Illuminating host defence against mycobacterial infection: interactions with autophagy and LC3-associated phagocytosis

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CHAPTER 1

General Introduction & Thesis outline

1.1 Mycobacteria and immune defence: a delicate balance

Tuberculosis (TB) is the world's leading infectious disease killer, causing a total of 1.25 million deaths in 2023. TB is present in all countries and age groups, though half of the cases are distributed among Bangladesh, China, India, Indonesia, Nigeria, Pakistan, Philippines and South Africa[1]. The bacterium *Mycobacterium tuberculosis* (*Mtb*) is the causative agent of TB. This pathogen has coevolved with humans over thousands of years[2,3], specialising in evasion, modulation and exploitation of the host immune response[4,5]. *Mtb* is transmitted through aerosol droplets from sick individuals. Once inhaled by the new host, *Mtb* is phagocytosed by alveolar macrophages, where, by undermining cellular defence mechanisms such as lysosomal trafficking, *Mtb* is capable of surviving intracellularly[6,7]. As such, bacteria can establish a replicative niche as well as exploit macrophages as vehicles for dissemination[8].

There are over 200 species in the *Mycobacterium* genus[9], and they are classified into different groups: TB-causing, leprosy-causing and nontuberculous mycobacteria (NTM). The TB-causing group includes species capable of causing disease in humans or animals, like *Mtb* and *M. bovis,* respectively. NTM can cause disease in immunocompromised individuals, like *M. marinum* (*Mm*) and *M. avium*[10]. These diseases manifest among others as lung infections, skin infections and disseminated infections. *Mm*, which causes skin infection in humans, is of particular interest because the type of disease that it causes in cold-blooded animals shows strong similarities to human TB. In this thesis, we use *Mm* infections in zebrafish larvae as a model to get a better understanding of host-pathogen interactions during the early stages of infection, where the pathogen establishes its replicative niche in macrophages.

Mycobacterial infection success is, above all, due to the ability of mycobacteria to survive in diverse intracellular environments. Several virulence factors mediate intracellular survival. For example, it has been shown that both *Mtb* and *Mm* avoid acidified compartments but also have acidic resistance, which enables them to withstand eventual exposure to acidification to some extent[11,12]. The mycobacterial membrane serine protease MarP is one of the virulence factors that enables evasion of acidification, as studies demonstrated that MarP-deficient mycobacteria are killed specifically in lysosomes[11]. Once degradation is avoided, bacteria can replicate, eliciting the macrophage's lytic cell death or necrosis. This allows the bacteria to infect other macrophages and disseminate.

To halt infection dissemination and promote mycobacterial elimination, the host creates a physical immunological barrier, the granuloma, the pathological hallmark of TB. Granulomas are organised aggregates of macrophages, multinucleated giant cells, epithelioid and foamy cells, neutrophils, and lymphocytes.[6] Granulomas succeed in restricting and containing the infection, which requires an effective adaptive immune response. Immunocompromised individuals with HIV infection present CD4$^+$T cell dysfunction, and therefore, they fail to form well-organised granulomas and develop active TB[13,14]. Mycobacteria have developed strategies to overcome the host defences in the granuloma and manipulate them for their growth. Research performed in the zebrafish-*Mm* infection model enabled the visualisation of early granuloma formation and the identification of host and bacterial factors contributing to this process[15-18]. In infected macrophages, the mycobacterial type VII secretion system ESX-1 and its effector protein early secreted antigenic target 6 kDa (ESAT-6) induce apoptotic cell death and recruitment of uninfected macrophages[19]. ESX-1/ESAT-6 has been proposed to stimulate the recruitment of new macrophages by inducing the secretion of matrix metalloproteinase 9 (MMP9) in epithelial cells surrounding the nascent granuloma, resulting in cellular expansion of the granuloma[20]. Newly recruited macrophages phagocytose dying infected cells and their bacterial content; with this, mycobacteria ensure the expansion of their replicative niche.

When infected macrophages in the granuloma die by necrosis instead of apoptosis, bacteria are released within the granuloma environment, forming an acellular necrotic core called a caseous core. In this region, bacteria are surrounded by layers of foamy macrophages that provide a niche for dormant bacteria[14,21]. At the necrotic caseum, the passive diffusion of antibiotics is limited, producing regions of suboptimal antibiotic concentrations and monotherapy, contributing to the selection of drug-resistant strains[22-24]. Drug-resistant *Mtb* strains are a major concern for global health. In 2023, 175,923 people were diagnosed and treated for Multidrug-resistant TB (MDR-TB)[1]. The appearance of drug-resistant *Mtb* variants has been blamed on inadequate implementation of control measures. Still, since MDR-TB evolve in well-functioning health systems, more factors need to be considered[25]. Elements of the host-pathogen interaction are increasingly recognised to influence the evolution of drug resistance[3,25]. Increased understanding of host-pathogen interaction could enable the development of better or new therapies for the prevention and treatment of TB[26,27].

