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

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Pulmonary tuberculosis

Pulmonary tuberculosis (TB) is a bacterial infection caused by *Mycobacterium tuberculosis* that in humans commonly enters the lung and preferably resides there inside cells of the host. In most cases the infection remains in the lungs or in the local lymphatics (primary complex). Alternatively, it can also disseminate via lymphatics and blood vessels to other organs (military tuberculosis). The pathogen is spread to other individuals by droplets through the air, when people who have an active TB infection cough, sneeze, or otherwise transmit respiratory fluids. Upon infection often no symptoms are observed, and this condition is known as latent tuberculosis. It is estimated that one third of the world population has a latent TB infection, that progresses to active TB in 5-10% of cases. TB has been responsible for a high mortality in human populations and is still a major health issue in the third world. Since the late 19th century, mortality rates declined in Europe and the United States. However, worldwide new cases of TB are diagnosed every second and one TB patient does not survive the infection every 20 seconds (WHO, 2013: http://www.who.int/tb/publications/en/).

During *Mtb* infection, the bacterium is taken up by host phagocytes that are present in the lung and it replicates within these cells (Eum et al., 2010; Repasy et al., 2013). As infection progresses, macrophages and other immune cell types are recruited to sites of infection, creating a structured aggregate of infected and uninfected cells which is called a granuloma (Philips and Ernst, 2012; Berg and Ramakrishnan, 2012). These granulomas consist of a tightly interdigitated inner core of macrophages termed 'epithelioid' macrophages, surrounded by additional immune cells, including T-cells, B-cells, dendritic cells and neutrophils (Philips and Ernst, 2012; Berg and Ramakrishnan, 2012; Ramakrishnan, 2012). The granuloma is the hallmark structure of tuberculosis and constitutes a crucial niche in which bacteria persist. Eventual rupture of granulomas is crucial for bacterial release into the lung and transmission of the disease to other individuals.

Mtb is believed to have existed for more than 70.000 years and seems to have co-evolved together with humans before the migration out of Africa (Comas et al., 2013). This indicates that *Mtb* had considerable time and opportunity to develop mechanisms for the manipulation of its host's immune response. *Mtb* caused more deaths in industrialized countries than any other disease during the 19th century. At that time, the majority of the urban populations of Europe and North America were infected with *Mtb*, and ~80% of patients who developed active TB did not survive this infection (Harvard University Library, open collections: http://ocp.hul.harvard.edu/contagion/tuberculosis.html).

Nowadays, TB is a major risk for immunosuppressed individuals like HIV-infected people and patients using TNF-alpha inhibitors or immunosuppressive

drugs. Although effective therapies declined the incidence of TB in recent years, the increase of drug-resistant forms of TB are of concern and several problems regarding the clinical treatment of TB still exist. The first successful antibiotic, streptomycin (purified from *Streptomyces griseus*), became available in the 1940s. It restricted *Mtb* growth in humans, but although the effects of this drug were astonishing at the start, within a few months resistant mutant strains began to appear. The last decades the intensive use of antibiotics and inefficient drug treatment have increased the occurrence of multi-drug resistant *Mtb* strains (Ottenhof, 2012). Currently, combinations of antibiotics are used and efficient therapy requires intensive long-term (over 6 months) treatment. In addition, the widely used TB vaccine is an attenuated strain of *Mycobacterium bovis*, bacillus Calmette–Guérin (BCG), which is highly effective against disseminated TB in children but has a low efficiency in adult populations (Ottenhoff and Kaufmann, 2012). Finally, in areas and countries with a poorly developed healthcare system, access to treatment remains challenging (Lienhardt et al., 2012). The desired development of novel therapeutic strategies requires a better understanding of the host-pathogen interactions during *Mtb* pathogenesis (Goldberg et.al., 2012; Ottenhoff, 2012).

Phagocytic cells and mycobacterial infection

The cells comprising our immune system are equipped with a large set of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD like receptors, mannose receptors and complement receptors. For example, macrophages use PRRs to recognize invading pathogens by pathogen-associated molecular patterns (PAMPs) on the surface of bacteria. Subsequently, the bacteria are internalized using one PRR or a combination of PRRs and an appropriate immune response is initiated dependent on the type of receptors that have recognized the pathogen (Medzhitov and Janeway, 2000; Kawai and Akira, 2005).

