF	↑ Innate and adaptive immunity	No improvement	[212]
Corticosteroids	↓ Inflammation	↑ Sputum conversion	[213]
PBA	↑ Cellular lipid homeostasis and proteostasis	↓ TB symptoms	[214]
L-Arginine	↑ RNS as substrate for NOS	↑ Sputum conversion ↓ TB symptoms	[207, 215]
Doxycycline	↓ MMP activity	↓ Pulmonary cavity volume	[216]
Metformin	↓ Inflammation ↑ Autophagy	↑ Sputum conversion ↓ Mortality ↓ LTBI reactivation	[217-219]

Thesis Scope And Outline

This thesis aimed to identify new host-directed therapies against intracellular bacteria, with an emphasis on *Mtb*. **Chapter 2** describes the development of an *in vitro* flow cytometry-based screening platform able to measure intracellular bacterial burden in cell lines. By applying this new screening tool on a Library Of 1260 Pharmacologically Active Compounds (LOPAC), several drugs were found to be capable of reducing intracellular burden of *Stm* or *Mtb* in an HDT fashion. Furthermore, the LOPAC screening results were fed into a machine learning algorithm that was then successfully used to predict new potential drugs against *Stm* and *Mtb* from the PubChem repository. The *in vitro* testing of these predicted drugs demonstrated that 55.6% and 50% were efficacious against intracellular infection of *Mtb* and *Stm*, respectively. This outcome outperformed the results of the screen itself, considering that 10% of the LOPAC had an HDT effect against *Mtb* and 14.6% against *Stm*. Interestingly, all predicted HDT drugs were receptor tyrosine kinases (RTKs) inhibitors. An independent *in vitro* screen targeting the host kinome of infected cells using small interfering RNAs (siRNAs), confirmed the RTK signalling pathway as an important regulator of intracellular *Mtb* survival.

Chapter 3 set out to identify improved versions of the RAC (Rho family)-alpha serine/threonine-protein kinase (AKT1/PKB) inhibitor H-89, previously identified as a HDT drug for *Stm* and *Mtb* infection. A library of 76 structural analogues of H-89 was constructed and screened using the flow cytometry-based screening model developed in chapter 2. An analogue, 97i, showed superior efficacy against intracellular *Mtb* infection *in vitro* and *Mm* infection *in vivo*, while maintaining similar high efficacy against *Stm* infection. Compared to H-89, 97i targets kinases other than AKT1, such as the PCTAIRE family of kinases, calcium/calmodulin dependent protein kinase type 1 (CAMK1) and phosphofructokinase, liver type (PFKL). *In vitro* 97i treatment of macrophages was shown to increase intracellular bacterial trafficking to the lysosome mediated by autophagy.

Considering the vital role of autophagy in intracellular bacterial infection, the flow cytometry-based screening assay (Chapter 2) was employed to probe a library of 96 known autophagy modulating compounds. **Chapter 4** describes the findings of 6 potent HDT drugs that lacked toxicity as result of this screen: Fluspirilene, Pimozide, Tamoxifen, Amiodarone, Chloroquine, and its metabolite, Hydroxychloroquine. Fluspirilene and Pimozide that are antipsychotic drugs and structural analogues of the diphenylbutylpiperidine-class were investigated into more detail. These compounds, Pimozide in particular, decreased intracellular infection *in vitro* by targeting multiple

mechanisms, including the lysosomal response, autophagy, reducing CISH presence and ROS/RNS generation.

Chapter 5 explored the mechanism of Tamoxifen (identified in Chapter 4) to reduce intracellular *Mtb* infection, both *in vitro* and *in vivo*. Unexpectedly, the effect of Tamoxifen treatment was independent of its estrogen receptor (ER) modulating activity. Instead, Tamoxifen appeared to increase the lysosomal response with consequential enhanced mycobacterial presence in the lysosome of macrophages, using RNA sequencing and microscopic colocalization studies.

Chapter 6 examined the role of epigenetic regulation in host-pathogen interactions between the macrophage and *Mtb*. *Mtb* infection was found to affect histone deacetylase (HDAC) expression, suggesting histone acetylation is modified significantly, and posing HDAC modulators as putative HDT drugs. Several HDAC inhibitors, both specific for class IIa HDACs as well as pan-class, decreased *Mtb* survival *in vitro*, correlating with a diminished pro-inflammatory cytokine response in both Mϕ1 and Mϕ2. These HDAC inhibitors additionally showed efficacy in the *in vivo* zebrafish embryo model for TB, supporting the feasibility of this approach.

In **Chapter 7**, the results of the above-mentioned studies are summarized and discussed.

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