Selective autophagy in host defense against mycobacterial infection

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Chapter 1

Introduction and outline of thesis

1. General introduction to Tuberculosis

1.1 Tuberculosis remains a global health threat

Tuberculosis (TB) has affected humans since ancient times and remains a dangerous infectious disease today. It usually affects the lungs, although it can also invade other organs of the body, such as the brain, the intestine, the kidneys, or the spine [1]. *Mycobacterium tuberculosis* (Mtb) is the causative agent of TB. There are two general types of TB infection based on the clinical symptoms: latent and active TB. TB spreads when people who have active TB cough, spit, speak, or sneeze in the vicinity of uninfected individuals, who then inhale the aerosols containing the bacteria [2]. Patients with latent TB do not manifest visible clinical symptoms, as bacteria can maintain a dormant state inside the host for a long period of time. However, latent TB can progress to clinically active TB because of various factors that can compromise the immune system of the host, such as malnutrition, diabetes, smoking, alcohol addiction, or reinfection. Overall, there is a chance of around 10% that latent TB becomes active [3]. The clinical signs of active TB include chronic coughing, pain in the chest, weakness or fatigue, weight loss, fever, and night-sweats. Evidence of TB infections in Europe can be tracked centuries back. Based on historic records, around 25% of the population died due to a TB epidemic in the 19th century, frequently referred to as 'consumption' due to the associated weight loss [1]. In modern times, TB remains a leading cause of morbidity and mortality in worldwide, with 10.4 million new cases and 1.7 million deaths in 2015 [4]. Moreover, TB infection often coincides with human immunodeficiency virus (HIV) infection, or immunocompromising chronic diseases such as diabetes mellitus. The resulting comorbidity and the increased occurrence of drug resistant Mtb strains have contributed to an increase in TB manifestations and associated mortality [5, 6]. According to the World Health Organisation (WHO), TB is a pandemic disease that represents a significant health burden for developing Countries. WHO estimated that more than one-third of the world's population is currently infected with Mtb and about 10-15% of this large number of carriers will progress to active TB. Furthermore, drug-resistant TB is a serious health threat for both developing and developed countries, as these strains are (becoming) resistant to the most frequently used first- and second-line anti-TB drugs [4].

1.2. Pathogenesis of tuberculosis

Mtb is a bacterial pathogen that can parasitize host immune cells. It was first described in 1882 by Robert Koch. In most cases, TB patients are infected by Mtb, but other strains of the Mtb complex can also cause TB, such as *M. bovis*, *M. africanum*, *M. canetti*, and *M. microti*. However, cases of TB caused by these other mycobacterial species have not been documented worldwide and are generally limited to regions with poor public health [7].

TB infections start when the mycobacteria-enclosed aerosols reach the pulmonary alveoli. Invading mycobacteria can be recognized by alveolar macrophages through several pattern recognition receptors (PRRs), including toll-like receptor (TLR) 1, TLR2, TLR4, and TLR9 [8-10]. Macrophages attempt to eliminate the bacteria from the infected tissue through phagocytosis [11]. During this process, Mtb is engulfed (phagocytosed) and temporarily resides in a membrane-bound vesicle, called a phagosome. Bacteria-containing phagosomes normally fuse with lysosomes to form phagolysosomes. Phagolysosome fusion presents a major anti-bacterial strategy by exposing engulfed bacteria to lysosomal acidic hydrolases [3]. The critical survival strategy of Mtb inside macrophages is to prevent the fusion between phagosomes and lysosomes. Furthermore, Mtb can also resist the acidic environment of lysosomes [12, 13] and initiate various countermeasures to protect itself against other host defense mechanisms, such as generation of reactive oxygen species (ROS) and nitrogen species (RNS) [14]. Thus, Mtb is able to survive and replicate inside macrophages and will eventually overgrow and kill the immune cells [12, 15]. To achieve this, Mtb not only resists the phagolysosomal pathway, but can also escape from phagosomes into the cytosol. Once inside the cytosol, the bacteria have access to sufficient nutrients, which improves its replication rate inside of macrophages [16]. This process requires the type VII secretion system ESX-1 (6-kDa early secretory antigenic target (ESAT-6) secretion system 1) [17]. This secretion system allows Mtb to resist host immune responses by exporting several effector proteins [18, 19]. A recent study showed that the ESX-1 secretion system directly affects the acidification of Mtb-containing phagosomes [20]. Furthermore, the ESX-1 secretion system has multiple other functions that contribute to the ability of Mtb to survive and replicate inside macrophages and to promote its cell-to-cell spreading [21].

Mtb infected macrophages produce inflammatory cytokines and chemokines to recruit other immune cells to form a compact and organized structure called a granuloma – the clinical hallmark of TB [22]. The recruitment of macrophages in the early stage of infection depends on the local production of ligands (CCL2/MCP-1, CCL12, and CCL13) that bind to the chemokine receptor CCR2 [23]. Adaptive immune cells, like T cells and B cells, are also recruited to the forming granuloma during later stages of the infection. Maintaining the structure of the granuloma requires the production of TNF-α by infected macrophages and T cells [24].

The granuloma is situated at the core of TB pathogenesis. This inflammatory structure functions to restrict mycobacteria in a limited area and provides a local environment where cells of the immune system can interact with the bacteria [22, 23]. However, recent studies have found that mycobacteria also utilize the granulomas to avoid killing by the host's immune response [24-26]. For example, macrophages in the granulomas have been shown to undergo an epithelioid transition that is characterized by downregulation of immune-related genes and upregulation of epithelial markers, a process which is induced by bacterial virulence factors [22, 24, 25, 27]. Formation of tight junctions between neighboring epithelioid macrophages further limits access to the granuloma core by newly recruited immune cells and prevents bacterial clearance [22, 24, 28]. Thus, macrophages and dendritic cells (DCs) inside of the granulomas are unable to delivery antigens to lymphocytes, effectively repressing the adaptive immune response [29, 30]. Mtb inside the granuloma can become metabolically dormant and persist for decades before reactivation occurs [22].

1.3. Prevention and control of tuberculosis

The “End TB strategy” was launched in 2014 by the WHO and achieved much progress in reducing new TB cases by improving treatment regimens and public health awareness [31]. TB prevention principally depends on the immunization of infants. Currently, Bacillus Calmette-Guérin (BCG) is the only TB vaccine used worldwide [32]. The BCG vaccine was generated as an attenuated live vaccine derived from a virulent strain of the *M. bovis* species by more than 200 times of consecutive passage [33]. The BCG vaccine effectively prevents forms of TB during childhood. However, BCG vaccination provides a highly variable level of protection against TB in

different populations and regions [34].

Both latent and active TB can be diagnosed and cured. Diagnostic tools for latent and active TB are readily available. Latent TB is efficiently detected by the Mantoux tuberculin skin test (TST) and the Interferon-gamma release assay (IGRA). The Mantoux test is based on a subcutaneous injection of tuberculin purified protein derivative (PPD), followed by a measurement of the resulting induration (palpable, raised, hardened area or swelling) as a read out of the level of immune recognition of tuberculin peptides. The interferon-gamma release assay is based on the quantification of Interferon-gamma production in response to the presence of TB antigens in the whole blood [35]. For active TB, chest radiography and bacterial cultures are efficient and rapid diagnostic methods [36].

