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The innate immune response against mycobacterial infection : analysis by a combination of light and electron microscopy

Hosseini, R.

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Author: Hosseini, Rohola
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CHAPTER 5

SUMMARY AND DISCUSSION

The immune response towards (myco)bacterial infection consists of complex intracellular signaling for the activation of effector molecules to eliminate the pathogen, and intercellular signaling that alerts other immune cells to the presence of infectious agents (Philips and Ernst, 2012; Berg and Ramakrishnan, 2012; Repasy et al., 2013). This work aimed to study both of these responses simultaneously using the zebrafish model. In this chapter the major findings of this study are summarized and discussed and perspectives for future studies are described.

The tail fin infection model

In this thesis the development of a novel infection method using the zebrafish as an animal model is described that enables *in vivo* visualization of intracellular structures by both light and electron microscopy. This tail fin infection model is introduced in **Chapter 2**. In this model, pathogens are injected directly in the tail fin of zebrafish larvae, resulting in a local infection in the tail fin in the case of a *M. marinum* (*Mm*) infection, which leads to the formation of single early granuloma structure. The tail fin is very suitable for high-resolution light microscopy imaging, because the tissue consists of only a few cell layers (Kimmel et al., 1995), and the infected cells are therefore located at a relatively short distance from the objective of the microscope. The relatively low amount of out of focus light enhances the contrast and the resolution of images of this tissue. For transmission electron microscopy this localized infection model has the major advantage that only a small tissue volume needs to be investigated in order to find and image the site of infection. When the larvae are imaged using light microscopy before fixation and TEM imaging, the images obtained by these two different techniques can be correlated. In our experiments, this correlation was performed based on the localization of the bacteria, which were fluorescently labelled and their morphology could easily be recognized in TEM images.

In future studies, the tail fin of zebrafish larvae may be suitable for super-resolution light microscopy techniques, because of the low level of out-of-focus light and the relatively small tissue volume that needs to be investigated. Advances in electron microscopy have been made, which enable studying cellular structures in their native state (i.e. cryo-genic conditions) using cryo-electron microscopy (Studer et al., 2008; Koning et al., 2014). Since zebrafish larvae are too large for cryo-fixation (high-pressure freezing), limited cryo-EM studies have been performed on zebrafish samples. However, high pressure freezing of just the tail fin would be a very interesting approach in future experiments.

The tail fin infection model offers new opportunities for studying host-pathogen interactions in zebrafish larvae. The interaction between the pathogen and the host immune system in vivo is complex as the host cell initially induces an intracellular response towards the pathogen, and subsequently cells migrate and signal to other immune cells, and eventually a collective immune response from different cell types takes place. Thus far multiple infection models were required to study these different aspects of infection. In this thesis, the tail fin infection model is presented, which provides opportunities for future studies on the role of host-pathogen interactions to be performed within the same infection model. This model can for example be valuable for screening of different knock-out zebrafish lines. The introduction of the CRISPR/Cas9 technology for generating knock-out lines is expected to revolutionize the field of zebrafish research, and the tail fin infection model may be instrumental in providing a valuable assay to study the phenotype of these lines.

Autophagy as an intracellular immune response against *Mm*

The autophagic response against *Mm* was investigated in transgenic zebrafish larvae expressing a GFP-Lc3 fusion protein, using LM and TEM in **Chapter 2**. Lc3 is a ubiquitin-like protein involved in the biogenesis of autophagosomal structures, and often used as a marker for autophagic compartments (Kabeya et al., 2000; Klionsky et al., 2012). Two types of GFP-Lc3-positive structures appeared upon infection of *Mm* in the tail fin. First, numerous relatively small vesicles (~1 µm) were observed that did not contain bacteria. These small vesicles are highly dynamic and can fuse with other compartments containing bacteria. Correlation of light and electron microscopy images showed that these small GFP-Lc3-positive vesicles in the vicinity of bacteria indeed had the appearance of an initial autophagic vacuole (Eskelinen, 2008). Second, larger GFP-Lc3 structures (~3 µm) were present that often contained phagocytized bacteria. The GFP-Lc3 signal of these larger structures could either originate from an autophagosome, which had taken up cytosolic bacteria, or from an autophagosome/autolysosome after fusion with a phagosomal compartment containing bacteria (as has been shown in cell culture studies). The autophagosomal nature of these vesicles was supported by correlative light and electron microscopy data showing that the larger GFP-Lc3 vesicle containing bacteria had the morphology of a degradative autophagic vacuole. The advantage of studying the infection process in a whole animal model was demonstrated by showing different autophagic responses in the immune cells compared to the other cell types. The larger bacteria-containing GFP-Lc3 vesicles were observed more often in leukocytes than in other cell types (mainly epithelial cells in the tail fin).

The tail fin infection model could be used to visualize different autophagic adaptor proteins to understand the basic mechanisms underlying autophagic response during infection. Alternatively, this model can be used for other pathogens than *Mm* to investigate their interaction with the autophagic machinery.