Toll-like receptors (TLR), especially TLR4 are associated with recognition of mycobacteria and play an important role in mycobacterial infection (Tjärnlund et al., 2006; Corr and O'Neill, 2009). Along with the recognition of pathogens, these receptors initiate a signalling cascade through several intracellular adaptor molecules, including Myeloid differentiation factor 88 (MyD88), to induce intracellular effectors and the initiation of an inflammatory response (Medzhitov and Janeway, 2000; van der Vaart et al., 2013). MyD88 is a key adaptor protein in the TLR signalling pathway since it is used by all TLRs (except TLR3) to initiate an inflammatory response (Takeda and Akira, 2004). The C-terminal part of MyD88 consists of a toll/interleukin-1 receptor (TIR) homology domain that enables interaction with TLRs. Its N-terminal death domain enables formation of a 'Myddosome' signalling complex. This complex consisting of interleukin-1 receptor

associated kinases (IRAK) plays a central role in inflammation and the host defense by activating nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling (Lin et al., 2010; Gay et al., 2011). The TLR and MyD88 signalling pathways are well conserved between human and the zebrafish (van der Sar et al., 2006).

Once macrophages have ingested mycobacteria, they encapsulate the bacteria in phagosomes and activate an array of intracellular effector mechanisms. These mechanisms are aimed at elimination of bacteria inside the phagosomes or upon fusion with lysosomes, and include acidification of compartments, and production of oxidants, nitrosylating agents, antimicrobials and proteases (MacMicking, 2014; Torraca et al., 2014). In response to the capability of the host cells to eliminate bacteria, pathogenic mycobacteria have evolved mechanisms for the manipulation of its host's immune response by secretion of virulence factors via specialized secretion systems (Baxt et al., 2013; Houben et al., 2014). They are able to prevent lysosomal fusion and acidification of phagosomal compartments (Armstrong and Hart, 1971; Tan and Russell, 2015). While a fraction of *Mtb* within a host cell is kept inside phagosomes, another fraction is able to escape from the phagosomes into the cytoplasm (van der Wel et al., 2007; Simeone et al., 2015).

Mtb has been shown to be sensitive to low pH, resulting in growth arrest at pH 5.0 and lower (Chapman and Bernard, 1962; Tan et al., 2010). Therefore lysosomal fusion with phagosomes has always been considered to be the sole mechanism in the host defence against mycobacterial pathogenesis. However, recent studies demonstrate additional mechanisms restricting mycobacterial proliferation. These include phagocytosis of dead cells containing pathogens (a process called efferocytosis), antimicrobial peptides as a part of the autophagic response and sequestration by autophagic vacuoles of escaped bacteria in the cytoplasm (Mostowy, 2013; Deretic, 2012; Weiss and Schaible, 2015).

Another classical view concerning the *Mtb* pathogenesis has recently been revised. A role for other phagocytic cells than only macrophages has been suggested during TB infection. In particular, neutrophils have been found to be infected in *in vivo* studies (Eum et al., 2010; Repasy et al., 2013), and it has been suggested that neutrophils play a protective role for the host during *Mtb* infection (Perskvist et al., 2000; Lowe et al., 2012).

The zebrafish as a model for mycobacterial infection

The zebrafish, and fish in general, are naturally susceptible to tuberculosis, caused by *Mycobacterium marinum* (*Mm*), which is genetically related to *Mtb*

and shows a similar pathogenesis to the human disease, including the formation of granulomatous lesions (Davis et al., 2002; Swaim et al., 2006). The high level of homology between the zebrafish and human immune system, which consist of comparable cell types, makes the zebrafish a suitable animal model to study host-pathogen interactions (Renshaw and Trede, 2012). Although fish (obviously) do not have lungs, the zebrafish model can be employed to understand the basic mechanisms of respiratory infectious diseases (Martin and Renshaw, 2009). The first advantage of using *Mm* as a tool in tuberculosis studies is its relatively short generation time. The generation time of *Mtb* is about twenty hours, while for *Mm* this takes about four hours, which enables faster and more productive studies. The second advantage is the fact that *Mm* is less harmful to humans, since its growth is restricted in human body temperatures. In case of *Mm* infection in humans, the infection tends to stay in the extremities of the body. As a result, the bacteria can be handled at a relatively low biosafety level.

The larval stage of the zebrafish provides additional advantages for mycobacterial research. First, during the first weeks of development, from embryonic to larval development, they are transparent enabling detailed *in vivo* imaging of the infection process in real time (Davis et al., 2002). Second, during this stage of development only the innate immune cells are present, i.e. macrophages and neutrophils, that have been demonstrated to be sufficient for the formation of early granuloma structures during mycobacterial infection (Davis et al., 2002). These cell types are the first line of defence against invading pathogens, and the cell-type-specific interactions with the bacteria can be studied by imaging in real time. Third, in addition to imaging the immune cell interactions with the bacteria, transcriptome profiles of specific cell types can be studied as well (Rougeot et al., 2014). Fourth, in zebrafish effective gene knockdowns using antisense morpholino oligonucleotides and relatively simple methods for generating specific gene knockouts lines using CRISPR/Cas9 are well established. (Bedell et al., 2011; Hwang et al., 2013).