Preventive therapy is necessary to lower the risk of disease progression from latent to active TB. The standard treatment of latent TB recommended by the WHO is an oral antibiotic regimen (e.g. isoniazid and/or rifampicin) for 6 to 9 months [35, 37]. However, the use of a single antibiotic frequently leads to development of drug resistance in active TB cases. Thus, treatment of active TB usually consists of a combination of antibiotics to kill the bacteria and lower the chance of Mtb developing drug resistance. Eradication of Mtb from the body by drug treatment is hard and time consuming, due to the special structure and chemical composition of the mycobacterial cell wall, which strongly inhibits the penetration of drugs and makes many antibiotics ineffective [38]. If TB patients do not receive sufficient treatment, it leads to a growing incidence of drug resistant strains: Multidrug-resistant (MDR), Extensively drug-resistant (XDR) and Totally drug-resistant (TDR) strains. This further reduces the treatment options and increases the incidence of death [39, 40]. In summary, there is a large variability in levels of protection provided by the current vaccine and Mtb is becoming increasingly resistant to many anti-TB drugs, which both stress the need for new therapeutics and more effective anti-TB treatments.

1.4. Zebrafish as a model to study mycobacterial pathogenesis

Researchers have frequently used – and are still using – *in vitro* Mtb cultures or Mtb infected macrophages to investigate the mechanisms of Mtb infection. However, results obtained from *in vitro* studies may be difficult to translate into human therapies, as two-dimensional cell cultures lack the TB granuloma characteristic of this disease [41]. Recently, successful attempts have been made to culture granulomas in three dimensions, using media that resemble the extracellular environment found in tissues [42]. In addition, reliable animal models are essential to improve our understanding of the mechanisms of TB pathogenesis. Artificially infected animal models are an indispensable approach to investigate the host and bacterial factors involved in TB pathology, and to select new candidates for drugs and vaccines [43].

Currently, several animal species are being used to study Mtb infections, which include mice, guinea pigs, rabbits and non-human primates. In murine infection models, it has been difficult to replicate human TB pathologies as the commonly used mouse strains do not develop the highly organized granuloma structures observed in humans and in non-human primates [22]. However, alternative mouse strain, such as C3HeB/FeJ, DBA/2 and CBA/J, can develop necrotic granulomas in the lungs following infection with Mtb [44-46]. These models were generated by selecting for mouse strains with increased susceptibility to Mtb, which resulted in the identification of genetic loci that prevent the formation of necrotic granulomas [46, 47]. These new TB models have been used to screen for anti-TB drugs and to test new vaccine candidates [45, 48]. Other mammalian models (such as guinea pigs and rabbits) have been developed that mimic human TB pathology, including the formation of necrotic granulomas [43]. However, both the guinea pig and rabbit models lack of immunological reagents available for mice and are difficult in genetic manipulation [49]. The primate infection models present similar clinical signs as human TB and form classical TB granuloma structures. However, the costs and ethical considerations arising from the use of these models imply that they can only be used sparsely. Thus, additional animal models are necessary to study TB pathogenesis.

During the last 10 years, the zebrafish has become a widely used alternative animal model to study mycobacterial pathogenesis [50-52]. Adult zebrafish have fully functional innate and adaptive

immunity, which is highly similar to the mammalian immune system [53]. Furthermore, zebrafish are naturally susceptible to *Mycobacterium marinum* (Mm), the causative agent of TB in ectotherms. As a close relative of Mtb, Mm shares many of its virulence factors [54, 55]. The zebrafish-Mm model presents additional advantages that are distinct from those of other TB models [56]. Zebrafish embryos and larvae are transparent, which allows intravital imaging of host-pathogen interactions following microinjection of Mm [57]. At 1 day post fertilization or at later stages, zebrafish larvae can form organized and compact Mm granulomas, which have high similarity to the early stages of granulomas generated by Mtb in primates [58]. Zebrafish transgenesis methods have been well established, and the recent breakthroughs in gene editing with CRISPR/Cas9 have facilitated the generation of knock-out and knock-in zebrafish [59]. On the bacterial side, Mm presents advantages over working with Mtb, including lower biosafety restrictions (BSL2 instead of BSL3) and a considerably shorter replication time [58]. In recent years, the insights from the Mm-zebrafish embryo infection model have contributed significantly to our understanding of TB pathogenesis [27, 59-62].

2. Autophagy: an important immune defense mechanism against Mtb

2.1 The basic function of autophagy

Autophagy is an evolutionary conserved lysosomal degradation pathway in eukaryotic cells that can degrade cytoplasmic materials and organelles. By removing unwanted cellular contents, autophagy functions in maintaining cellular homeostasis. This physiological phenomenon was first discovered by Christian De Duve around 55 years ago [63]. The process is genetically well-defined and many of the factors involved are conserved in eukaryotes from yeast to humans [64]. Autophagy is recognized as a survival mechanism in response to different types of stress, including nutrient deficiency, growth factor deficiency, and hypoxia [65]. The autophagic degradation of cytoplasmic material recycles amino acids and other nutrients, e.g. to fuel metabolic pathways in nutrient-deficient conditions. Autophagy can also be stimulated by other stress factors that include diseases and infections [66]. Activation of autophagy in these contexts generally follows upon an increased transcriptional activation and/or post translational protein modification of autophagy-related factors and regulators by the host cells [67].

Three main categories of autophagy are identified in mammalian cells, based on the mechanisms used to capture cytosolic cargo. These include macroautophagy, microautophagy, and chaperone-mediated autophagy. All of them rely on proteolytic degradation of cytosolic materials in lysosomes [68]. Macroautophagy is characterized by capturing cargo in a double membrane-bound structure. During this process, the isolation membrane (or autophagophore) undergoes expansion and elongation to form a double membrane vesicle, known as an autophagosome, which eventually fuses with a lysosome to generate an autophagolysosome [69] . The term "autophagic flux" is used to describe the whole process from autophagosome formation to the degradation of the cytoplasmic cargo by hydrolases [70]. Microautophagy is a non-selective degradation process during which cytosolic components are directly engulfed by lysosomes [71]. Finally, chaperone-mediated autophagy (CMA) was identified in 1981 and is quite different from macro- and microautophagy in terms of its selectivity and mechanism of cargo degradation [72]. CMA only eliminates targeted proteins and delivers them to the lysosomes via a process assisted by chaperone proteins/heat shock cognate proteins, such as Hsc-70. Hsc-70 can be recognized by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), which leads to degradation of the Hsc-70 protein complex [73]. Of the three types of autophagy, macroautophagy is the most abundant process and is therefore also the most extensively studied form of autophagy.

