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

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CHAPTER 6

General Discussion and Conclusion

6.1 Introduction and goal

Despite substantial progress in understanding tuberculosis (TB), the disease's diversity at the levels of the bacillus *Mycobacterium tuberculosis*¹ (*Mtb*), the host², and the environment³ has made the eradication of the TB epidemic still far from within reach⁴. Genetic and phenotypic variation endow *Mtb* with structural resilience and metabolic flexibility, enabling it to survive in diverse microenvironments and establish a successful infection⁵. *Mtb* has evolved multiple survival, evasion, and subversion strategies to drive transmission, infection and disease progression⁶. The initial host response is mediated primarily through innate immune effector cells, including granulocytes and macrophages, which employ a variety of defence mechanisms to restrict *Mtb* infection, such as *Mtb*-containing phagosome damage repair, LC3-associated phagocytosis, autophagy, apoptosis, and granuloma formation⁷. The host and pathogen are engaged in a battle for survival, in which their dynamic interactions determine the outcome of the infection, which could result in clearance, asymptomatic latent TB, or active TB. Understanding these host-pathogen interactions is essential for developing a comprehensive approach to TB control. A closely related mycobacterial pathogen, *Mycobacterium marinum* (*Mm*), shares important virulence mechanisms with *Mtb*. This mycobacterial species is often used to investigate specific aspects of the host-pathogen interplay that are less accessible using conventional *Mtb* models, for example, how infected cells behave during the initial stages of disease. In this thesis, we use *Mm* infection in *Danio rerio* (zebrafish) larvae as a model to investigate early host-pathogen interactions, with a particular focus on autophagy-mediated degradation.

6.2 Autophagy's Role in Anti-Mycobacterial Immunity: Impact of PIKfyve Inhibition

Autophagy is a crucial mechanism for maintaining cellular health, as it clears damaged or worn-out cellular parts as well as microbial invaders⁸⁻¹⁰. These cellular parts or microbes are captured into double membrane vesicles, called autophagosomes, which fuse with lysosomes to degrade the contents. The involvement of autophagy in restricting *Mtb* infection although demonstrated several years ago¹¹⁻¹³, has remained controversial. Within the field, it is debated whether autophagy activation is a sizeable contribution to the immune response against *Mtb* infection¹⁴⁻¹⁷. Research has uncovered different pathogenic strategies by which *Mtb* can evade autophagic degradation and even benefit from this host defence mechanism^{18,19}. *Mtb* is capable of disturbing autophagy initiation as well as autophagosome fusion with lysosomes^{20,21}.

Hence, additional studies are essential to determine the impact of autophagy's regulatory contribution during mycobacterial infections.

As reviewed in **Chapter 2**, research in the zebrafish infection model has contributed to clarifying the innate host defence function of autophagy during mycobacterial infection. Clinically approved drugs with autophagy modulating activity, have been found to induce autophagy and reduce mycobacterial burden in the Zebrafish-*Mm* model^{22,23}. This supports that the autophagy pathway can be targeted as a host-directed therapy against mycobacterial infections. Likewise, autophagy activation by *p62*, *optn* and *dram1* overexpression in zebrafish embryos resulted in improved resistance to infection, while deficiencies in these autophagy-related genes increased susceptibility^{24,25}. In **Chapter 3**, using the Zebrafish-*Mm* model, we characterised in vivo the autophagic targeting of *Mm* from the onset of phagocytosis by macrophages up to the dissemination of infection through the zebrafish host. The tail fin infection (TFI) technique enabled us to reproduce the whole-organism infection context, as opposed to cell-based in vitro systems, and observe in vivo the intracellular dynamics of Lc3 associations with *Mm*. We characterised four phenotypes: Lc3-positive puncta, spacious, compact, and compound vesicles. These highly dynamic, heterogeneous structures correlate with in vitro results obtained using *Mtb* and human iPSC-derived macrophages^{26,27}. Our observations could substantiate mycobacterial phenotypic heterogeneity within the host cell²⁸ and highlight the value of the TFI technique for in vivo imaging of subcellular processes.