The zebrafish model for TB has increased our understanding of mycobacterial pathogenesis (van der Vaart et al., 2012; Ramakrishnan, 2013; Cronan and Tobin, 2014). In the classical view of the granuloma structures, it was assumed to be a protective static structure that was driven by the host. However, studies in zebrafish have shown that the secretion of virulence factors is required for the efficient formation of granulomas (Volkman et al., 2004; Stoop et al., 2011). Thus, mycobacteria also benefit from these granuloma structures, which are required for their dissemination within the host as well as for the spread from human to human (Flynn and Chan, 2005; Davis and Ramakrishnan, 2009).

During mycobacterial infection the formation of granuloma structures and the recruitment of phagocytic cells by the host are a highly balanced processes.

Zebrafish larvae with a deficient or a hyperactive immune system are both more susceptible to mycobacterial infection (van der Vaart et al., 2013; Kanwal et al., 2013). A balanced expression of tumor necrosis factor (TNF) plays an important role in maintaining an optimal state. (Clay et al., 2008; Roca and Ramakrishnan, 2013). Therefore, modulating the TNF expression appeared to increase the resistance towards mycobacterial infection (Roca and Ramakrishnan, 2013).

Recent findings in zebrafish larvae suggest a crucial role for TLR-MyD88 signalling pathway in the response to mycobacterial infections. The mycobacterial cell-wall component phthiocerol dimycoceroserate (PDIM) suppresses its recognition by TLRs and thereby inhibits MyD88-mediated pro-inflammatory signalling (Cambier et al., 2014). The activation of TLR-Myd88 signalling has been shown to play a crucial role in host defence against mycobacterial infection, including the induction of autophagic response mediated by DRAM1 (van der Vaart et al., 2014).

The role of neutrophils in mycobacterial pathogenesis is still not well known. In the zebrafish model, neutrophils were observed to have a direct interaction with mycobacteria (Meijer et al., 2008), and inhibition of neutrophil recruitment to the infection site resulted in an enhanced mycobacterial burden (Yang et al., 2012). Neutrophils have been shown to become infected upon recruitment to established granulomas and were able to actively eliminate the mycobacteria by means of oxidative mechanisms (Yang et al., 2012). Enhanced production of reactive nitrogen species (RNS) by neutrophils through the transcription factor hypoxia-inducible factor 1-alpha (Hif-1α) plays an important role in mycobacterial elimination (Elks et al., 2013). These results indicate that innate immune cell types other than macrophages also contribute to host defence against mycobacterial infections.

Recent advances in microscopy techniques

The immune response towards mycobacterial infection consists of complex intracellular signalling for the activation of effectors molecules to eliminate the pathogen, and intercellular signalling that alerts other immune cells of an organism to the presence of infectious agents. Both of these responses can be studied using the zebrafish model. Although the dynamic behavior of cells and intercellular interactions can be visualized using (fluorescence) light microscopy, often higher resolution (electron microscopy) imaging is required for the visualization of intracellular structures, such as the double membrane of autophagic compartments. In the following paragraphs the possibilities of these microscopy techniques will be discussed.

Light microscopy

Light microscopy has been extensively used in biology since the discovery of microorganisms by Antoni van Leeuwenhoek in the 16th century. Innovations in the 20th century have led to phase contrast and differential interference contrast (DIC) microscopy that allow detailed imaging of living cells or even entire organisms without any staining , due to the enhanced contrast provided by these techniques. The successful imaging of tissue or entire organisms using these techniques is mainly dependent on transparency (or translucency), which makes the zebrafish an ideal animal model for light microscopy studies. In zebrafish embryos and larvae, even individual phagocytic cells and pathogens have been visualized using light microscopy techniques (Herbomel et al., 1999; Davis et al., 2002).

Fluorescence microscopy adds the ability to visualize structures with molecular specificity using fluorescently labeled antibodies or fluorescent proteins, such as green fluorescent protein (GFP) (Taylor and Salmon, 1989). Fluorescent proteins can be fused with a protein of interest, which enables the observation of its localization and dynamics. In addition, fluorescent proteins can be fused to a promoter/enhancer region of a gene of interest to generate a reporter construct for the activity of this promoter/enhancer. Promoter fusions are often used to label specific tissues or cell types when promoters are used that are specifically active in a certain cell type. In zebrafish, promoter fusions are often used to generate transgenic lines in which specific cell types are fluorescently labelled, including neutrophils and macrophages (Renshaw et al., 2006; Hall et al., 2007; Ellett et al., 2011).