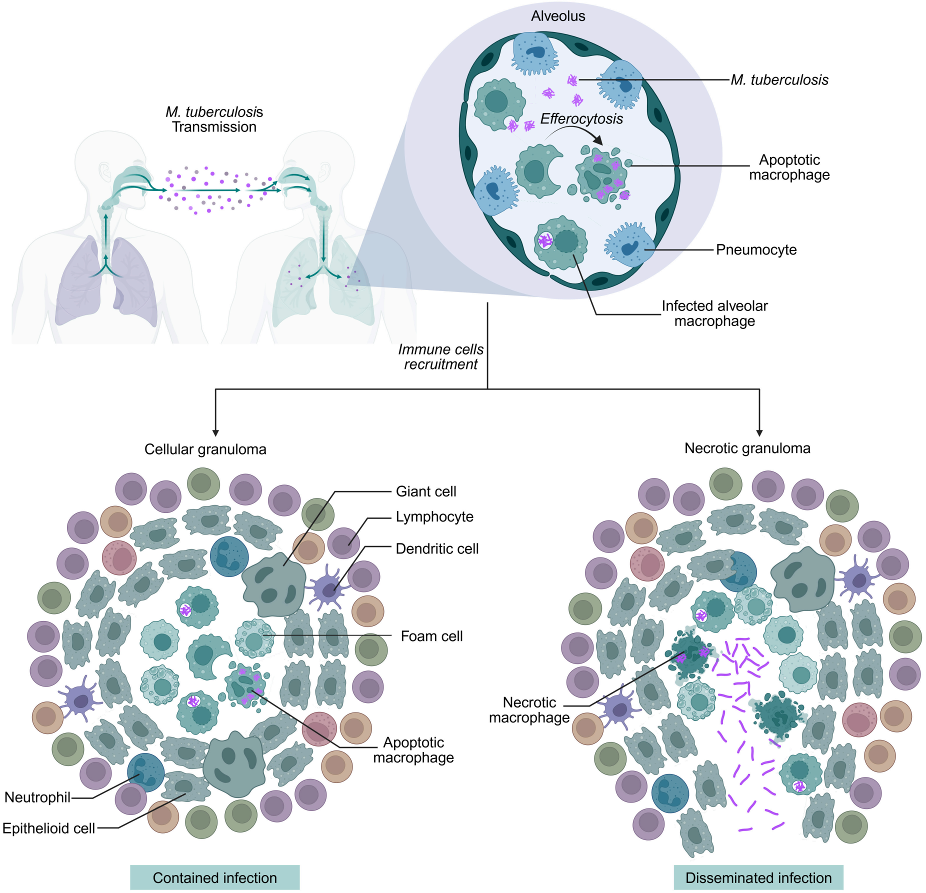

Figure 1.1: *Mycobacterium tuberculosis* pathogenesis.

Mtb is transmitted through aerosol droplets from sick individuals. Once inhaled by the new host, *Mtb* is phagocytosed by alveolar macrophages. Infected macrophages die by apoptosis, causing the recruitment of immune cells. Newly recruited macrophages phagocytose dying infected cells and their bacterial content; with this, mycobacteria ensure the expansion of their replicative niche. To halt infection dissemination and promote mycobacterial elimination, the host creates a physical immunological barrier, the granuloma. When infected macrophages in the granuloma die by necrosis, bacteria are released within the granuloma environment. Finally, the granuloma collapses, and the infection is disseminated.
Schematic created with BioRender.

1.2 One beneficiary, many obstructions: How mycobacteria halt intracellular vesicle trafficking

Macrophages are the first responders against invading microbes, including infection by mycobacterial species. Pathogen-associated molecular patterns (PAMPs) on the *Mtb* surface, such as glycolipids, lipoproteins, and carbohydrates, activate host cell surface and intracellular pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and cyclic GMP-AMP synthase (cGAS)-STING[28,29]. These receptors are involved in the activation of innate immune response mechanisms against mycobacterial infection, consisting of the phagolysosomal pathway, autophagy, inflammasome activation, and apoptosis[30]. Mycobacteria are phagocytosed in a phagosome, whose aim is to traffic the bacteria to lysosomal degradation. Effective pathogen transport relies on a highly dynamic interaction between phagosomes and intracellular vesicles, where phagosomes undergo structural and compositional changes referred to as phagosome maturation.[31] Phosphoinositides (PtdIns) play a significant role in phagolysosome formation and maturation. Through recruitment and interaction with effector proteins, they regulate the rearrangement of the actin cytoskeleton, ion and organelle transport, signal transduction, and membrane fusion-fission events mediating phagosome maturation and fusion with lysosomes[32-34]. After phagocytosis, early phagosomes fuse with early endosomes (EE), acquiring the RAS-associated protein RAB5A (Rab5) GTPase, which regulates fusion and traffic of EE. GTP-bound Rab5 recruits effector vacuolar protein sorting 34 homolog (Vps34), a phosphatidylinositol (PI) 3-kinase (PI3K), that generates phosphatidylinositol-3-phosphate (PtdIns3P)[35]. Rab5 and PtdIns3P recruit EEA1, which docks early phagosomes and EE together, priming them to fuse via soluble NSF attachment protein receptor (SNARE). Conversion of early to late phagosomes requires the recruitment of RAS-associated protein RAB7A (Rab7) GTPase, which catalyses the fusion with LE and lysosomes. Finally, phagosome-lysosome fusion occurs via SNAREs associated with lysosomes. As the phagolysosome is shaped, it acquires v-ATPase $H^{\pm}$ and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activities, which contribute to the acidification and the production of reactive oxygen species (ROS) in the phagolysosome, respectively[31].