Recent studies have focused on core components of the autophagy pathway to reaffirm its role in *Mtb* control. Research in human macrophages showed that ATG7 and ATG14 are required for *Mtb* restriction²⁹. More specifically, ATG7 and ATG14 knockout infected cells displayed higher bacterial burden and necrotic cell death compared to wild-type infected cells. In addition to the requirement for autophagy, ATG14 was associated with the maturation of *Mtb*-phagosomes, independent of its role in autophagy. Other than studying core autophagy components, in **Chapter 4** we directed our attention to the still poorly understood membrane phospholipid dynamics, required for the trafficking of pathogens through phagosomal or autophagosomal degradation pathways. We observed that chemically inhibiting the enzymatic function of PIKfyve, and with that, the production of PtdIns(3,5)P₂, affected the autophagic response to mycobacterial infection, in that PtdIns(3,5)P₂ drives the autophagosome-lysosome fusion³⁰⁻³². PIKfyve inhibition resulted in reduced association of *Mm* with the autophagy marker Lc3 and reduced *Mm*-phagosome acidification. Furthermore, we observed that infected macrophages showed increased cell death. Although it remains to be established if this cell death is apoptotic or necrotic in nature, our results

suggest that PIKfyve is required to protect cells against host-detrimental cell death triggered by *Mm* in early infection. This correlates with observations in bone marrow-derived murine macrophages carrying mutations in autophagy genes (ATG5, ATG16L1, and ATG7), which showed a higher bacterial burden and increased necrotic cell death upon infection¹⁶.

Our results demonstrate that PIKfyve inhibition influences the fate of infected cells by affecting the maturation of mycobacteria-containing vesicles in the autophagy pathway and likely also in the phagolysosomal pathway. Therefore, not only does manipulating core autophagy components impact the infection outcome, but also manipulating precursor molecule synthesis can have a similar effect. Interestingly, the host-protective role of PIKfyve identified in our study correlates with studies on Coxiella^{33,34} and Legionella infections³⁵, but is in sharp contrast with its effect on Salmonella infection, as PIKfyve has been found to expand the replicative niche of this pathogen^{36,37}. Moreover, PIKfyve has been found to facilitate the entry of SARS-CoV-2 virus into host cells³⁸. It will therefore be of great interest to gain further understanding of how PIKfyve-mediated vesicle trafficking determines the outcome of different infections.

6.3 Rubcn as a Protective Factor in Early *Mycobacterium marinum* Infection

Besides autophagy, a related process called LC3-associated phagocytosis (LAP) also contributes to the immune response to *Mtb* infection within macrophages^{11,39-42}. While autophagy targets intracellular bacteria invading the cytosol or present in damaged bacteria-containing vesicles, LAP is triggered by extracellular pathogens binding to surface receptors⁴³. As a result, the membrane of the phagosome is decorated with LC3. By acting as a bridge between the phagocytic and autophagic machinery, LAP facilitates the host lysosomal trafficking process and the degradation of various pathogens⁴⁴. However, as for autophagy, *Mtb* has developed strategies to subvert LAP^{5,6}. The molecular mechanisms by which LAP influences the host immune response to infections with *Mtb* or other mycobacterial pathogens are still poorly understood. In **Chapter 5**, we studied the role of the RUN domain Beclin 1-interacting and Cysteine-rich domain containing protein (Rubicon or Rubcn) in the Zebrafish-*Mm* infection model. Rubcn is of particular interest because it has a dual function: it inhibits autophagy and stimulates LAP^{45,46}. Rubcn's function as a regulatory switch is mediated by differential protein-protein interactions in the class III phosphatidylinositol 3-kinase (PI3KC3) complex during autophagy and LAP⁴⁷⁻⁵⁰. Additionally, during LAP, Rubcn uniquely interacts with the

NADPH-oxidase (NOX2), thereby stimulating the ROS production that triggers LC3 recruitment to the phagosomal membrane⁵¹.