The availability of fluorescent proteins has spurred the development and improvement of more advanced imaging modalities, including confocal laser scanning microscopy (CLSM) or in short confocal microscopy. Confocal microscopes have a more complicated design compared to traditional fluorescent microscopes. Excitation of fluorophores occurs using an intense spot of laser light which is moved over the specimen. Detection of the emission light is performed by electronic detectors, so-called photomultiplier tubes (PMTs). Finally, a pinhole is introduced in the light path to eliminate out-of-focus signals, which results in fluorescent images from an optical section instead of an entire specimen. By acquiring and combining images from different focal planes, a 3-dimensional reconstruction of the specimen can be realized (Claxton and Fellers, 2006).

Besides confocal microscopy, more recent high-resolution fluorescence microscopy techniques have been developed that improve spatial resolution bypassing Abbe's diffraction limit of 0.2 micrometers (Egner et al., 2002; Hell, 2007). Eric Betzig, Stefan W. Hell and William E. Moerner were awarded the Nobel Prize in Chemistry in 2014 for achieving nanometer scale resolution using fluorescence

microscopy, thereby bypassing Abbe's diffraction limit of 0.2 micrometers. One of these techniques is called stochastic optical reconstruction microscopy (STORM), which relies on the use of blinking fluorophores, that can be detected at the single-molecule level and subsequently localized with a high positional accuracy (Rust et al., 2006). The second technique is stimulated emission-depletion (STED) that uses a laser exciting all the fluorescent molecules, while another light source quenches fluorescence from all molecules except those in a nanometer-sized volume in the middle (Hell and Wichmann, 1994).

Electron microscopy

The curiosity to observe objects that could not be visualized with light microscopy led to the development of electron microscopy, which is an imaging technique that uses accelerated electrons, instead of light waves, as a source of illumination. Since the wavelength of an electron is much shorter than that of visible light, the structure of smaller objects can be determined using an electron microscope. However, the better resolution of EM is compromised by time resolution, since the specimen in EM is always required to be fixed and the imaging is performed in vacuum, so visualization of dynamic processes is impossible. Currently, two main forms of electron microscopes exist. The original form is the transmission electron microscope (TEM), whereas the second form is the scanning electron microscope (SEM).

A TEM, which was invented by Ernst Ruska in 1933, can achieve a resolution of ~1 nm for biological samples, whereas most light microscopes are limited by the diffraction limit of Abbe of 0.2 micrometers. The transmission electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope. The electron beam is accelerated by an anode and transmitted through the specimen that is partly transparent to electrons and partly scatters electrons out of the beam. The non-scattered electrons transmitted through the specimen carry the information and after magnification by the objective lens the projection of a specimen can be visualized as an image. The projection can be visualized onto a fluorescent screen and recorded onto a photographic film or a charge-coupled device (CCD) camera.

Transmission electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens. Using accelerated electrons requires a vacuum, and therefore a biological specimen typically needs to be chemically fixed and dehydrated, which introduces artefacts in the images acquired from the specimen compared to their natural appearance. A second disadvantage of the TEM for biological samples is the need for extremely thin (~ 100 nm) sections of the specimens, in order for the electrons to be transmitted through the specimen. The ultrathin sectioning also requires embedding of the biological specimens in a polymer resin for stabilization, in addition to the fixation and dehydration. Finally,

these thin sections require treatment with heavy atoms to label lipids and proteins in order to achieve a sufficient contrast in the images.

The SEM generates images of the surface of a specimen and uses a focused electron beam instead of an accelerated electron beam. The focused electron beam probes an area of the specimen by scanning as a raster to produces images. Upon interaction of the electron beam with the specimen, it loses energy by a variety of mechanisms. Subsequently, the lost energy is converted to alternative forms that can be observed as emission of heat, light, x-rays, low energy secondary electrons and high-energy backscattered electrons. These emitted signals provide the information about the properties of the specimen surface. Typically, images are constructed from signals detected by a conventional secondary electron detector, providing a characteristic 3-dimensional appearance of the structures on the surface of a specimen. Similar to TEM, images can be produced from sections using the signals from back-scattered electrons, still by probing the surface of the sections.

Recent innovations have equipped a SEM with an ultramicrotome inside the SEM chamber. Ultramicrotomes are traditionally used to produce ultra-thin sections of biological specimen for TEM imaging. The modified ultramicrotome inside the SEM is able to remove material from the surface of the specimen, revealing a fresh surface for imaging using the focused electron beam. Serial subsequent imaging and sectioning inside the microscope allows high-resolution 3-dimensional imaging of biological samples. This imaging technique is called serial block-face scanning electron microscopy (SBF-SEM) (Denk and Horstmann, 2004; Peddie and Collinson, 2014). Complementary methods have been developed using similar technology, such as Focused ion beam (FIB) SEM, which uses a focused ion beam to remove a thin layer of material from the surface of specimen instead of an ultramicrotome.