Mtb has evolved virulence factors that alter phagosome dynamics and maturation, allowing it to replicate within infected macrophages. *Mtb* is capable of blocking phagosome maturation by affecting phagosome acidification, the acquisition of hydrolytic enzymes and antimicrobial peptides[6,8,14]. To interfere with this pathway at the Rab GTPases level, *Mtb* secretes the nucleotide diphosphate kinase (Ndk) that inactivates Rab5 and Rab7[36]. Furthermore, the recruitment of Rab22a inhibits the phagosome fusion with LE, affecting the Rab5-Rab7 conversion[37]. As a result of these effects on Rab GTPase function and localisation, *Mtb* phagosome maturation is arrested[38]. This arrest is further sustained because *Mtb* mannose-capped lipoarabinomannan (ManLAM) present in the bacterial envelope can block the increase in cytosolic Ca$^{+2,}$ blocking PtdIns3P production by VPS34 in EE[39,40]. *Mtb* also produces the secreted acid phosphatase (SapM), which degrades PtdIns3P[40-42]. These impacts on phosphoinositide production will eliminate Rab GTPase and EEA1 recruitment, and thereby *Mtb* prevents PtdIns3P-mediated phagosome-lysosome fusion.

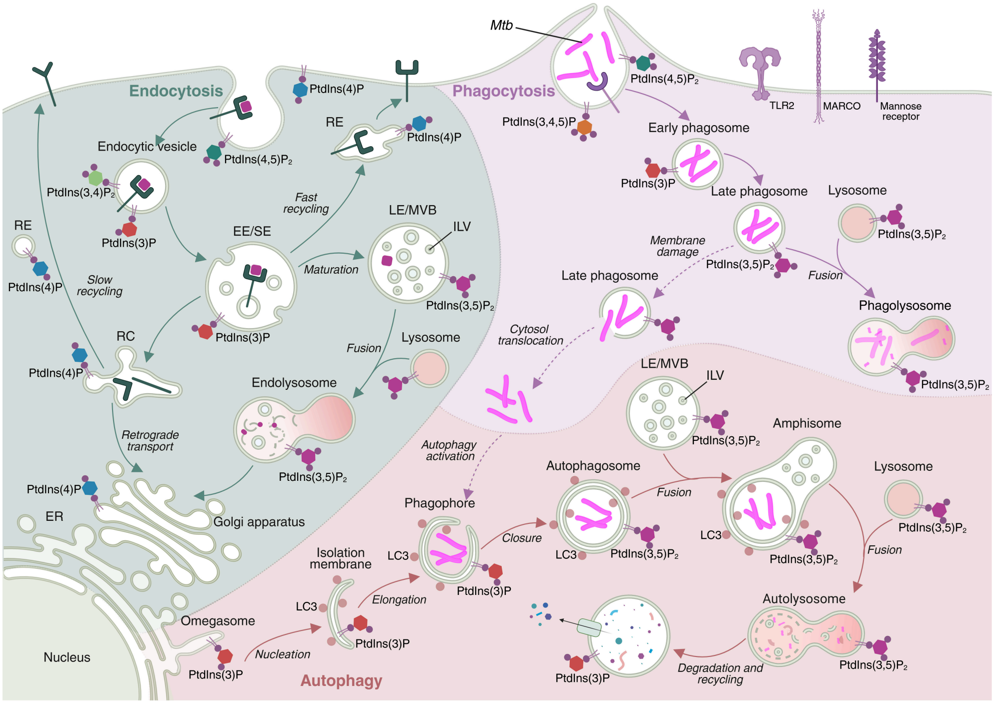

Figure 1.2: Phosphoinositides in vesicle trafficking of mycobacterium-infected cells.

