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

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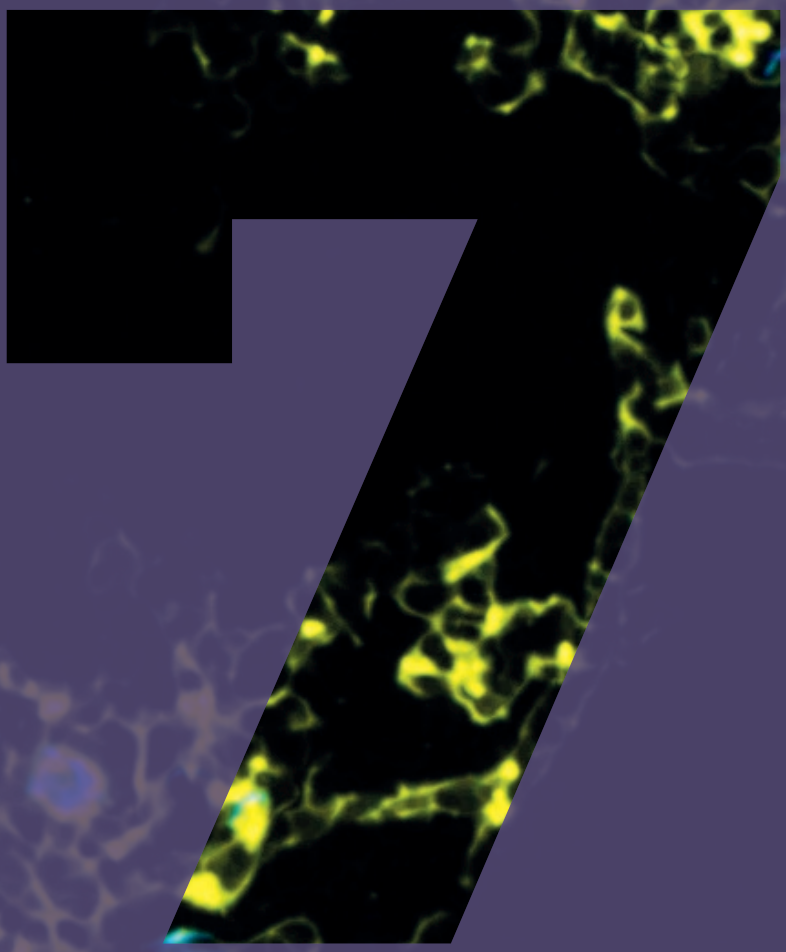

CHAPTER 7

Summary – Samenvatting

7.1 Summary

Despite substantial progress in understanding tuberculosis (TB), the diversity of the disease makes the eradication of the TB epidemic still far from reach. *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, has evolved multiple strategies to survive in host cells and evade or subvert the immune response, which allows it to promote transmission, infection, and disease progression. The initial host response is primarily mediated by innate immune effector cells, including granulocytes and macrophages, which employ various defence mechanisms to restrict *Mtb* infection, such as preventing phagosomal escape, LC3-associated phagocytosis (LAP), autophagy, apoptosis, and granuloma formation. Host and pathogen engage in a dynamic battle for survival, with interactions determining infection outcomes: clearance, asymptomatic latent TB, or active TB. Understanding these host-pathogen interactions is essential for developing improved TB control strategies. A closely related mycobacterial pathogen, *Mycobacterium marinum* (*Mm*), shares key virulence mechanisms with *Mtb* and is often used to study aspects of host-pathogen interplay that are less accessible with conventional *Mtb* models. When combined with transparent zebrafish larvae, *Mm* infection is highly suitable for examining the behaviour of infected immune cells during early stages of infection.

This thesis focuses on advancing knowledge of host immune responses during early mycobacterial infection, with a particular emphasis on autophagy-mediated mycobacterial degradation. This is achieved by combining chemical and genetic interventions with the zebrafish-*Mm* infection model to: (i) investigate early host-pathogen interactions between *Mm* and the autophagic machinery in macrophages; (ii) study the role of the lipid kinase PIKfyve in the maturation of *Mm*-containing vesicles; and (iii) examine the function of Rubcn, a key regulator linking autophagy and LAP, in host defence against mycobacterial infection.