Generally, the TEM produces images of biological specimens with much higher resolution compared to the images acquired using a SEM. This difference in resolution is at least an order of magnitude. Although recent advances have enabled acquiring images from a large field of view in TEM (Faas et al., 2012), SEM was already able to produce images from a large field of view since it scans the surface of a specimen point-by-point. Next to the difference in resolution, the main difference between a TEM and a SEM for imaging biological specimen is the ability of acquisition high-resolution images from large volumes. In TEM, the volume is reconstructed after imaging the ultra-thin sections separately and requires intensive image processing afterwards. In contrast, in SBF-SEM and FIB-SEM the images for volume reconstruction are acquired automatically, sometimes by imaging for weeks without interruptions (Peddie and Collinson, 2014).

Outline of the thesis

This thesis focuses on studies on mycobacterial infection in zebrafish using a combination of light and electron microscopy observations . The complex interaction between the intracellular architecture of the host cell and the bacteria, and the dynamics of the intercellular interactions between infected cells are investigated. In order to provide a quantitative analysis of these complex interactions during *Mm* infection *in vivo*, a tail fin infection model of zebrafish larvae was developed, as described in **chapter 2**. In this chapter, the tail fin infection model was used to visualize the autophagic engulfment of *Mm* in host cells. The GFP-Lc3 transgenic zebrafish line, *Tg(CMV:EGFP-map1lc3b)* enabled the visualization of autophagosomal structures using light microscopy. In order to confirm the autophagic nature of these structures, correlative light and electron microscopy (CLEM) was performed on *Mm* infected GFP-Lc3 transgenic zebrafish larvae. We were able to show that the GFP-Lc3-positive structures observed surrounding the bacteria are indeed autophagic in nature and that the smaller (~1 um) GFP-Lc3-positive compartments in the vicinity of *Mm* are autophagosomes. In addition, we quantified in early granulomas the presence of intracellular bacteria in different intracellular compartments, which showed that the majority of bacteria were present as large aggregates and approximately 5% of bacteria were engulfed in autophagic vacuoles.

The role of different phagocytic cells and their contribution to the mycobacterial infection is to a large extent unknown. In **chapter 3,** transgenic zebrafish lines with fluorescently labeled neutrophil and macrophages were used to explore the intercellular dynamics and the fate of infected macrophages and neutrophils during the course of *Mm* infection using the tail fin infection model. The macrophages and neutrophils seem to have distinct functions during infection. The macrophages are mainly responsible for efferocytosis, while the infected neutrophils show reverse migration away from the infection site. Extrusion of epithelial cells after efferocytosis of dead infected immune cells appears to contribute in restriction of bacterial burden, while burst of highly infected macrophages contributes to bacterial growth and dissemination of *Mm*.

The MyD88-mediated signalling plays a major role in mycobacterial infection. MyD88 deficient zebrafish and mice have been shown to be more susceptible to infection by bacteria. In **chapter 4**, the infection progression and the early granulomas were investigated in MyD88-deficient zebrafish larvae. These larvae have an altered immune response against *Mm*, since after phagocytosis of a pathogen the intracellular signalling cascade is blocked. The recruitment of other

phagocytic cells was reduced as well, probably because the MyD88 deficiency also blocks the efficient activation of NF-κB-induced pro-inflammatory signals. Transmission electron microscopy images show that the increase of the bacterial infection is mainly associated with extracellular growth. The quantification of the intracellular compartments containing *Mm* show that probably efferocytosis by macrophages plays an important role in restricting bacterial growth in the mutant larvae.

Finally, the findings in this thesis are discussed and perspectives are proposed for further research on the host-pathogen interactions using the zebrafish as an animal model in **chapter 5.**

References

Armstrong, J.A., and P.D. Hart. 1971. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J. Exp. Med.* 134:713–740. doi:10.1084/jem.134.3.713.

Baxt, L.A., A.C. Garza-Mayers, and M.B. Goldberg. 2013. Bacterial subversion of host innate immune pathways. *Science*. 340:697–701. doi:10.1126/science.1235771.

Bedell, V.M., S.E. Westcot, and S.C. Ekker. 2011. Lessons from morpholino-based screening in zebrafish. *Brief Funct Genomics*. 10:181–188. doi:10.1093/bfgp/elr021.

Berg, R.D., and L. Ramakrishnan. 2012. Insights into tuberculosis from the zebrafish model. *Trends in Molecular Medicine*. 18:689–690. doi:10.1016/j.molmed.2012.10.002.

Cambier, C.J., K.K. Takaki, R.P. Larson, R.E. Hernandez, D.M. Tobin, K.B. Urdahl, C.L. Cosma, and L. Ramakrishnan. 2014. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature*. 505:218–222. doi:10.1038/nature12799.