Macroautophagy (hereafter referred to solely as autophagy) is historically regarded as a non-specific pathway. However, it has become clear that this process can also be used to selectively remove material from the cytoplasm. In that case, so called selective autophagy receptors (SLRs) identify and capture targets for autophagosomal degradation based on a molecular tag, such as ubiquitin [74]. Ubiquitination is a highly regulated process that is conserved in all eukaryotes. Ubiquitination can delivery covalently tagged substrates to (1) the proteasome, (2) the lysosome or, (3) the autophagosome [75]. The crosstalk between ubiquitination and autophagy relies on SLRs, which act like a bridge by simultaneously binding to ubiquitinated cargos and the forming autophagophore [76]. The selective autophagic degradation of misfolded proteins is called aggrephagy, that of mitochondria is called mitophagy, while the selective elimination of invading microbes is called xenophagy [77].

Xenophagy (also known as bacterial or anti-microbial autophagy) is considered a cell-autonomous defense mechanism against invading pathogens [77]. Deficiency of intracellular nutrients due to competition from invading pathogens is one of the signals sensed by eukaryotic cells to identify microbial invading and to diminish invading pathogens via autophagy [66]. Anti-microbial autophagy was first described in response to *Streptococcus pyogenes* (group A Streptococcus) infection [78]. The bacteria are sequestered into autophagosomes and fuse with lysosomes to form autophagolysosomes. This process results in elimination of most of the bacteria [78]. Around the same time, another study confirmed that stimulating autophagy can inhibit Mtb survival in infected macrophages [79]. This study has shown that either physiological or pharmacological induction of autophagy decreased the viability of Mtb, while induction of autophagy was beneficial for the maturation of Mtb-containing phagosomes [79]. Until now, autophagy has been shown to be able to directly target a diverse spectrum of pathogens, including various bacteria, viruses, and intracellular parasites [80].

2.2 The components of the autophagy machinery

Autophagy is a dynamic process which requires a series of distinct steps to complete. Autophagy is activated with the formation of a structure called the isolation membrane, also known as a phagophore. This lipid bilayer is thought to originate from the endoplasmic reticulum (ER) and/or the trans-Golgi network and endosomes [81]. The phagophore then elongates and expands around the cargo, sequestering the cytoplasmic material into a double membrane structure. This double membrane structure defines the autophagosome. The autophagosome eventually matures and undergoes fusion with lysosomes, which promotes the degradation of the autophagosomal contents by lysosomal acid proteases [68] (Fig1). This process is driven by autophagy-related proteins, which are controlled by a number of signaling pathways in response to cellular stress factors, such as the mammalian target of rapamycin (mTOR) signaling pathway for nutrient sensing and pattern recognition receptor (PRR)-signaling for invading microbes [66, 82]. The importance of autophagy is well established in mammals and other vertebrates, but the underlying molecular mechanisms have been uncovered using genetic analysis of yeast. Currently, more than 41 different ATGs have been revealed and identified in yeast by genetic

screening, followed by identification of homologs in higher eukaryotes [83].

Starvation, a classical inducer of autophagy, can result in low nutrient and amino acid levels, which induces autophagy by inhibiting the function of mTOR [84]. In turn, mTOR then relieves its inhibition of unc-51-like kinases 1/2 (ULK1 and ULK2) [84, 85], which are recruited to the phagophore to bind with the autophagy related gene 13 (ATG13) and FAK family kinase-interacting protein of 200kDa (FIP200) [85]. ULK1 and ULK2 have significant homology both in the C terminal and N-terminal regions. The C-terminal regions of ULK1 and ULK2 are required for interactions with ATG13 and FIP200, and for the translocation of ULK1 to nascent phagophores [84]. Assembly of this complex is essential for autophagy, because it allows the attraction of other ATG proteins to the phagophore assembly site (PAS) and activates several downstream targets through phosphorylation [67, 86]. The activation of the ULK1/2 complex results in binding with Beclin1 (ATG6 in yeast) and ATG14L, which attracts additional proteins to the PAS for initiation of phagophore formation [68]. This process requires the class III phosphatidylinositol-3 kinase (PI3KC3), resulting in the generation of phosphatidylinositol 3-phosphate (PI3P) by vesicular protein sorting 34 (Vps34) and recruitment of other effectors of the autophagy pathway [87]. PI3P is strictly required for elongation of phagophore and attracts other ATG proteins to the phagophore [88, 89].

The subsequent elongation and closure of the phagophore requires the recruitment of two ubiquitin-like proteins, ATG8/LC3 and ATG12 [90]. ATG8/LC3 (Microtubule-associated protein 1 light chain 3; hereafter referred to as LC3) can occur in two forms: LC3-I, which resides freely in the cytoplasm; and LC3-II, which is the membrane bound form of LC3. LC3-II is formed when LC3-I is conjugated to the lipid phosphatidylethanolamine (PE). Upon activation of autophagy, ATG12 is conjugated to the crucial autophagy factor ATG5. The ATG12-ATG5 conjugate forms a complex with ATG16L1, which lipidates LC3 to direct its localization in the membrane of the forming autophagosome. To date, at least 6 selective autophagy receptors (SLRs) have been identified, namely Sequestosome 1 (SQSTM1/p62), Neighbor of BRCA1 gene1 (NBR1), Nuclear dot protein 52 kDa (NDP52), Optineurin (OPTN), BCL2-interacting protein 3 like (BNIP3L), and NDP52-like receptor TAX1-binding protein (TAX1BP1) [76, 91, 92]. The common feature of these

receptors is that they contain an LC3 interaction region (LIR) motif and a ubiquitin binding domain (UBD). The LIR enables the targeting of selective receptors to LC3 (or other homologs of the LC3 family) attached to the membrane of a forming autophagosome [93]. The UBDs (diverse ubiquitin binding domains in each receptor) can recognize and bind ubiquitin [94]. UBDs ensure that selective receptors bind to ubiquitinated cargos to target them for autophagy [95].

Eventually, during the maturation of autophagosomes into autophagolysosomes, the tail-anchored SNARE syntaxin 17 recruited to the membrane of autophagosome allows fusion with lysosomes [96]. This process also requires lysosomal membrane proteins LAMP1 and LAMP2 [97]. The result of autophagolysosomal fusion is the degradation of sequestered cargo by lysosomal hydrolases (Fig1)