Quantification of intracellular structures containing *Mm* in early granulomas

In **chapter 2** transmission electron microscopy was performed on early granuloma structures to quantify intracellular structures sequestering bacteria. It was confirmed that at this stage the bacteria resided in different cell types and that they could occur in the extracellular matrix. We quantified the number of intracellular bacteria residing individually in phagosomes, the cytoplasm, autophagic vacuoles, or lysosomes, or residing in aggregates or acidic aggregates. Only a very small fraction (~0.4%) of bacteria was found inside an initial autophagic vacuole with a double membrane, which is most likely due to the highly transient nature of these structures. These autophagosomes generally contain a single bacterium. The fraction of bacteria in degradative autophagic vacuoles is considerably larger (~4.5%). Another population of bacteria resides in phagosomal compartments and this fraction of bacteria (~11%) has been taken up most recently from the extracellular space or has succeeded in blocking lysosomal fusion. For *Mm* it has been shown in cell cultures that they are able to escape the phagosomal compartment, which most likely explains the ~13% of bacteria residing freely in the cytoplasm. This fraction of bacteria is an obvious target for autophagy. These data were confirmed in larvae in chapter 4.

Macrophage and neutrophil dynamics and function during the course of a *Mm* infection

Macrophages and neutrophils contribute strongly to the host defence against intracellular pathogens, including *Mm* (Eum et al., 2010; Yang et al., 2012; Srivastava et al., 2014). These phagocytic cells are the first responders against invading mycobacteria and their specific dynamic interaction with the pathogen is to a large extent uncomprehended (Srivastava et al., 2014; Cronan and Tobin, 2014). We used the tail fin infection model to study the complex interactions of macrophages and neutrophils with each other and *Mm* during the course of an infection using confocal imaging in **chapter 3**. We show that initially both macrophages and neutrophils are recruited to the infection site. Unlike previously reported for systemic infection models, we observed that neutrophils are efficient in phagocytosis of *Mm* and that they also play a role in the dissemination of

Mm in the host, similarly to macrophages Actually, at the initial stages of infection, neutrophils were observed to have even a higher contribution to dissemination of bacteria than macrophages. In addition to phagocytosis of *Mm* by neutrophils, we have also observed their association with unphagocytized bacteria. This type of interaction of neutrophils with the pathogen (or with infected macrophages) occurs for a few minutes without engulfment of the bacteria. We observed neutrophils undergoing netosis when the bacterial aggregate was too large for phagocytosis.

The macrophages appear to play a major role in efferocytosis, a process that is defined as phagocytosis of dead cells, in this case containing bacterial content. As a result, during the course of infection large bacterial aggregates accumulate within macrophages that are unable to contain the *Mm* aggregates and eventually undergo a burst event. Upon such a burst event, other macrophages and neutrophils are recruited that take up the bacterial content. This sequence of events results in the dissemination of *Mm* through the host and an increase in the bacterial burden in the tail fin. Alternatively, macrophages (also neutrophils) containing high bacterial content can be extruded out of the tail fin, and this process is mediated by epithelial cells.

Correlating cellular dynamics and ultrastructure

Visualizing the interactions between the phagocytic cells and *Mm* is complicated by the highly dynamic nature of simultaneous intercellular and intracellular responses. To visualize intercellular interactions time-lapse imaging was performed using confocal microscopy and for the visualization of intracellular structures serial block face scanning electron microscopy (SBF-SEM) was performed on the same samples in **chapter 3**. We used SBF-SEM to image the region of interest in the infected tail fin in 3D at EM resolution. The alignment of the 3D EM images with the images obtained by confocal microscopy was performed using the localization of the bacteria. After alignment the fluorescently labelled macrophages and neutrophils that had been observed in the confocal images were identified in the EM images. Using this approach the EM images of the infected macrophages and neutrophils could not only be correlated with the confocal images but the dynamic behaviour of these cells in the previous hours was known as well.

Although mycobacteria are notorious for their ability to block apoptosis of their host cell (Repasy et al., 2013), our EM images showed macrophages containing a large bacterial content showing early apoptotic morphology with clear chromatin condensation in the nucleus. The confocal images show that this cell had phagocytized a large aggregate of bacteria ~ 30 minutes earlier. One macrophage appeared in the EM

images to contain bacteria in a single compartment together with dead cell remains, as a result of efferocytosis. This cell showed no sign of cell death, which was surprising since the confocal images show that this macrophage phagocytized the aggregate ~1.5 hour earlier.