Chapman, J.S., and J.S. Bernard. 1962. The tolerances of unclassified mycobacteria. I. Limits of pH tolerance. *Am. Rev. Respir. Dis.* 86:582–583.

Clay, H., H.E. Volkman, and L. Ramakrishnan. 2008. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity*. 29:283–294. doi:10.1016/j.immuni.2008.06.011.

Comas, I., M. Coscolla, T. Luo, S. Borrell, K.E. Holt, M. Kato-Maeda, J. Parkhill, B. Malla, S. Berg, G. Thwaites, D. Yeboah-Manu, G. Bothamley, J. Mei, L. Wei, S. Bentley, S.R. Harris, S. Niemann, R. Diel, A. Aseffa, Q. Gao, D. Young, and S. Gagneux. 2013. Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. *Nat Genet*. 45:1176–1182. doi:10.1038/ng.2744.

Corr, S.C., and L.A.J. O'Neill. 2009. Genetic variation in Toll-like receptor signalling and the risk of inflammatory and immune diseases. *J Innate Immun*. 1:350–357. doi:10.1159/000200774.

Cronan, M.R., and D.M. Tobin. 2014. Fit for consumption: zebrafish as a model for tuberculosis. *Dis Model Mech*. 7:777–784. doi:10.1242/dmm.016089.

Davis, J.M., and L. Ramakrishnan. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell*. 136:37–49. doi:10.1016/j.cell.2008.11.014.

Davis, J.M., H. Clay, J.L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan. 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity*. 17:693–702.

Denk, W., and H. Horstmann. 2004. Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. *Plos Biol*. 2:e329. doi:10.1371/journal.pbio.0020329.

Deretic, V. 2012. Autophagy: An Emerging Immunological Paradigm. *The Journal of Immunology*.

Egner, A., S. Jakobs, and S.W. Hell. 2002. Fast 100-nm resolution three-dimensional microscope reveals structural plasticity of mitochondria in live yeast. *Proc. Natl. Acad. Sci. U.S.A.* 99:3370–3375. doi:10.1073/pnas.052545099.

Elks, P.M., S. Brizee, M. van der Vaart, S.R. Walmsley, F.J. van Eeden, S.A. Renshaw, and A.H. Meijer. 2013. Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism. *PLoS Pathog*. 9:e1003789. doi:10.1371/journal.ppat.1003789.

Eum, S.-Y., J.-H. Kong, M.-S. Hong, Y.-J. Lee, J.-H. Kim, S.-H. Hwang, S.-N. Cho, L.E. Via, and C.E. Barry. 2010. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*. 137:122–128. doi:10.1378/chest.09-0903.

Faas, F.G.A., M.C. Avramut, B.M. van den Berg, A.M. Mommaas, A.J. Koster, and R.B.G. Ravelli. 2012. Virtual nanoscopy: generation of ultra-large high resolution electron microscopy maps. *The Journal of Cell Biology*. 198:457–469. doi:10.1083/jcb.201201140.

Flynn, J.L., and J. Chan. 2005. What's good for the host is good for the bug. *Trends Microbiol.* 13:98–102. doi:10.1016/j.tim.2005.01.005.

Gay, N.J., M. Gangloff, and L.A.J. O'Neill. 2011. What the Myddosome structure tells us about the initiation of innate immunity. *Trends in Immunology*. 32:104–109. doi:10.1016/j.it.2010.12.005.

Goldberg, D.E., R.F. Siliciano, and W.R. Jacobs. 2012. Outwitting evolution: fighting drug-resistant TB, malaria, and HIV. *Cell*. 148:1271–1283. doi:10.1016/j.cell.2012.02.021.

Hell, S.W. 2007. Far-field optical nanoscopy. *Science*. 316:1153–1158. doi:10.1126/science.1137395.

Hell, S.W., and J. Wichmann. 1994. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt Lett*. 19:780–782.

Herbomel, P., B. Thisse, and C. Thisse. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development*. 126:3735–3745.

Houben, E.N.G., K.V. Korotkov, and W. Bitter. 2014. Take five - Type VII secretion systems of Mycobacteria. *Biochim. Biophys. Acta*. 1843:1707–1716. doi:10.1016/j.bbamcr.2013.11.003.

Hwang, W.Y., Y. Fu, D. Reyon, M.L. Maeder, S.Q. Tsai, J.D. Sander, R.T. Peterson, J.-R.J. Yeh, and J.K. Joung. 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* 31:227–229. doi:10.1038/nbt.2501.