The schematic illustrates the subcellular enrichment of PtdIns in steps of endocytosis and phagocytosis/autophagy pathways targeting *Mtb*. The presence of different PtdIns, converted by kinase and phosphatase activities, characterises the membrane of each vesicle and regulates its functions. The localisation of PIKfyve kinase (Chapter 4) products PtdIns(3,5)P$_2$ and PtdIns(5)P, as well as the precursor PtdIns(3)P, is depicted. PtdIns(3,5)P$_2$ localises in LE, MVB, lysosomes, phagolysosomes and autolysosomes. EE: early endosome; ER: endoplasmic reticulum; LE: late endosome; MVB: multivesicular body; RE: recycling endosome. Schematic created with BioRender.

Considering the importance of PtdIns in phagolysosome formation, the enzymes catalysing PtdIns production play critical roles in the innate immune response. As previously mentioned, PtdIns3P, together with Rab5, recruits EEA1 and facilitates fusion events between early phagosomes and EE. PtdIns3P can be removed from the phagosome due to its conversion by the enzyme phosphoinositide kinase, FYVE-type zinc finger containing lipid kinase (PIKfyve), which converts PtdIns(3)P to PtdIns(3,5)P$_2$[43]. PIKfyve inhibition impairs phagosome maturation, resulting in reduced phagosome-lysosome fusion and degradative capacity[44,45]. PIKfyve has been implicated in the pathogenesis of intracellular pathogens, such as *Coxiella burnetii*[46,47], *Legionella pneumophilia*[48], and *Salmonella* Typhimurium[49,50]. In these infections, PIKfyve inhibition restricted the expansion of the pathogen-containing vacuoles, indicating that these pathogens exploit PIKfyve to transform the phagosome into a replicative niche. The role of PIKfyve in mycobacterial infections has remained unknown and is addressed in this thesis.

1.3 Autophagy to the rescue?: The need for timely intervention in anti-mycobacterial immunity

Inside the phagosome, *Mtb* focuses on ensuring a successful intracellular lifecycle. Like many other aspects of *Mtb* virulence, this is determined by the ESX-1 type VII secretion system[51]. ESX-1 mediates phagosomal membrane damage that enables *Mtb* to access nutrients and deliver effectors to the cytosol. Damaged *Mtb* phagosomes expose their intraluminal content, activating the cytosolic surveillance pathway. Galectins target host glycans inside the phagosome[52,53], and bacteria are labelled with ubiquitin by SMURF[54] and parkin[55] ubiquitin ligases. Galectins and ubiquitin are recognised by selective autophagy adaptors, such as Nuclear Domain 10 Protein 52 (NDP52), optineurin (OPTN), and sequestosome 1 (SQSTM1, also known as p62), and are directed to autolysosomal degradation[56]. Phagosomal damage also allows *Mtb* molecules to be released into the cytosol and recognised by cytosolic PRRs. *Mtb* DNA[57,58] and cyclic-di-adenosine monophosphate[59] (c-di-AMO) are recognised by cytosolic PRRs, cyclic GMP-AMP synthase[60-62] (cGAS) and stimulator of interferon genes protein[63] (STING), inducing type I IFN[64] response and autophagy.

Autophagy is a cellular pathway important for maintaining cellular homeostasis as well as for immunity. By delivering cytoplasmic contents to the lysosome for degradation, it provides adaptation to nutrient starvation, selectively degrades proteins and foreign substances, clears damaged organelles, and counteracts ageing[65]. In the immune system, autophagy plays protective roles during microbial infections as a cell-autonomous defence mechanism of innate immune cells, by controlling inflammation, and by generating peptides for antigen presentation[66,67]. Upon mycobacterial infection, selective autophagy is part of the host macrophages' immune response. As discussed above, mycobacteria are labelled with ubiquitin[68,69] and recognised by autophagy adapters like sequestosome 1 (SQSTM1)[70] and optineurin (OPTN)[71,72]. These adapters recruit the microtubule-associated protein 1 light chain 3 (LC3), which coats the double membrane of nascent phagophores (cytosolic isolation membranes), causing the engulfment of the bacteria into autophagosomes. In the same way as in phagosome maturation, PtdIns play a role in autophagosome maturation. Autophagosomes form at parts of the endoplasmic reticulum (ER) enriched in phosphatidylinositol-3-phosphate (PtdIns3P). FAK family kinase-interacting protein of 200 KDa (FIP200) interacts with the ER-enriched domain[73] and induces the assembly of the Unc-51-like autophagy activating kinase 1 (ULK1) complex[74], comprised of FIP200, autophagy-related proteins ATG13 and ATG101, and ULK1. ULK1 phosphorylates and activates phosphatidylinositol 3-kinase class III complex 1 (PI3KC3-C1)[75], allowing the phagophore expansion. PI3KC3-C1 is composed of five subunits: VPS34, PI3-kinase regulatory subunit 4 (VPS15), Beclin 1 (BECN1), ATG14, and nuclear receptor-binding factor 2 (NRBF2). Phagophore elongation and closure to a mature autophagosome require phosphatidylethanolamine (PE) to be conjugated to LC3[76]. Autophagosome closure is catalysed by the endosomal sorting complex required for transport complex (ESCRT)[77,78], and regulated by Ras-related protein Rab-5A (Rab5)[79,80]. Consequently, pleckstrin homology domain-containing family M member 1 (PLEKHM1), ectopic P-granules autophagy protein 5 Homolog (EPG5) and RAB7[81] tether autophagosomes with lysosomes. Ultimately, two SNARE complexes, syntaxin 17 (STX17)-synaptosome-associated protein 29 (SNAP29)-vesicle-associated membrane protein 7 (VAMP7/8), and synaptobrevin homolog YKT6 (YKT6)-SNAP29-STX7, trigger the autophagosome fusion with the lysosome to degrade its content[66,76,82].