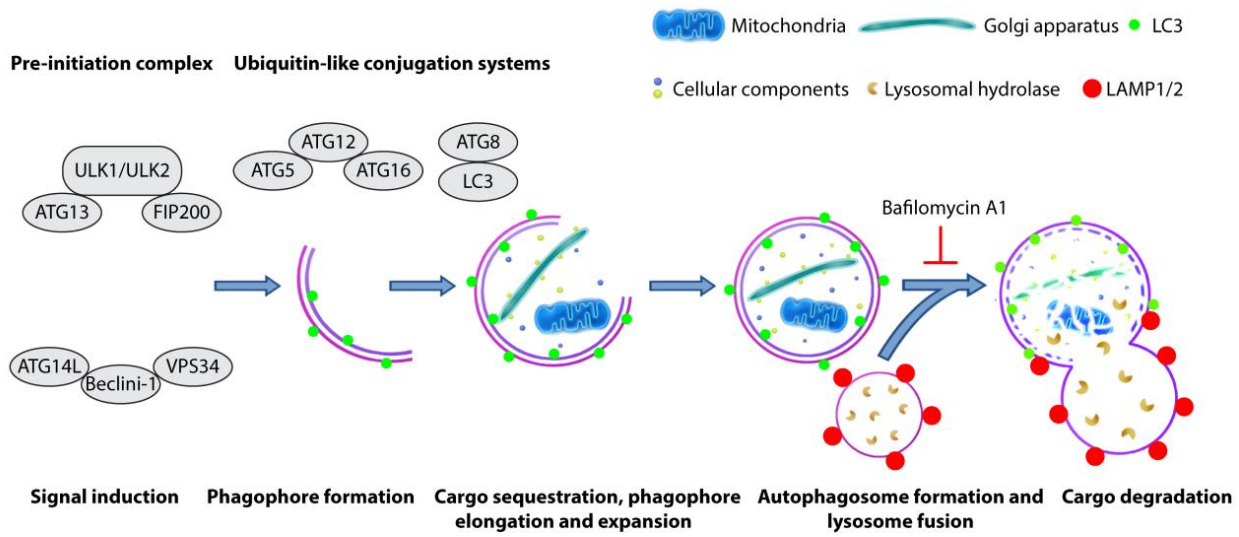

Figure 1: Schematic representation of the autophagy pathway

The basal level of autophagy activity is low under healthy conditions. To maintain homeostasis, autophagy is activated upon sensing cellular stress signals, such as nutrient deprivation or intracellular infections. Autophagy is induced through the activation of the ULK1/ULK2 complex which also contains ATG13 and FIP200. This complex subsequently interacts with the VPS34-Beclin1-Atg14L complex, contributing to the initiation of the isolation membranes (phagophores) from endomembrane sources such as endoplasmic reticulum (ER), Golgi apparatus, the mitochondria and the plasma membrane-derived endocytic organelles. Phagophores can sequester cargo via selective or autonomous recognition. The elongation and expansion of phagophores containing cargo requires the

involvement of the ATG5-ATG12-ATG16 and ATG8-LC3 ubiquitin-like conjugation complexes to form double-membraned autophagosomes. The maturation of autophagosomes involves fusion with lysosomes to form autophagolysosomes. This event requires the lysosomal membrane proteins LAMP1/2. After fusion, the sequestered cargo is degraded into amino acids and other small molecules by lysosomal and acidic hydrolases. The degraded material can be recycled and utilized as a source of energy for maintenance of cellular functions under the various stresses. LC3 is widely used as a general marker for autophagic activity and is involved in the entire process of autophagy.

2.3 Role of autophagy in immunity

Recent studies have demonstrated that defects in autophagy are associated with many diseases, including neuro-degenerative diseases, diabetes, cancer, and infectious diseases [98]. In this thesis, we focus on the function of autophagy in immunity, and in particular on its role in defense against the intracellular mycobacterial pathogens that cause TB. The main functions of autophagy in innate and adaptive immunity can be classified as follows: elimination of invading pathogens; control of pro-inflammatory signaling; antigen presentation to activate the adaptive immune system; and secretion of immune mediators [66].

Autophagy is a prominent innate immune mechanism by which an infected cell eliminates intracellular pathogens [66]. Invading microbes are recognized by pattern recognition receptors (PRRs), such as toll like receptors (TLRs) and NOD like receptor (NLRs). These receptors can recognize pathogen-associated molecular patterns (PAMPs), which are derived from microbes. PAMPs consist, for instance, of nucleic acids (e.g. bacterial DNA, double and single stranded RNA), or other molecules that are specific for invading pathogens (e.g. flagellin) [99]. For instance, recognition of lipopolysaccharide (LPS) – the outer membrane constituent of Gram-negative bacteria – by TLR4 leads to activation of autophagy [100]. Furthermore, LPS-induced autophagy enhanced the colocalization between mycobacteria and autophagosomes in cultured macrophages [100]. The function of autophagy in host defense against infection is well established for a number of invading microbes, including Mtb, Salmonella *Typhimurium*, *Shigella flexneri*, *Listeria monocytogenes* and *Streptococcus pyogenes* [101].

There is also increasing evidence that the process of autophagy participates in reduction and

modulation of inflammatory responses [102]. For instance, single nucleotide polymorphisms (SNPs) in genes central to the autophagy machinery significantly increase the susceptibility for Crohn's disease, which is characterized by uncontrolled inflammation in the gastrointestinal tract [103]. These results implicate that autophagy can affect the outcome of inflammatory disorders like Crohn's disease. One explanation for this is derived from the fact that autophagy controls the homeostasis and development of immune cells, such as macrophages, neutrophils and lymphocytes. These cells are all necessary for host immune and inflammatory responses and secrete cytokines and chemokines. Thus, defects in autophagy could indirectly result in poorly controlled inflammatory responses [66, 104].

However, a direct effect of autophagy on inflammatory processes has also been uncovered. Saitoh et al. (2008) first described that the loss of a central component in the autophagy machinery (ATG16L1) increased the production of pro-inflammatory cytokines when macrophages were stimulated with the endotoxin LPS. Their results demonstrated that autophagy directly controls the activity of the inflammasome, a multiprotein structure that promotes the maturation of interleukin 1 beta (IL1b) and interleukin 18 (IL18) and pyroptosis, an inflammation-associated type of programmed cell death [102]. Other studies expanded on these important findings, and it is now clear that autophagy controls the activity of inflammatory cytokines at the transcriptional level [105]; at the inflammasome-dependent processing step [102]; and during the excretion of mature cytokines [106]. This immune function of autophagy is highly relevant to TB pathogenesis, since nonresolving inflammation during mycobacterial infection fuels the generation of TB granulomas [107].

Several studies have demonstrated that autophagy is also involved in adaptive immune responses, including the regulation of antigen processing and presentation [66]. Inside antigen-presenting cells (APCs), autophagy can deliver cytoplasmic and nuclear antigens to lysosomes, which can then be presented to cells of the adaptive immune system ($CD4^+$ T cells) through the major histocompatibility complex class II (MHC-II) molecules [108]. This function of autophagy is also relevant to TB prevention, as it has been shown that stimulating autophagy-mediated antigen presentation increases the efficacy of BCG vaccination [109].

2.4 Autophagy as defense mechanism against Mtb infections

Susceptibility to active TB is partially genetically determined and variations in genes involved in the autophagic pathway have been identified that might disturb the host response to Mtb infection. A genome-wide association study has revealed a link between certain polymorphisms in ATGs and predispositions to TB in human patients. Three autophagy-related genes were identified from this screen, namely ATG16L1, IRGM, and VDR [110]. Multiple other studies have experimentally demonstrated the involvement of autophagy factors in controlling Mtb infections in cultured cells [79, 111, 112], including the demonstration of an important role for IRGM in the elimination of intracellular mycobacteria [113].

During Mtb infection, bacteria prevent phagosome maturation and are able to permeabilize the phagosomal membrane using region of difference 1 (RD1)-dependent virulence factors, which are secreted through the bacterial ESX-1 system [114]. This enables the pathogen to escape into the cytoplasm, which activates selective autophagy following recognition of the bacteria by PRRs. Even when Mtb remains inside a permeabilized and immature phagosome, its extracellular bacterial DNA can still leak from the phagosome and be recognized by the cytosolic DNA sensor STING (stimulator of interferon genes) [115]. Recognition by STING results in the labeling of bacteria with ubiquitin, which requires the ubiquitin ligases PARK2 (Parkin) and SMURF1 [116, 117]. This subsequently targets Mtb, or Mtb-containing immature phagosomes, for autophagolysosomal degradation via the ubiquitin-binding selective autophagy receptors p62 and NDP52 [115] (Fig2).