Kanwal, Z., A. Zakrzewska, J. den Hertog, H.P. Spaink, M.J.M. Schaaf, and A.H. Meijer. 2013. Deficiency in hematopoietic phosphatase ptpn6/Shp1 hyperactivates the innate immune system and impairs control of bacterial infections in zebrafish embryos. *The Journal of Immunology*. 190:1631–1645. doi:10.4049/jimmunol.1200551.

Kawai, T., and S. Akira. 2005. Pathogen recognition with Toll-like receptors. *Current Opinion in Immunology*. 17:338–344. doi:10.1016/j.coi.2005.02.007.

Lienhardt, C., M. Raviglione, M. Spigelman, R. Hafner, E. Jaramillo, M. Hoelscher, A. Zumla, and J. Gheuens. 2012. New drugs for the treatment of tuberculosis: needs, challenges, promise, and prospects for the future. *J. Infect. Dis.* 205 Suppl 2:S241–9. doi:10.1093/infdis/jis034.

Lin, S.-C., Y.-C. Lo, and H. Wu. 2010. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature*. 465:885–890. doi:10.1038/nature09121.

Lowe, D.M., P.S. Redford, R.J. Wilkinson, and A. O'Garra. 2012. Neutrophils in tuberculosis: friend or foe? *Trends in ….*

MacMicking, J.D. 2014. Cell-autonomous effector mechanisms against mycobacterium tuberculosis. *Cold Spring Harb Perspect Med*. 4. doi:10.1101/cshperspect.a018507.

Martin, J.S., and S.A. Renshaw. 2009. Using in vivo zebrafish models to understand the biochemical basis of neutrophilic respiratory. *Biochem. Soc. Trans.*

Medzhitov, R., and C. Janeway. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol.* 8:452–456.

Meijer, A.H., A.M. van der Sar, C. Cunha, G.E.M. Lamers, M.A. Laplante, H. Kikuta, W. Bitter, T.S. Becker, and H.P. Spaink. 2008. Identification and real-time imaging of a myc-expressing neutrophil population involved in inflammation and mycobacterial granuloma formation in zebrafish. *Developmental & Comparative Immunology*. 32:36–49. doi:10.1016/j.dci.2007.04.003.

Mostowy, S. 2013. Autophagy and bacterial clearance: a not so clear picture. *Cellular Microbiology*. 15:395–402. doi:10.1111/cmi.12063.

Ottenhoff, T.H.M. 2012. New pathways of protective and pathological host defense to mycobacteria. *Trends Microbiol.* 20:419–428. doi:10.1016/j.tim.2012.06.002.

Ottenhoff, T.H.M., and S.H.E. Kaufmann. 2012. Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog*. 8:e1002607. doi:10.1371/journal.ppat.1002607.

Peddie, C.J., and L.M. Collinson. 2014. Exploring the third dimension: volume electron microscopy comes of age. *Micron*. 61:9–19. doi:10.1016/j.micron.2014.01.009.

Perskvist, N., L. Zheng, and O. Stendahl. 2000. Activation of human neutrophils by Mycobacterium tuberculosis H37Ra involves phospholipase C gamma 2, Shc adapter protein, and p38 mitogen-activated protein kinase. *J Immunol*. 164:959–965.

Philips, J.A., and J.D. Ernst. 2012. Tuberculosis pathogenesis and immunity. *Annu Rev Pathol*. 7:353–384. doi:10.1146/annurev-pathol-011811-132458.

Ramakrishnan, L. 2012. Revisiting the role of the granuloma in tuberculosis. *Nat Rev Immunol*.

Ramakrishnan, L. 2013. Looking within the zebrafish to understand the tuberculous granuloma. *Adv. Exp. Med. Biol.* 783:251–266. doi:10.1007/978-1-4614-6111-1_13.

Renshaw, S.A., and N.S. Trede. 2012. A model 450 million years in the making: zebrafish and vertebrate immunity. *Dis Model Mech*. 5:38–47. doi:10.1242/dmm.007138.

Repasy, T., J. Lee, S. Marino, N. Martinez, D.E. Kirschner, G. Hendricks, S. Baker, A.A. Wilson, D.N. Kotton, and H. Kornfeld. 2013. Intracellular bacillary burden reflects a burst size for Mycobacterium tuberculosis in vivo. *PLoS Pathog*. 9:e1003190. doi:10.1371/journal.ppat.1003190.

Roca, F.J., and L. Ramakrishnan. 2013. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. *Cell*. 153:521–534. doi:10.1016/j.cell.2013.03.022.

Rougeot, J., A. Zakrzewska, Z. Kanwal, H.J. Jansen, H.P. Spaink, and A.H. Meijer. 2014. RNA sequencing of FACS-sorted immune cell populations from zebrafish infection models to identify cell specific responses to intracellular pathogens. *Methods Mol. Biol.* 1197:261–274. doi:10.1007/978-1-4939-1261-2_15.