Chapter 1 introduces the molecular pathogenesis of TB and the involvement of autophagy and LAP in the immune response against mycobacterial infection. The zebrafish TB model, based on *Mm* infection, is presented as a unique system to study host-pathogen interactions in a whole-organism physiological context.

Chapter 2 reviews how zebrafish infection models provide in vivo evidence for the crucial role of autophagy and LAP in defending against intracellular bacterial pathogens. Using transgenic zebrafish expressing fluorescent LC3, a marker for vesicles generated by autophagy or LAP, these studies have enhanced understanding of autophagy-associated host-pathogen interactions and support the development of strategies to modulate autophagy for pathogen degradation. Further studies are necessary to fully elucidate the regulatory contribution of autophagy during mycobacterial infections.

Autophagy is essential for cellular health, clearing invading microbes by capturing them into double-membrane vesicles called autophagosomes, which fuse with lysosomes for degradation. **Chapter 3** describes high-resolution live imaging of the zebrafish-*Mm* model to characterise in vivo the autophagic targeting of *Mm* from macrophage phagocytosis to infection dissemination. The tail fin infection (TFI) technique reproduces a whole-organism infection context and enables observation of intracellular LC3 dynamics. Phagocytosed *Mm* clusters and LC3-positive *Mm*-containing vesicles are visualised within the first hour of infection, revealing four phenotypes: LC3-positive puncta, spacious vesicles, compact vesicles, and compound vesicles. LC3 associations with *Mm*-vesicles are transient and heterogeneous, highlighting mycobacterial phenotypic diversity within host cells and the value of TFI for in vivo subcellular imaging.

Macrophages are the first responders against mycobacterial infection. Mycobacteria are phagocytosed and engulfed in phagosomes, which traffic them to lysosomal degradation. Effective pathogen transport requires phagosome maturation. Phosphoinositides (PtdIns) play crucial roles in phagolysosome formation and maturation, and the enzymes that generate PtdIns are vital for innate immunity. **Chapter 4** examines the role of PIKfyve, a phosphoinositide kinase that catalyses PtdIns(3,5)P_2 production, essential for autophagosome maturation and lysosomal function. Using chemical inhibitors YM201636 and Apilimod in the zebrafish-*Mm* model, we observe that PIKfyve inhibition reduces LC3 association with *Mm* and phagosome acidification, while increasing infected macrophage cell death. These results indicate that PIKfyve is necessary to protect host cells during early infection by supporting vesicle maturation in autophagy and likely in phagolysosomal pathways, emphasising the significance of precursor molecule synthesis in infection outcomes.

Besides autophagy, LC3-associated phagocytosis (LAP) contributes to the macrophage immune response. While autophagy targets intracellular bacteria in the cytosol or damaged vesicles, LAP is triggered by extracellular pathogens binding to surface receptors, decorating phagosomes with LC3. Acting as a bridge between phagocytosis and autophagy, LAP facilitates lysosomal trafficking and pathogen degradation. **Chapter 5** addresses Rubcn, a negative regulator of autophagy and activator of LAP. Using morpholino knockdown, mRNA overexpression, and a CRISPR-Cas9 zebrafish mutant, we show that Rubcn deficiency increases infection burden, reduces LC3 recruitment to *Mm*-containing vesicles, and diminishes *Mm*-Rab5 association, suggesting impaired phagosome maturation and enhanced pathogen survival. Rubcn overexpression reduces bacterial load, supporting a protective role in early *Mm* infection and highlighting LAP-mediated defence.

In conclusion, this thesis exploits the zebrafish-*Mm* model to advance the understanding of autophagy machinery participation in early mycobacterial pathogenesis in a living host. By combining 3D imaging analysis and quantification with chemical and genetic modulation, we reveal essential functions of PIKfyve and Rubcn in host resistance. PIKfyve promotes maturation of *Mm*-containing vesicles in the autophagy pathway, a process necessary for lysosomal fusion and bacterial degradation, while Rubcn likely facilitates phagosome maturation as an upstream LAP activator. Pharmacological stimulation of PIKfyve or Rubcn may provide a basis for host-directed therapies against mycobacterial infections.