A previous study using *M. abscessus* (*Mabs*) infection showed that bacterial burden was lower under conditions of RUBCN deficiency in human lung macrophages. The *Mabs* protein MAB_0676c was identified as a virulence factor that contributes to bacterial survival by inhibiting autophagic flux via RUBCN expression and IL10 signalling activation⁵². On the other hand, the virulence protein CpsA from *Mtb* has been found to inhibit LAP rather than autophagy⁴¹. Confirming the interaction with the LAP pathway, the attenuated infection phenotype of Δ *cpsA* mutant bacteria was restored in macrophages lacking RUBCN or other LAP components like NOX2. CpsA acts upstream of NOX2, but is not currently known to interact directly with RUBCN. An immunoregulatory host cytokine, IL-27, produced by infected macrophages upon *Mtb* infection, has also been shown to interfere with LAP. *Mtb*-infected macrophages displayed an increased expression of RUBCN and NOX2, which is consistent with LAP activation, and this expression was inhibited by IL-27 exposure. In addition to suppressing anti-mycobacterial cytokine production, IL-27 was found to affect the expression of v-ATPase, critical for LC3 recruitment and bacterial clearance in the LAP pathway⁵³. While the results in *Mabs* infection can be explained by RUBCN's function in autophagy inhibition, the results obtained in *Mtb* infection are explained by its stimulation of LAP-mediated *Mtb* degradation. These studies illustrate the importance of understanding the differential roles of RUBCN in different infection models.

Our results on Rubcn function during *Mm* infection of zebrafish correlate with previous findings in *Mtb*-infected macrophages⁵³. In the absence of Rubcn, we observed higher infection levels in systemic and localised infections, while *rubcn* overexpression generated lower bacterial burden. Furthermore, we determined that Lc3 recruitment to *Mm*-containing vesicles was reduced in Rubcn-deficient larvae. Our results suggest that the host defence against *Mm* is Rubcn dependent, which would be consistent with a main effect on the LAP pathway. Rubcn deficiency also affected the *Mm*-Rab5 association volume, which was lower for highly infected cells. Since Rab5 is known to control early endosomes/phagosomes trafficking⁵⁴, this observation suggests that Rubcn deficiency affects the maturation of *Mm*-containing phagosomes, bolstering *Mm* survival and replication. This idea is consistent with research in *A. fumigatus* infection, which concluded that Rab5 is required for LAP signal transduction, based on observations that Rab5c-deficient macrophages displayed reduced V-ATPase assembly and LC3 lipidation, making them more susceptible to infection⁵⁵. Our results suggest that the lack of Rubcn affects *Mm* normal vesicle trafficking, impairing host defence. While additional studies will be necessary to determine the activity of the LAP pathway during *Mm*

infection in zebrafish, our model provides evidence of Rubcn's protective role in early *Mm* infection.

6.4 From Z-Stacks to Insight: Quantifying Host–Pathogen Interactions in 3D

For the past two decades, the zebrafish model has served as an alternative for studying mycobacterial infection in mouse or cell culture models^{56,57}. The Zebrafish TB model is based on the natural host-pathogen interaction between Zebrafish and *Mm*⁵⁶. The external development and transparency of the embryos and larvae enable microinjection of fluorescently labelled *Mm* strains into transgenic zebrafish, facilitating in vivo imaging analysis of early host-pathogen interactions^{58,59}. This system provides a unique opportunity to investigate not only the cellular and molecular mechanisms of infection but also the timing and sequence of these events within a living host. Studying the temporal and modular aspects of host-pathogen interactions, including the induced immune responses, has yielded unprecedented insights into the infection process, notably leading to a renewed vision on the role of early granulomas in cell-to-cell spreading of mycobacterial pathogens^{60,61}. In the zebrafish–*Mm* model, these approaches allow us to visualise and characterise the dynamic sequence of early events, from initial bacterial recognition to the activation of autophagy-mediated degradation pathways.