Rust, M.J., M. Bates, and X. Zhuang. 2006. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Meth*. 3:793–795. doi:10.1038/nmeth929.

Simeone, R., F. Sayes, O. Song, M.I. Gröschel, P. Brodin, R. Brosch, and L. Majlessi. 2015. Cytosolic access of Mycobacterium tuberculosis: critical impact of phagosomal acidification control and demonstration of occurrence in vivo. *PLoS Pathog*. 11:e1004650. doi:10.1371/journal.ppat.1004650.

Stoop, E., T. Schipper, and S. Huber. 2011. Zebrafish embryo screen for mycobacterial genes involved in the initiation of granuloma formation reveals a newly identified ESX-1 component. *Disease models & …*.

Swaim, L.E., L.E. Connolly, H.E. Volkman, O. Humbert, D.E. Born, and L. Ramakrishnan. 2006. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect. Immun.* 74:6108–6117. doi:10.1128/IAI.00887-06.

Takeda, K., and S. Akira. 2004. Microbial recognition by Toll-like receptors. *J. Dermatol. Sci.* 34:73–82. doi:10.1016/j.jdermsci.2003.10.002.

Tan, M.P., P. Sequeira, W.W. Lin, W.Y. Phong, P. Cliff, S.H. Ng, B.H. Lee, L. Camacho, D. Schnappinger, S. Ehrt, T. Dick, K. Pethe, and S. Alonso. 2010. Nitrate respiration protects hypoxic Mycobacterium tuberculosis against acid- and reactive nitrogen species stresses. *PLoS ONE*. 5:e13356. doi:10.1371/journal.pone.0013356.

Tan, S., and D.G. Russell. 2015. Trans-species communication in the Mycobacterium tuberculosis-infected macrophage. *Immunological Reviews*. 264:233–248. doi:10.1111/imr.12254.

Taylor, D.L., and E.D. Salmon. 1989. Basic fluorescence microscopy. *Methods Cell Biol.* 29:207–237.

Tjärnlund, A., E. Guirado, E. Julián, P.-J. Cardona, and C. Fernández. 2006. Determinant role for Toll-like receptor signalling in acute mycobacterial infection in the respiratory tract. *Microbes Infect.* 8:1790–1800. doi:10.1016/j.micinf.2006.02.017.

Torraca, V., S. Masud, H.P. Spaink, and A.H. Meijer. 2014. Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model. *Dis Model Mech*. 7:785–797. doi:10.1242/dmm.015594.

van der Sar, A.M., O.W. Stockhammer, C. van der Laan, H.P. Spaink, W. Bitter, and A.H. Meijer. 2006. MyD88 innate immune function in a zebrafish embryo infection model. *Infect. Immun.* 74:2436–2441. doi:10.1128/IAI.74.4.2436-2441.2006.

van der Vaart, M., C.J. Korbee, G.E.M. Lamers, A.C. Tengeler, R. Hosseini, M.C. Haks, T.H.M. Ottenhoff, H.P. Spaink, and A.H. Meijer. 2014. The DNA damage-regulated autophagy modulator DRAM1 links mycobacterial recognition via TLP-MYD88 to authophagic defense. *Cell Host and Microbe*. 15:753–767. doi:10.1016/j.chom.2014.05.005.

van der Vaart, M., H.P. Spaink, and A.H. Meijer. 2012. Pathogen recognition and activation of the innate immune response in zebrafish. *Adv Hematol*. 2012:159807. doi:10.1155/2012/159807.

van der Vaart, M., J.J. van Soest, H.P. Spaink, and A.H. Meijer. 2013. Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. *Dis Model Mech*. 6:841–854. doi:10.1242/dmm.010843.

van der Wel, N., D. Hava, D. Houben, D. Fluitsma, M. van Zon, J. Pierson, M. Brenner, and P.J. Peters. 2007. M. tuberculosis and M. leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. *Cell*. 129:1287–1298. doi:10.1016/j.cell.2007.05.059.

Volkman, H.E., H. Clay, D. Beery, J.C.W. Chang, D.R. Sherman, and L. Ramakrishnan. 2004. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. *Plos Biol*. 2:e367. doi:10.1371/journal.pbio.0020367.

Weiss, G., and U.E. Schaible. 2015. Macrophage defense mechanisms against intracellular bacteria. *Immunological Reviews*. 264:182–203. doi:10.1111/imr.12266.

Yang, C.-T., C.J. Cambier, J.M. Davis, C.J. Hall, P.S. Crosier, and L. Ramakrishnan. 2012. Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host and Microbe*. 12:301–312. doi:10.1016/j.chom.2012.07.009.