Besides directly targeting intracellular bacteria for xenophagy, p62 also contributes to defense against mycobacteria by delivering ubiquitinated cytosolic proteins to autophagolysosomes, where they are proteolytically converted into products capable of eliminating Mtb [118]. Thus, selective autophagy via the ubiquitin-binding receptor p62 presents an effective defense mechanism against intracellular mycobacterial infections via at least two mechanisms of action.

Despite the strong evidence – mostly from *in vitro* studies – demonstrating a role for autophagy in host defense against mycobacteria, the *in vivo* relevance of these mechanisms has recently

been questioned [119]. In a seminal study, Watson et al. (2012) previously found that mice with a monocyte/macrophage-specific deficiency in ATG5 were highly sensitive to Mtb infection and displayed elevated lung tissue damage. ATG5 is required for the early stage of autophagosome formation and ATG5 deficiency therefore affects the basal levels of autophagy [120, 121]. The study by Kimmey et al. (2015) recently showed that macrophage-specific ATG5 depletion indeed resulted in increased Mtb infection, but this was mostly due to an overstimulated inflammatory response, rather than to impaired autophagy. Furthermore, macrophage-specific depletion of other autophagy factors – including ULK1, ULK2, ATG4B, and p62 – did not affect the outcome of Mtb infection in mice. Instead, the authors of this paper suggest that autophagy-associated proteins may function independent of xenophagy to influence bacterial pathogenesis [119].

The discrepancies between *in vitro* and *in vivo* studies illustrate the need for further investigations into the role of autophagic defense against mycobacteria in animal models for TB. In this light, work using the zebrafish TB model can help to bridge the gap between mechanistic findings in cell culture models and their implications for disease outcome [52, 54, 58]. For instance, a study from our laboratory that combined *in vitro* and *in vivo* experiments, demonstrated the relevance of a novel signaling pathway controlling autophagic defense against mycobacterial infections [62]. In this study, analysis in Mtb infected human macrophages and the zebrafish model for TB revealed that the DNA damage-regulated autophagy modulator 1 (DRAM1) is activated downstream of pathogen recognition by TLRs. Signaling via the TLR-MYD88-NFκB innate immune sensing pathway activated DRAM1 and promoted selective autophagy against the bacteria. Transient knockdown of *dram1* in the zebrafish TB model leads to increased mycobacterial infection, whereas transient overexpression of Dram1 reduces infection by activation of autophagy. Finally, DRAM1-mediated selective autophagic defenses required the cytosolic DNA sensor STING and the selective autophagy receptor p62 [62].

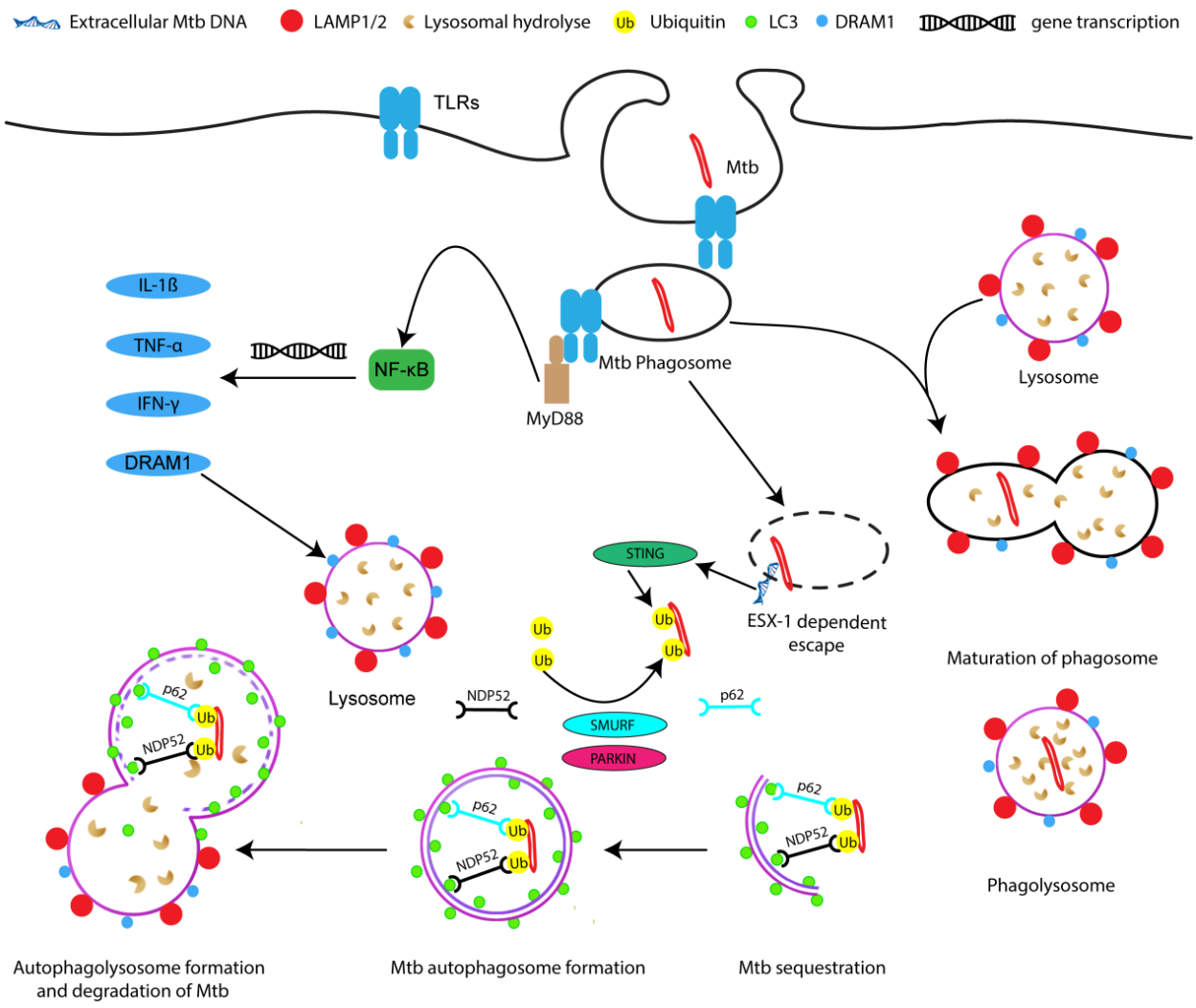

Figure 2: Schematic diagram of elimination of Mtb infection via the phagocytosis and autophagy pathways

The activation of autophagy via diverse signaling pathways plays an important function in the clearance of intracellular Mtb. Maturation of Mtb containing phagosomes partially attributes to killing of Mtb. However, Mtb inhibits phagosome maturation via multiple virulence mechanisms. For instance, Mtb utilizes its ESX-1 secretion system to escape from phagosomes into the cytosol. Cytoplasmic bacterial DNA can be recognized by the DNA sensor STING and promotes ubiquitination of Mtb. In this process, the ubiquitin ligases PARKIN and SMURF support the recruitment of ubiquitin to Mtb. Ubiquitin receptors, such as p62 and NDP52, recognize ubiquitinated Mtb and recruit LC3, contributing to the activation of autophagy to degrade Mtb. Finally, multiple factors stimulate autophagic clearance of Mtb. Mtb infection induces *DRAM1* as well as cytokine genes via a TLR-MYD88-NFκB signaling pathway. DRAM1 is thought to localize to the membrane of lysosomes to promote their fusion with autophagosomes.