While autophagy is important as a host defence mechanism against mycobacterial infection[83-85], *Mtb* has evolved ways to evade autophagic degradation and benefit from this host defence mechanism. *Mtb* disturbs autophagy initiation and flux, as well as autophagosome fusion with lysosomes. Furthermore, *Mtb* can also escape from autophagosomes back into the cytosol[86]. Since autophagy initiation depends upon PI3P, the effectors discussed above that impair PI3P production, such as ManLAM, have also been found to impair autophagy. In vitro studies showed that ManLAM reduces LC3 trafficking to phagosomal membranes[87]. Additionally, *Mtb* activates the host F-actin-binding protein Coronin 1a (CORO1A), which blocks the p38 MAPK pathway required for autophagy induction through TLR signalling[88]. Regarding autophagic flux, it has been shown that *Mtb* induces the expression of the host microRNA miR-33, which in turn downregulates the expression of autophagy genes including ATG5, ATG12, LC3, and lysosome-associated membrane glycoprotein 1 (LAMP1). This leads to a reduction in autophagic flux, as evidenced by a decrease in the recruitment of p62 and LC3[89]. Moreover, *Mtb* secretes enhanced intracellular survival (Eis), which negatively modulates autophagy, and this inhibition depends on ROS production[90,91]. Lastly, autophagosome-lysosome fusion blockage by *Mtb* is linked to Rab7[92] and requires, among others, the lipid phosphatase SapM[93,94]. SapM interacts with Rab7, inhibiting its recruitment to autophagosomes, blocking the maturation of *Mtb*-containing autophagosomes into autolysosomes[95].

Accumulating evidence establishes a crucial protective role for autophagy in the immune response against early mycobacterial infections[96-99]. As a result, more research is needed to elucidate the regulatory roles of autophagy during *Mtb* infection and to consider autophagy as a potential therapeutic target for TB control[83,100].

1.4 Is the alternative better? LC3-associated phagocytosis in anti-mycobacterial immunity

As a substitute for the autophagy pathway or a parallel pathway, LC3-associated phagocytosis (LAP)[101,102] is a mechanism of phagocytic cells, including macrophages, where a subset of autophagy machinery is used in the LC3 lipidation to the single membrane of the phagosome (forming a LAPosome)[101,103,104]. In comparison to intracellular autophagic cargo, LAP cargo comes from the extracellular environment[105], where it binds surface receptors such as fragment crystallizable gamma receptor (FcγR[106]), TLRs[107], C-type lectin domain family 7 member A (CLEC7/dectin-1[108]), or T-cell immunoglobulin mucin receptor 4 (TIM4)[109]. Following receptor ligation, phagosome cup development is regulated by the same mechanism that regulates phagocytosis (previously discussed[35]), and is, in contrast to phagophore formation in autophagy, independent of the ULK1 initiation complex. LAP requires the assembly of the PI3K3 complex, but its composition differs from the autophagy complex. Formation of the LAP complex uniquely requires ultraviolet radiation resistance-associated gene protein (UVRAG) and RUN domain and cysteine-rich domain-containing, Beclin 1-interacting protein (Rubicon or Rubcn), and shares with autophagy the presence of Beclin1, VPS34 and VPS15[110]. VPS34 lipid kinase produces PI3P on the surface of the closed phagosome, on which ROS production and LC3 lipidation depend[111]. ROS production in the phagosomal lumen is carried out by NADPH oxidase-2 complex (NOX2)[112], which associates with PI3P on the phagosome membrane through its p40^{phox} subunit[113]. Furthermore, NOX2-generated ROS are required for the assembly of V-ATPase subunits, allowing the interaction with ATG16L1, which drives LC3 conjugation to the phagosomal membrane[114]. Thus, both NOX2 activity and PI3P generation are needed for the assembly of the LC3 conjugation machinery, where LC3 is transferred from ATG3-LC3 to the PE lipid by the vATPase-ATG16L axis[115,116]. Following the lipidation of LC3 on the phagosomal surface, the now denominated LAPosome fuses with the lysosome, and the phagocytosed cargo becomes degraded.