Based on light microscopy observations, the rapid signal disappearance of neutrophils was previously suggested to be a characteristic of apoptotic cell death (Loynes et al., 2010). However, based on our correlated light and EM images, we suggest that the type of cell death characterized by a rapid disappearance of the fluorescent signal (<1 minute) is a form of necrotic cell death. Interestingly, necrotic cell death appears to be very efficient in protein denaturation as observed for fluorescent proteins, probably as a result of fusion of lysosomal compartments with the cytoplasm and the release of abundant peroxides. One necrotic neutrophil we observed in our EM images showed a round nucleus and an irregularly shaped plasma membrane and no apoptotic features. In the confocal images this neutrophil showed a compacted bacterial content and the disappearance of the fluorescent signal was observed within a time period of 1 min, approximately 30 minutes before fixation. Finally, a neutrophil was observed in the EM images that instead of phagocytosis was undergoing netosis, a process previously observed in cell culture studies in which neutrophils selectively release extracellular traps in response to large pathogens (Francis et al., 2014). In the confocal time-lapse images this neutrophil was observed to be interacting with a large aggregate of *Mm,* which had previously been sequestered by another neutrophil, over the last 30 min before fixation. In future studies it will be interesting to investigate how these mechanisms are regulated and initiated and which cell death mechanism reflects the most adequate response against mycobacterial infections.

Additionally, in the BF-SEM images we observed *Mm* infected epithelial cells in the process of undergoing extrusion. These cells did not show a fluorescent signal associated with macrophages or neutrophils. Instead, using the 3D information provided by this method, we could observe that the bacterial aggregate was entirely engulfed by an epithelial cell. The process of extrusion by epithelial cells in lungs is still unexplored. Since TB infection is spread through the air and typically encounters the lungs of humans, extrusion of bacterial content via the lung epithelium might have an important role in the innate immune defence against mycobacterial infection. On the other hand, extrusion may be advantageous to the mycobacteria, since after extrusion they still reside inside epithelial cells, thereby avoiding recognition and the subsequent inflammatory response. Further studies could shed a light on the contribution of extrusion to the progression of infection within the host as well as the dissemination of mycobacteria from human to human.

Mm early granuloma development and ultrastructural analysis in MyD88-deficient zebrafish larvae

Myd88-mediated signalling plays an important role during *Mm* infection (van der Vaart et al., 2014; Cambier et al., 2014). The early granuloma development and its ultrastructure in MyD88-deficient mutant larvae were investigated by both light and electron microscopy using the tail fin infection model in **chapter 4**. The mutants showed an increased bacterial burden, which is consistent with previous studies in Myd88-deficient larvae using other infection models (van der Vaart et al., 2013). Additionally, a clearly different phenotype of infection was observed in the tail fin of the mutant compared to the wild type larvae. The mutant showed more compacted growth of *Mm* in large aggregates at the site of infection. Furthermore, a lower number of leukocytes were observed at the site of infection compared to wild type. This was most likely due to a reduced recruitment towards the pathogen in *myd88*$^{-/-}$ larvae, and not to increased cell death because less TUNEL-positive cells were found in the mutant. However, the recruitment of leukocytes towards *Mm* infection is not dependent on *myd88*-mediated signalling at the initial stages of infection, as shown in *Mm* infection in the hindbrain. Apparently, there is a difference in MyD88 dependency between the initial and the long-term leukocyte response to *Mm* infection. The long term recruitment has been shown to be dependent on pro-inflammatory mediators like mmp9, TNF and other cytokine signalling, while the initial rapid recruitment of leukocytes towards *Mm* is not MyD88-dependent (Cambier et al., 2014).

TEM images of the infection sites in wild type and mutant larvae revealed that the majority of bacteria were located extracellularly in the mutant. This extracellular growth may be due to the lower level of recruitment of phagocytic cells to the site of infection. In other experimental approaches in which the immune system is compromised, for example knockdown of TNFα, increased extra cellular growth of *Mm* was observed as well (Roca and Ramakrishnan, 2013). Additionally, we studied the intracellular bacteria that resided in the immune cells of the mutant and the wild type. We already showed in chapter 2 that the largest fraction of *Mm* in wild type larvae was observed in acidic aggregates. Interestingly, a distinct difference was observed in the nature of compartments in which intracellular *Mm* was present in the mutant larvae. In mutant larvae a much smaller fraction was found in these acidic aggregates and a larger fraction in non-acidic aggregates and phagosomal compartments. It seems that acidification of these larger aggregates is highly MyD88 dependent. Alternatively, the differences could be explained by the presence of *Mm* in different cell types than leukocytes in the mutant.

Conclusion

In this thesis the zebrafish tail fin infection model is presented, which enables the study of a complex immune response towards (myco)bacterial infection using a combination of light and electron microscopy. The induction of autophagy upon a mycobacterial infection as an important innate immune response was visualized using correlative light and electron microscopy. Studying the role of leukocyte dynamics and function during the course of infection provided new insights into the complex host-pathogen interactions. Using a *myd88* mutant zebrafish line it was shown that the recruitment of leukocytes towards the site of infection and subsequent phagocytosis of bacteria is dependent on MyD88-mediated signaling. With the advancement of medical translational studies using zebrafish disease models, the tail fin infection model may provide new opportunities to develop novel therapies against pathogenic infections like tuberculosis.

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