3. The DRAM family of proteins

3.1 DRAM family proteins are regulators of autophagy

As described above, autophagy is orchestrated by several core proteins that are involved in all autophagic responses. In addition, autophagy regulators have been identified that are not critical components of the core autophagy machinery, but that play roles in regulating autophagy in specific situations or in response to specific stimuli. These autophagy regulators include the members of the DNA damage-regulated autophagy modulator (DRAM) family of proteins. DRAM1 was identified by Crighton et al. around one decade ago [122]. Until now, four other family members were identified and characterized as DRAM2/TMEM77, DRAM3/TMEM150B, DRAM4/TMEM150C and DRAM5/TMEM150A (Table 1). Currently, six Dram family members have been identified in zebrafish: Dram1, Dram2a, Dram2b, Dram3/Tmem150b, Dram4/Tmem150c and Dram5/Tmem150a. The DRAM family is conserved from humans to teleost fish, including zebrafish, with the exception that a DRAM1 homolog has not been identified in Coelacanth (*L. chalumnae*) yet (Fig3).

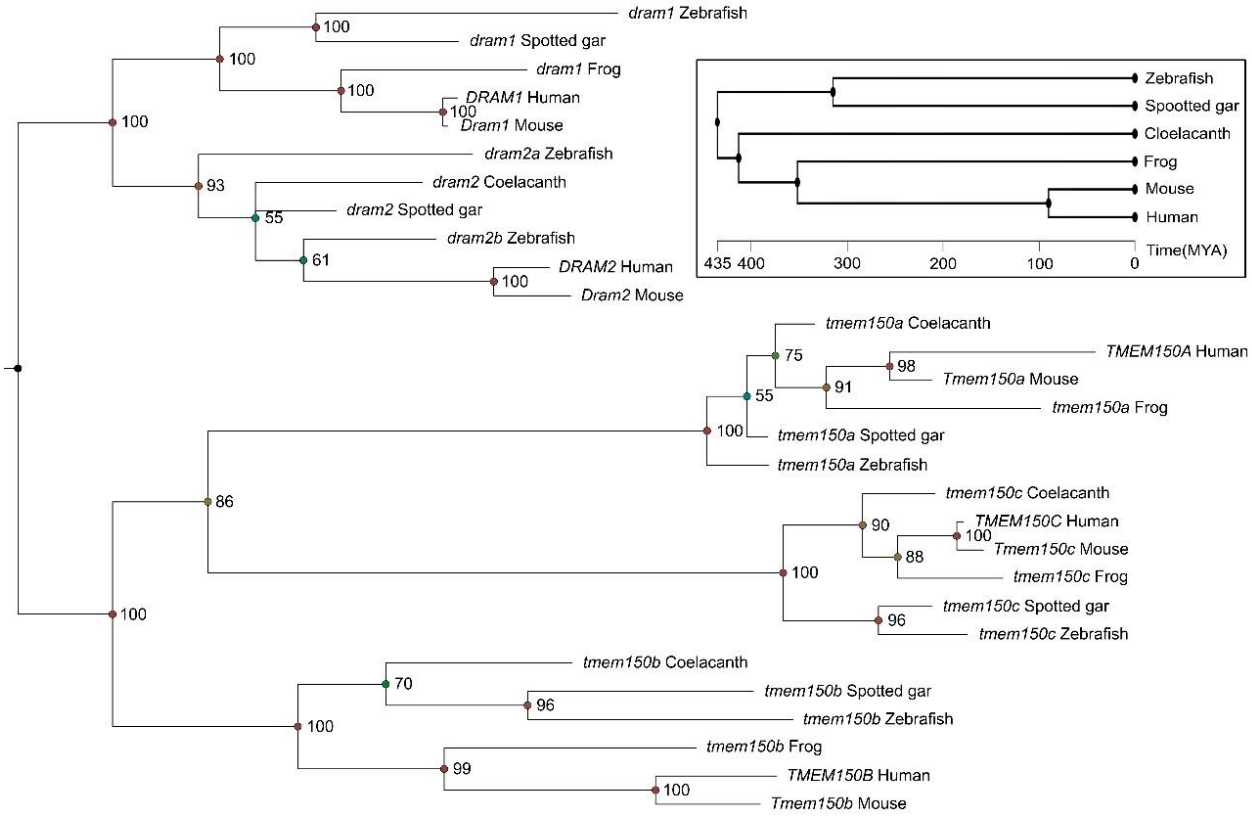

Figure 3: Phylogenetic comparison of DRAM family protein sequences from different species. Species include zebrafish (*Danio rerio*), spotted gar (*Lepisosteus oculatus*), coelacanth (*Latimeria chalumnae*), frog (*Xenopus)*, mouse (*Mus musculus*) and human (*Homo sapiens*). The protein sequences of DRAM1 (ENST00000258534.12), DRAM2 (ENST00000286692.8), DRAM3/TMEM150B (ENST00000326652.8), DRAM4/TMEM150C (ENST00000449862.6) and DRAM5/TMEM150A (ENST00000306353.7) were obtained from Ensembl. The phylogenetic tree was constructed with 4 independent MarKov chain Monte Carlo runs in MrByers 3.2.1 and each run consisted of 1,000,000 iterations sampled once every 200 iterations.

The human *DRAM1* gene encodes a protein that consists of 236 amino acids [122]. Protein domain analysis suggests that DRAM1 contains an endoplasmic reticulum (ER) targeting signal and six hydrophobic transmembrane regions (Table 1). DRAM1 is predominately found on lysosomes [122]. However, its presence in other compartments has also been described, including endosomes, peroxisomes, autophagolysosomes, the endoplasmic reticulum, and the Golgi apparatus [123]. Expression of *DRAM1* can be regulated by the tumor suppressing transcription factors p53 [122], p73 [124], and E2F1 [125], as well as the immunity-related transcription factor NF-κB [62]. It has been reported that DRAM1 is involved in the regulation of various cellular processes, including autophagy, apoptosis, immunity, and cellular differentiation [126]. For instance, DRAM1 is required to initiate autophagy and cell death downstream of p53-activation [122, 124]. Nonetheless, DRAM1 protein interactions remain poorly characterized. A direct interaction between DRAM1 and the apoptosis regulator BAX has been demonstrated [127], but the evidence for interactions with, for instance, p62 remains circumstantial [62, 128]. Given the many cellular functions DRAM1 is involved in, it is not surprising that this autophagy and cell death regulator has been implicated in several human diseases, including cancer [122, 124, 128-130], HIV[131] and tuberculosis [62].