Pathogens have been shown to induce or suppress LAP activation[117]. Research on fungi such as *Candida albicans*[118] and *Aspergillus fumigatus*[119-121] has established a melanin-mediated LAP blockage across species[122,123], underlining LAP's relevance in fungal infection clearance[124]. Studies in bacteria have shown species-specific mechanisms involved in LAP recognition and activation, with a significant molecular overlap[110]. Among the bacteria targeted by LAP are *Legionella dumoffii,*[125] *Listeria monocytogenes*[126,127], *Burkholderia pseudomallei*[128,129], *Shigella flexneri*[130,131], *Salmonella* Typhimurium[114,132,133]. Each species has evolved unique strategies to deal with LAP-mediated degradation[134]. Little is known about the role of LAP in mycobacterial infection. In vitro studies have shown that *Mm* resides transiently in single membrane compartments and that LC3 recruitment depends on the ESX-1 secretion system[135]. The *Mtb* virulence factor CpsA has been shown to inactivate NADPH oxidase[136], protecting bacteria from LAP elimination. Further research is needed to elucidate the underlying mechanisms which initiate LAP and lead to LC3 recruitment onto mycobacteria-containing phagosomes, as well as the relevance of LAP as a defence against mycobacterial infection.

Rubcn is of special interest in this regard, as it has been characterised as an autophagy inhibitor by impeding PI3P production and LC3 lipidation[137-139], and at the same time, as a LAP activator and essential component[101]. In LAP, Rubcn stabilises NOX2 complex through direct binding with p22^{phox} subunit, thus facilitating ROS production and stimulating the subsequent interaction between v-ATPase and ATG16L1, which drives LC3 conjugation to the phagosomal membrane[114,140]. In autophagy, Rubcn interacts with VPS34 via its RUN domain, suppressing its lipid kinase activity and negatively regulating autophagosome formation[137,141,142]. Lastly, Rubcn interacts with UVRAG through its coiled-coil domain. UVRAG interacts with the C-VPS/HOPS complex in late endosomes, which in turn activates Rab7 by promoting GTP binding[143]. Rab7 activation is crucial for the transition from early to late endosomes in endosome maturation. UVRAG-Rubcn association in early endosomes, where Rubcn is enriched, impedes UVRAG-C-VPS/HOPS binding and, consequently, Rab7 activation. As a result, UVRAG sequestration by Rubcn negatively regulates endocytic trafficking and autophagy[144].

Rubicon has been shown to promote host defence against several pathogens, including *Listeria monocytogenes*[126,127], *Aspergillus fumigatus*[119,120] and *Salmonella* Typhimurium[132]. In addition, it has been reported that the maturation of *Mtb*-containing phagosomes is negatively regulated by Leucine-rich repeat kinase 2 (LRRK2) through recruiting the PI3KC3 complex and Rubcn[145]. However, the role of Rubcn in mycobacterial infection remains to be further established.

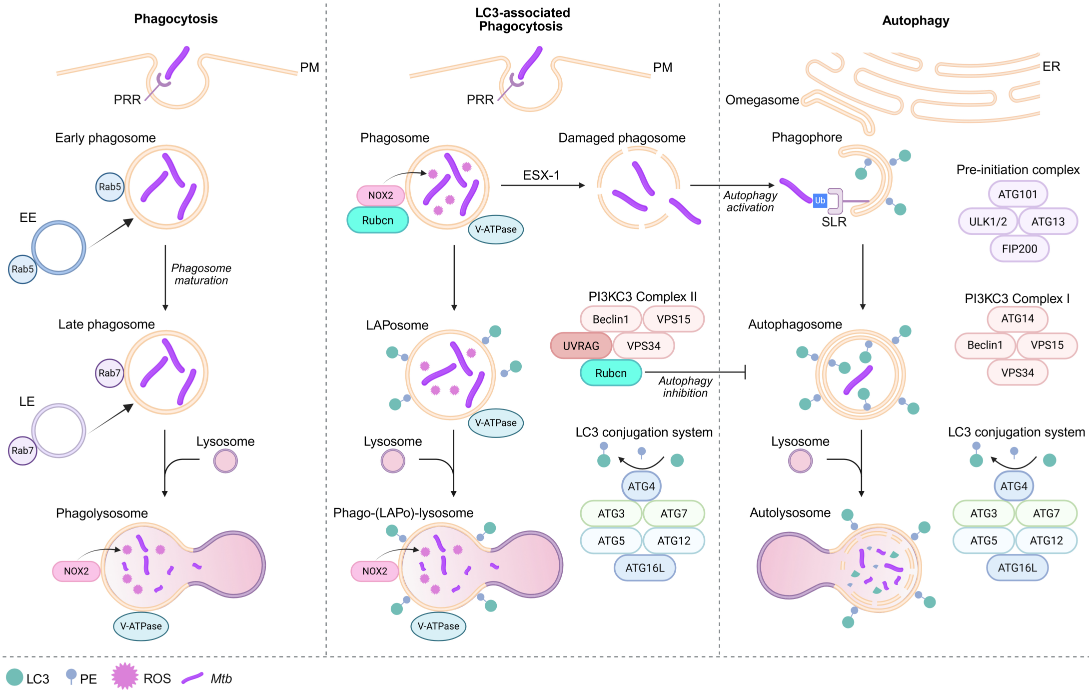

Figure 1.3: Molecular mechanisms of phagocytosis, LC3-associated phagocytosis and autophagy during *Mtb* infection.