In this thesis, we employed an image-based approach to infection biology in the zebrafish model. Using the TFI technique in combination with confocal microscopy, we obtained optical access to the entire infected tissue volume, enabling three-dimensional (3D) spatial analysis of infected cells and subsequent quantitative comparisons. To extract information from these datasets, we used two bioimage analysis platforms: ImageJ⁶² and Imaris (Oxford Instruments). Both allow 3D reconstruction of confocal microscopy images captured at multiple depths along the z-axis (z-stacks)⁶³. We quantified fluorescent signals within these 3D reconstructions, focusing on infected cells and/or mycobacterial clusters. We quantified fluorescent signals within these 3D reconstructions, focusing on infected cells and/or mycobacterial clusters.

To analyse spatial relationships in **Chapter 4**, we used DiAna, an open-source plugin for ImageJ, designed for 3D object segmentation, object-based colocalization, distance metrics, and statistical significance testing. This approach was well-suited for measuring fluorescent signals from LysoTracker-labelled lysosomes and GFP-Lc3-positive vesicles in proximity to discrete, segmented bacterial clusters, where proximity itself was a key biological parameter. Because

the experimental signals differed in their spatial characteristics and labelling patterns, we employed distinct segmentation algorithms for each analysis. In **Chapter 5**, the focus shifted to assessing the colocalization between bacterial clusters and cytoplasmic/membrane-bound proteins. To do this, it was first necessary to define the cellular volume of infected cells. We achieved this using the machine-learning-based segmentation tool in Imaris, which used the bright-field channel, capturing the overall morphology of the cells, to identify cell borders, and applied its advanced 3D visualisation capabilities to refine the segmentation. Within these segmented cell volumes, we then quantified the association between bacterial clusters and cytoplasmic/membrane-bound proteins.

Having reproducible and reliable methods to interpret biological images is essential^{64,65}. To achieve this, the algorithms and parameters used must remain consistent across analyses. Accurate, unbiased quantification of host–pathogen interactions strengthens the validity of conclusions, and the bioimage analysis software employed in our studies was instrumental in achieving that goal. Both Imaris and DiAna offer distinct advantages, and our choice of platform was guided by the nature of the biological question, balancing time efficiency with precision. Nonetheless, parameter testing and careful visual validation of segmentation outputs remain critical, as only the researcher can ultimately determine whether segmented objects accurately reflect the intended biological structures, an assessment that relies on both technical skills and deep biological insight.

6.5 Conclusion and Future Perspectives

The advantages of microscopic imaging, down to the subcellular level, motivated the choice of the zebrafish larval model in this thesis. These imaging possibilities are optimised in the TFI technique, where *Mm* infection is locally introduced in the thin tissue of the tail fin^{66,67}. Studies on the interactions of *Mm* with the autophagy machinery highlight that the TFI model has a lot of potential to examine how bacterial subpopulations contribute to mycobacterial survival in diverse intracellular environments⁶⁶ (**Chapters 3-5**). However, despite that macrophages and neutrophils are rapidly attracted to the infection site, the relatively simple tissue context is a limitation of the tail fin system. Therefore, in **Chapter 5**, we complemented the results of the TFI model with systemic infection studies, which yielded consistent results supporting the host-protective roles for Rubcn. Although 3D imaging in systemically infected larvae is more challenging, in future work, it would be feasible to perform quantitative analyses also in this more complex tissue, using the bioimage analysis platforms explored in this thesis.