DNA damage regulated autophagy modulator 2 (DRAM2) is closely related to DRAM1. Both DRAM1 and DRAM2 consist of six putative transmembrane domains and localize primarily to lysosomes [132, 133]. As is the case for DRAM1, overexpression of DRAM2 induces autophagic structures [134]. Moreover, silencing DRAM2 interferes with starvation-induced autophagy [134], which also implicates DRAM2 in regulation of autophagy. Like DRAM1, DRAM2 is also required for p53-dependent cell death, and overexpression of both DRAM1 and DRAM2 together was found to be sufficient to induce apoptosis [133]. DRAM2 was shown to interact with BECN1 and

UVRAG, essential components of the autophagy machinery, leading to the displacement of RUBCN from the BECN1-complex and promoting the activity of the class III phosphatidylinositol 3 kinase (PtdIns3K) [135]. DRAM2 also interacts with LAMP1 and LAMP2 to facilitate autophagosome maturation [135]. Although the transcriptional regulation of *DRAM2* remains to be determined, *DRAM2* mRNA levels have been identified as direct targets of down regulation by micro RNA (miRNA) 125b and miRNA144* [135, 136]. Downregulation of *DRAM2* is linked to human disease, as its expression was found to be reduced in ovarian cancers [133], and downregulation of *DRAM2* by miRNA125b promoted retino blastoma growth[136]. DRAM2 has also been implicated in tuberculosis, as further discussed below.

DRAM3 has an amino acid sequence overlap of 30% and a sequence similarity of 43% with DRAM1 [126, 137]. Like DRAM1, DRAM3 contains a signal peptide and several transmembrane domains. *DRAM3* has been detected in a range of normal tissues and tumor cells, but unlike *DRAM1*, its expression is not induced by p53 [137]. Similar to DRAM1, DRAM3 localizes to (auto)lysosomes and endosomes. However, it also localizes to the plasma membrane, which DRAM1 does not. The initial characterization of DRAM3 function revealed that it regulates autophagic flux and cell survival in response to starvation, but its effect on cell survival occurred independent of autophagy [137]

To date, DRAM4/TMEM150C and DRAM5/TMEM150A have been identified *in silico* as DRAM-family members but remain poorly characterized. DRAM5/TMEM150A was reported as the functional homologue of yeast Sfk1 [138]. DRAM5 forms a complex with PI 4-kinase type IIIα (PI4KIIIα) at the plasma membrane to regulate the generation of phosphatidylinositol 4,5-biphosphate $PI(4,5)P_2$ [138]. DRAM4 could also be detected at the plasma membrane but is primarily localized to lysosomes [138]. Clearly, the two remaining DRAM-family members are eagerly awaiting further characterisation.

Table 1: Interactions and functions of DRAM-family proteins in relation to human diseases

DRAM-family proteins	Protein domains	Protein localization	Interaction partners	Genetic regulation	Cellular functions	Involved diseases	First reported (year)
DRAM1	6 Transmembrane domains[122], Endoplasmic reticulum signal peptide[122]	Lysosomes [122, 139], Autolysosomes[123], Endosomes[123], Peroxisomes[123], Endoplasmic reticulum[123], Golgi apparatus[123]	p62[62], Bax[127]	p53[122], p73[124], NF-kB[62], E2F1[125], miRNA-26b[140]	Autophagy[122], Cell death[122], Cellular differentiation[129]	Cancer[122] APL[129] Ewing Sarcoma[130], Glioblastoma[128], HIV[131], Tuberculosis[62],	2006
DRAM2 (TMEM77)	6 transmembrane domains[133]	Lysosomes[133], Autophagosomes[134], Phagosomes[135]	DRAM1[133], Beclin1[135], UVRAG[135], Rubicon[135], LAMP1[135], LAMP2[135],	miRNA125b[136], miRNA144*[135],	Autophagy[134], Cell death[133],	Tuberculosis[135], Cancer[136],	2009
DRAM3 (TMEM150B)	Signal peptide[137], 6 Transmembrane domains[137]	Lysosomes[137], Autolysosomes[137], Endosomes[137], Plasma membrane[137]	-	-	Autophagy[137], Cell death[137],	-	2015
DRAM4 (TMEM150C)	-	Lysosomes[138], Plasma membrane[138],	-	-	-	-	2015
DRAM5 (TMEM150A)	-	Plasma membrane[138]	PI4KIIIα[138], EFR3[138]	-	Generation of $PI(4,5)P_2$[138]	-	2015

3.2 DRAM1 and DRAM2 play an important role in restricting mycobacterial infection

DRAM1 was first reported as a factor involved in host-pathogen interactions by Laforge et al. in 2013, who implicated DRAM1 in host defense against HIV infection via regulation of lysosome membrane permeabilization and subsequent cell death. This function of DRAM1 is dependent on activation of the p53 pathway and silencing of DRAM1 is shown to increase HIV infection[131]. Shortly thereafter, our group discovered that zebrafish Dram1 functions independently of p53 in host defense against intracellular mycobacteria [62]. As described before, we could demonstrate that mycobacterial infection induces zebrafish *dram1* and human *DRAM1* via a TLR-MYD88-NFκB signaling pathway. The autophagic defense against mycobacterial infection inferred by activation of zebrafish Dram1 also required Sting and the selective autophagy receptor p62 [62]. Furthermore, Dram1 promoted the fusion between bacteria-containing compartments and

lysosomes. Since expression of zebrafish *dram1* can also be induced by injection of the endotoxin LPS, we proposed that DRAM1 functions in defense against a spectrum of bacterial pathogens. This hypothesis was later confirmed by Masud et al. [141], who demonstrated that Dram1 also provides protection against infection by *Salmonella typhimurium*. A recent study using Mtb infected human macrophages revealed that DRAM2 also functions in defense against mycobacterial infections [135]. In this study, it was demonstrated that DRAM2 is required for acidification of Mtb-containing phagosomes. DRAM2 was shown to physically interact with a complex of autophagy regulators, including BECN1 and UVRAG, to remove the autophagy-inhibiting protein RUBICON from this complex and activate autophagy.

Concluding, both DRAM1 and DRAM2 have been demonstrated to participate in the immune response to mycobacterial infections, either *in vivo* using the zebrafish infection model (Dram1), or in vitro using human cell culture studies (both DRAM1 and DRAM2) [62, 135]. Interestingly, expression of human and zebrafish *DRAM1/dram1* is induced upon Mtb or Mm infection, while induction of miRNA144* reduces expression of *DRAM2* in response to Mtb infection [135]. The latter observation suggests that Mtb has evolved mechanisms to counteract the host's autophagy defenses. The interplay between the two DRAM-family members in defense against bacterial pathogens remains to be investigated.