Mtb activates host cell surface PRR and is phagocytosed in a single membrane phagosome. After phagocytosis, early phagosomes fuse with EE, acquiring Rab5. Sequential recruitment of Rab7 by fusion with LE catalyses the fusion with lysosomes. In LAP, NOX2, stabilised by Rubcn, assembles on the *Mtb*-phagosome, inducing ROS production in the phagosomal lumen. ROS allow the assembly of V-ATPase and its interaction with ATG16L1, which drives LC3 conjugation to the phagosomal single membrane, forming the LAPosome. *Mtb* virulence factor ESX-1 promotes phagosomal damage, activating the cytosolic surveillance pathway. *Mtb* are labelled with ubiquitin and recognised by autophagy adapters (SLR). These adapters recruit LC3 to the double membrane of the phagophore. This elongates and closes, forming the autophagosome, which is targeted to lysosomal degradation. EE: early endosome; LE: late endosome; PRR: pattern recognition receptor; SLR: sequestosome 1-like receptor, also known as selective autophagy receptor. Schematic created with BioRender.

1.5 When one door closes, a transparent window opens: Illuminating mycobacterial host-pathogen interactions in zebrafish

Even though TB has afflicted human populations for millennia, many unknowns persist about this highly successful pathogen. Technological progress has made it possible to design more complex experiments in order to reduce the gap between disease models and human disease. In this context, studies using animal models are crucial in that they have provided information on the disease mechanism, while at the same time, they have been used for pre-clinical testing of TB treatments. Animal models used for TB research include *Mus musculus* (mouse), *Oryctolagus cuniculus* (rabbit), *Cavia porcellus* (guinea pig), and non-human primates (NHP). Although no animal model can recapitulate TB in its entirety, their collective contributions have been significant[146]. Among these, the mouse has been the most commonly used model for vaccine research[147] and drug testing against TB[148]. However, differences in immunological responses between the mouse model and humans, such as difficulties in mimicking caseating granuloma formation, have promoted the development of new animal models. For the past two decades, *Danio rerio* (zebrafish) has become an alternative for studying mycobacterial infection[149,150]. Human and zebrafish have similar innate and adaptive immune systems, and more than 80% of human disease-associated genes are present in zebrafish[151,152]. The Zebrafish TB model is based on the Zebrafish-*Mm* natural host-pathogen interaction. *Mm* and *Mtb* genomes share an 85% identity[153], and *Mm* has been shown to cause infection conducive to granuloma formation in larvae[154] and adult individuals[155]. The embryo/larva model is used to study the innate immune response against mycobacterial infection in isolation from adaptive immunity. The embryo and larva external development and transparency enable microinjection of fluorescently labelled *Mm* strains into transgenic zebrafish (with fluorescently labelled macrophages or neutrophils)[156], facilitating imaging and analysis of early host-pathogen interactions in vivo. The Zebrafish-*Mm* infection model has contributed to many aspects of the host-pathogen interaction, including the understanding of the role of granuloma formation[19,157,158] and vascularisation[159,160], infection dissemination[161-163], and the intracellular mechanisms of infection[11,164] and host defence[71,165-167]. Significantly, the use of the model led to understanding that many of the cellular pathological effects observed in mycobacterial infection, including the initiation of granulomas, are dependent on the ESX-1 secretion system[168,169].

As discussed in detail in Chapter 2, the zebrafish model has proven very useful to investigate autophagy's role in infection[170,171]. Its capacity for whole-organism in vivo imaging provides a unique advantage for the autophagy field, enabling the observation of host-pathogen interactions in a physiological context at an intracellular level.