The host-protective role that we identified for PIKfyve in *Mm* infection suggests that mycobacteria do not have a virulence mechanism to subvert this lipid kinase, in contrast to other pathogens that use PIKfyve to their advantage for expanding their replicative niche^{36,37}. Further studies are required to determine if the host-protective role of PIKfyve can be extended to other mycobacterial pathogens, including *Mtb*. Furthermore, genetic inhibition would provide a valuable addition to the chemical inhibition approaches used in **Chapter 4**. In our experience, CRISPR-mediated knockout of PIKfyve affected zebrafish viability, and therefore, inducible knockout approaches should be considered in future work. Despite the current limitations, our results support the hypothesis that PIKfyve contributes to anti-mycobacterial host defence by mediating the maturation of mycobacteria-containing autophagosomes. This sheds new light on an underexplored question in mycobacterial research. However, it is just a beginning towards understanding the complexity of membrane phospholipid dynamics underlying phagosome maturation during mycobacterial infection. One interesting question is the possible involvement of PIKfyve in LAP, which is plausible because PtdIns(3,5)P₂ has been found to stimulate V-ATPase assembly, which is a main driver of LAP^{68,69}. A valuable tool for future research in these areas is a recently developed PtdIns(3,5)P₂ reporter, which enables visualising PIKfyve activity in real time⁷⁰. This reporter, currently developed for use in *Dictyostelium*, could be expressed in the zebrafish macrophage lineage, making it ideally suited for combination with the TFI model and for investigating PIKfyve function not only in autophagy but also in other lysosomal degradation pathways.

Besides marking autophagosomes, LC3 can associate with phagosomes in the LAP pathway. A recent study on *Mtb* suggests that LAP could be a transient initial response to damage of the phagosome, followed by enclosure of the pathogen in a multimembrane structure⁷¹. This study illustrates that LAP and autophagy may be closely interconnected responses, difficult to disentangle. LAP activity has been shown to dominate the host defence of zebrafish larvae against *Salmonella* infection^{72,73}. However, thus far, all studies on *Mm* infection in zebrafish highlighted autophagy as the main defence pathway, specifically the receptor-mediated process referred to as xenophagy^{24,25,74,75}. Results in this thesis

indicate that LAP is also active during *Mm* infection in zebrafish. First, during imaging in **Chapter 3**, we observed LC3-decorated spacious vesicles, which likely have a phagosomal rather than autophagosomal origin. Furthermore, we found that genetic deletion or inhibition of Rubcn increased *Mm* infection burden. This phenotype is consistent with the known role of Rubcn as a positive regulator of LAP. However, a formal demonstration that LAP occurs during *Mm* infection is still needed. To this end, chemical or genetic inhibition studies should be performed on factors that can distinguish between the autophagy and LAP pathways, such as ATG13/ATG14, which are specific for autophagy and NOX2, which is specific for LAP. In addition, it must be noted that recently several pathways distinct from the Rubcn-dependent LAP pathway have been identified, where LC3 is conjugated to single membranes. These pathways, including LAP, are now collectively referred to as conjugation of ATG8s to single membranes (CASM)⁷⁶⁻⁷⁹. Further study will be required to dissect the requirement of Rubcn and understand the specificity of various CASM pathways in response to mycobacteria or other pathogens.

In conclusion, in this thesis, we have exploited the zebrafish-*Mm* model to increase understanding of the role of the autophagy machinery during early mycobacterial pathogenesis in a living host. We developed 3D imaging analysis methods for quantification of interactions between the pathogen and the host molecule LC3, a marker for vesicles derived either from autophagy or from the related process LAP. By combining 3D image analysis with chemical or genetic modulation approaches, we identified new roles for two signalling factors, PIKfyve and Rubcn, showing their requirement for host resistance to mycobacterial infection. We provide evidence that PIKfyve, a lipid kinase modulating membrane phospholipid composition, promotes the maturation of *Mm*-containing vesicles in the autophagy pathway, a process necessary for lysosomal fusion and eventual bacterial degradation. A similar host-protective function was identified for Rubcn, which likely promotes the maturation of *Mm*-containing phagosomes as an upstream activator of LAP. These results suggest that pharmacological stimulation of PIKfyve or Rubcn activities could be considered for developing host-directed therapies against mycobacterial infections.

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