4. Prospects of DRAM1 as a target for host-directed therapy against tuberculosis

4.1 Host-directed therapies as adjuvant for TB treatment

The rapid emergence of drug-resistant Mtb strains and co-morbidity caused by, for instance, HIV co-infections makes it difficult to treat TB patients [5]. Thus, the development of new and effective treatment regimens for TB is urgently needed. Currently, host-directed therapy (HDT) has gained interest as a complementary approach to antibiotic treatment. HDTs could transform traditional antibiotic therapies into more effective treatments and reduce the length of TB treatment regimens [142].

HDTs do not act like traditional antibiotics that directly target the pathogens and thereby put selective pressure on them. Therefore, application of these strategies might also reduce the development of drug resistance [5]. HDTs can increase host cellular responses to pathogens, counteract the cellular effects of disease-causing virulence factors, and activate immune responses (i.e. activation of autophagy, production of anti-microbial peptides, reactive oxygen species or cytokines), or reduce the pathological consequences of excessive inflammation [38, 142].

A range of candidate host-directed TB therapies have been developed aiming either at reducing the abundant inflammation and lung tissue damage typical of TB pathology, or at augmenting the specific innate and adaptive immune processes which directly target Mtb [38]. On the latter front, the most promising strategies for development of HDTs include 1) targeting the mechanisms of granuloma formation, 2) the induction of phagolysosomal fusion and autophagy, and 3) the modulation of cell-mediated immune responses [5, 38, 143].

Various pro-inflammatory cytokines are produced in response to Mtb infection, including TNF-α, IL1-b, IL-12, IL-17 and interferon gamma (IFN-γ) [144]. Inflammation functions as a double-edged sword during TB infection, and the levels of pro- and anti-inflammatory cytokine production can strongly affect the outcome of Mtb infections [14]. The balance of host inflammatory responses is also controlled by the production of lipoxin A4 (LXA4) and leukotriene B4 (LTB4): increased LXA4 levels are beneficial for a balanced inflammatory response and control of TB, while increased LTB4 levels produce the opposite effect with hyperinflammation and exacerbated infection [145, 146]. The inflammation induced by Mtb infection starts from the very early stages, and continues during the progression to active TB, until complete eradication [146]. This is at the basis of the current concept of modulating the inflammatory response as an HDT to reduce the lung tissue damage and adjust the host immune response [147]. For instance, a clinical trial has revealed that an IFN-γ adjuvant therapy can improve the outcome of TB treatment, resulting in significantly reduced respiratory symptoms and lung tissue damage, as well as reduced mortality compared to chemotherapy regimens without IFN-γ supplement. However, IFN-γ adjuvant therapy has also resulted in side effects, such as fever and headaches [148, 149].

The granuloma plays a central role in Mtb pathogenesis, encapsulating the bacteria to avoid Mtb

spreading into deeper tissue. As a side effect, granulomas also limit the effectiveness of anti-TB treatment due to poor penetration of antibiotics. TNF-α is known to be essential for granuloma maintenance and host defense against TB [150-152]. Hence, patients undergoing anti-TNF treatment for inflammatory diseases are at risk of activation of latent TB[151]. Nevertheless, neutralizing TNF-α during TB treatment with antibiotics could be promising strategy, as this was found to disrupt the architecture of granulomas and improve drug efficacy against Mtb [153]. However, the role of the granuloma in TB pathogenesis is not completely understood yet, which still restricts the use of this HDT in the clinic [26].

Given that autophagy is a critical immune defense mechanism against Mtb infection, this process is also a promising therapeutic target for TB treatment [154]. In fact, autophagy inducers were identified as hits in several drug screens for HDTs using Mtb infected human cells [155-157]. Furthermore, it was demonstrated that autophagy is required for effective anti-mycobacterial drug action of the first line drugs, such as isoniazid and pyrazinamide [156]. Both isoniazid and pyrazinamide treatment clearly induced autophagosome formation and co-localization of Lc3 with Mt.b in primary murine bone marrow-derived macrophages (BMDMs) [156]. However, it is a risk to induce canonical autophagy by non-selective drugs, due to the involvement of autophagy in diverse cellular functions. Rapamycin is a general autophagy-inducing drug which acts by inhibiting mTOR. Strikingly, treatment of zebrafish larvae with this drug increased susceptibility to mycobacterial infection, rather than decreasing it [62]. This could potentially be explained by the fact that Rapamycin is also known for its immunosuppressive effects on the host [158]. Thus, targeting autophagy to combat infectious diseases requires the development of specific modulators of autophagy.

4.2. Prospects of Dram-family members as host directed therapy against TB

Killing Mtb in infected macrophages in the early stages of infection is a key approach to avoid progression of TB disease. Two out of five DRAM family members (DRAM1 and DRAM2) have been implicated in anti-mycobacterial defense by enhancing autophagy and the microbicidal function of lysosomes either *in vitro* or *in vivo* [62, 135]. Thus, these DRAM family members are potential targets for HDTs that stimulate killing of Mtb by host-autonomous mechanisms.

Activation of zebrafish Dram1 leads to a significantly improved disease outcome following mycobacterial infection. The current bottleneck is to dissect how to pharmacologically stimulate DRAM1 in animal models or human patients. Based on our previous study, LPS injection is a strong inducer of *dram1* expression *in vivo* [62]. However, this approach carries severe risks in a clinical situation, as LPS injections can result in hyperactivation of inflammatory processes, or even toxic shock.

Another approach would be to directly inject DRAM1 recombinant protein into TB patients to elevate DRAM1 protein levels. DRAM1 protein could directly participate in defense against Mtb. However, it will be difficult to ensure that DRAM1 ends up at the appropriate location in infected cells to carry out its function. Thus, the more valid approach is to continue our study into the *in vivo* working mechanisms of DRAM1 (and other members of the DRAM family), to identify endogenous modulators that can serve as drug targets to stimulate DRAM1 activity. Identifying those might help to bring this research closer to clinical applications.

5. Outline of the thesis

The aim of the work described in this thesis was to exploit the benefits of the embryonic and larval zebrafish TB model to further study the function of selective autophagy in defense against mycobacterial infections. To this end we created null mutants for zebrafish Dram1, and the selective autophagy receptors p62 and Optineurin. The generated mutant lines were then used to study the role of these proteins in autophagic defense, as well as their potential effect on bacterial pathogenesis outside of autophagy.

This introductory **Chapter 1** provides background information about TB and autophagy and highlights that the DRAM family of proteins could be promising targets for host-directed therapy to modulate autophagy and eliminate mycobacterial infection.

Chapter 2 describes how mutation of the *dram1* gene leads to increased susceptibility to mycobacterial infection and highlights that the absence of Dram1 induces Caspase-1 dependent cell death of infected macrophages.

Chapter 3 reports on a transcriptome analysis of *dram1* mutants in the absence and presence of infection. This study revealed that deficiency in Dram1 has major effects on the expression of genes in pathways involved in metabolism, lytic cell death, and Toll-like receptor signaling.

Chapter 4 describes that mutation of the genes for the selective autophagy receptors Optineurin and p62 results in increased susceptibility to mycobacterial infection. These proteins mediate an autophagic defense response against mycobacterial infection by sequestering ubiquitin-labeled bacteria into autophagosomes.

Chapter 5 summarizes and discusses the findings presented in this thesis in relation to the latest scientific advances in TB and autophagy research.

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