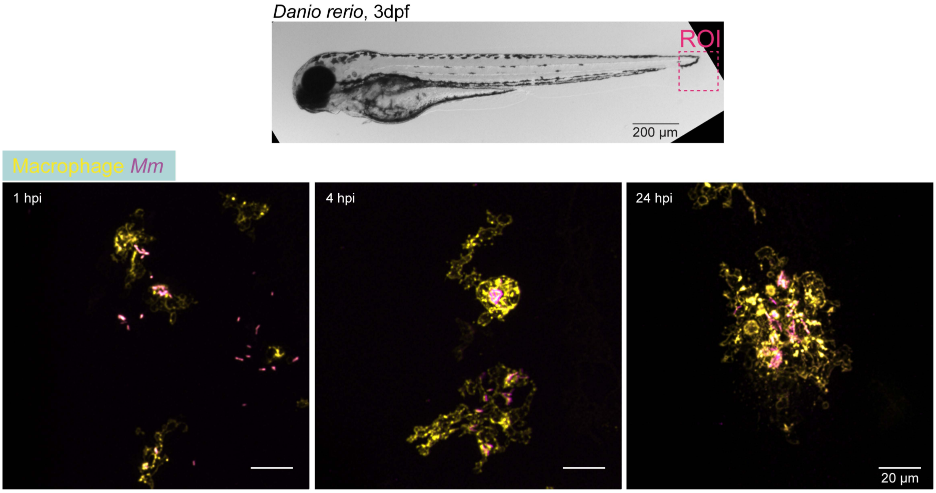

Figure 1.4: Zebrafish-*Mm* tail fin infection model.

Microinjection of fluorescently mCrimson-labelled *Mm* (150 CFU, pseudo colour magenta) strains into the tail fin area of 3dpf zebrafish larvae carrying the construct (*mpeg1:mCherry-F)* that labels macrophages (pseudo colour yellow). Confocal laser-scanning microscopy images of 1, 4, and 24 hpi. At 1 hpi, macrophages are already recruited at the site of infection and phagocytosis of bacteria can be observed. At 4 hpi, all the bacteria clusters in the site of infection appeared to be phagocytosed, and *Mm*-phagosomes are visible. At 24 hpi, the bacterial cluster sizes and numbers are increased. Scale bars: 20 µm. hpi: hours post-infection.

1.6 Thesis Outline

Since *Mtb* was identified as the causative agent of TB almost 150 years ago, research has been moving unceasingly to find a solution to this never-ending pandemic. Although much progress has been made on the understanding of the molecular pathogenesis of Mtb and related mycobacterial pathogens, several aspects remain unknown, limiting novel therapeutic development. This thesis focuses on adding knowledge to the host immune response during early mycobacterial infection, focusing on mycobacteria's autophagy-mediated degradation. This is achieved by coupling chemical and genetic inhibition to the zebrafish model of tuberculosis.

In **Chapter 1**, we present TB molecular pathogenesis and the involvement of autophagy and LAP in the immune response against mycobacterial infection.

Additionally, we introduce the zebrafish TB model as a unique model to study host-pathogen interactions in a whole-organism physiological context.

In **Chapter 2**, we review how research using zebrafish infection models has provided in vivo evidence of the significant role of autophagy and LAP as part of the defences against intracellular bacterial pathogens. These studies have increased the understanding of autophagy-associated host-pathogen interactions, which could favour the design of autophagy-modulating strategies oriented towards the degradation of specific pathogens.

In **Chapter 3**, we report on high-resolution live imaging data from the zebrafish TB model to characterise mycobacteria-autophagy interactions during the early stages of infection. By microinjecting fluorescent *Mm* into the tail fin tissue of GFP-Lc3 transgenic zebrafish larvae, we were able to visualise phagocytosed *Mm* clusters and LC3-positive *Mm*-containing vesicles within the first hour of infection. LC3 associations with *Mm*-vesicles were transient and remarkably heterogeneous, alternating between spacious, compact and composite morphologies.

In **Chapter 4**, we focus on the involvement of the phosphoinositide kinase PIKfyve in mycobacterial infection. PIKfyve catalyses the generation of PtdIns(3,5)P_2, which is critical for the maturation of autophagosomes and the proper function of lysosomes. We studied the role of PIKfyve in the zebrafish TB model using two chemical inhibitors of the enzymatic activity of PIKfyve, YM201636 and Apilimod. We found that in the infected macrophages, PIKfyve mediates maturation of (auto)phagosomal *Mm*-containing vesicles, increasing the acidification, and ultimately enhancing the cell autonomous resistance to mycobacterial infection.

In **Chapter 5**, we address the role of the RUN domain-containing protein Rubicon (Rubcn) in mycobacterial infection. Rubcn negatively regulates autophagy and stimulates LAP. Since mycobacterial virulence factors target autophagy and LAP, the efficacy of these pathways as host defence mechanisms remains unknown. We studied the role of Rubcn in the zebrafish-TB model by affecting *rubcn* transcript levels by morpholino KD and *rubcn* mRNA overexpression. We also generated a CRISPR-Cas9 zebrafish mutant line. Our results suggest that the lack of Rubcn affects *Mm* normal vesicle trafficking, impairing host defence. Our model provides evidence of Rubcn's protective role in early *Mm* infection.

In **Chapter 6**, the results obtained in this thesis are summarised and discussed in a broader context.

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