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Autophagy and Lc3-associated phagocytosis in host defense against Salmonella

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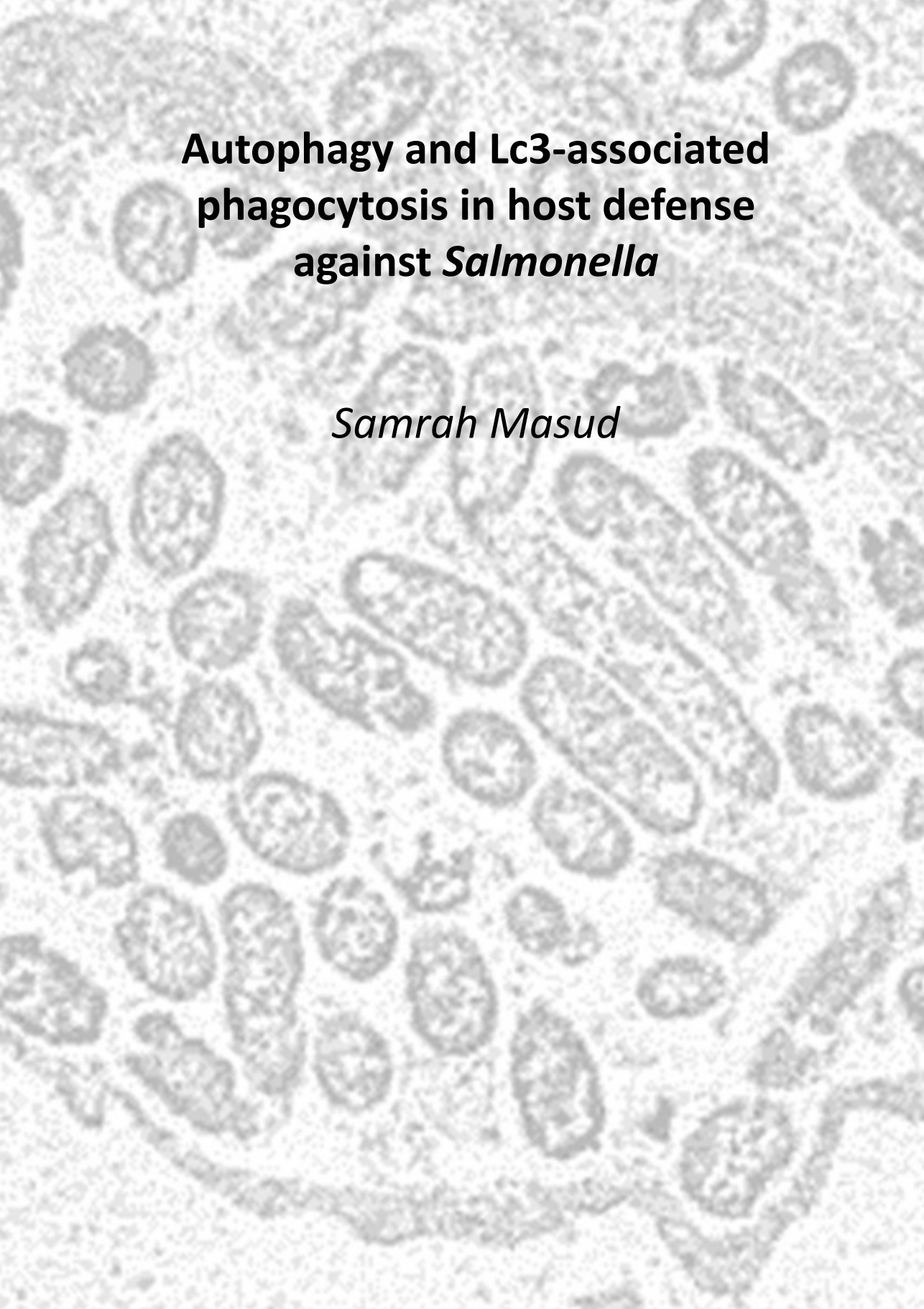
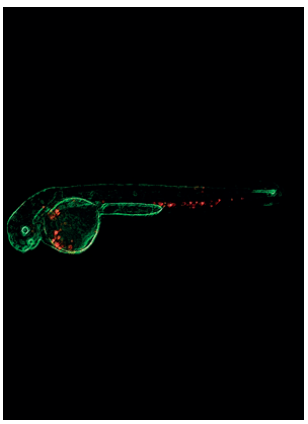
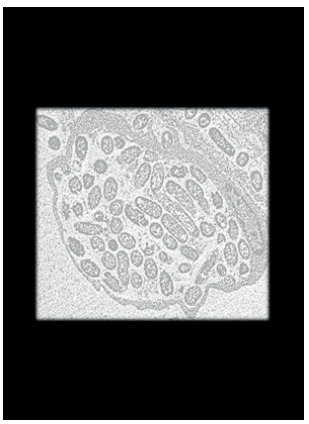
Autophagy and Lc3-associated phagocytosis in host defense against *Salmonella*

Samrah Masud







Autophagy and Lc3-associated phagocytosis in host defense against *Salmonella*

Cover (front): A two days old zebrafish embryo expressing a GFP-tagged Lc3 autophagy marker (green), systemically challenged with *mCherry*-expressing *Salmonella* Typhimurium (red), imaged at 4 hours post infection under a stereo fluorescence microscope. The image is shown in pastel smooth art.
Cover (back): A transmission electron micrograph of a *Salmonella*-infected macrophage in a two days old zebrafish embryo. The image is shown in pastel smooth art.

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Autophagy and Lc3-associated phagocytosis in host defense against *Salmonella*

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To my source of inspiration, my MOM, the one who believes in me,
my DAD, and my lovely kids, ZOYA, ABDULLAH, and AHMED

Table of Contents

Chapter 1 **Introduction and outline of the thesis** **7**

Chapter 2 **Modelling infectious diseases in the context of a developing immune system** **45**

Chapter 3 **Macrophages target *Salmonella* by Lc3-associated phagocytosis in a systemic infection model** **109**

Chapter 4 **Increased virulence of *Salmonella* mutants in Rubicon-deficient zebrafish** **151**

Chapter 5 **Autophagy modulator Dram1 promotes Lc3-associated phagocytosis of *Salmonella*** **181**

Chapter 6 **Summary and discussion** **203**

Dutch Summary / Samenvatting **223**

Curriculum vitae **231**

List of Publications **235**

Chapter 1

Introduction and outline of the thesis

Introduction and outline of the thesis

Our immune system has been shaped through millions of years of co-evolution with microbial pathogens, which have developed a wide variety of virulence strategies for evading host defense mechanisms. Some of these pathogens have the ability to survive intracellularly in host cells, even within the cells of the innate immune system that are armed with strong microbicidal mechanisms (Thi et al., 2012). Bacteria of the genus *Salmonella* are examples of facultative intracellular pathogens, capable of replicating inside epithelial cells as well as innate immune cells, such as macrophages and dendritic cells (Watson & Holden, 2010). There are several clinical manifestations of *Salmonella* infections (Salmonellosis), ranging from self-limiting gastroenteritis (food poisoning) to life-threatening typhoid (enteric) fever and bacteremia. Salmonellosis is one of a growing number of bacterial infectious diseases that are becoming more difficult to treat due to emerging antibiotic resistances (Kingsley et al., 2009; Wain et al., 1999). This emphasizes the need for novel therapeutic strategies and more research into the pathogenesis of *Salmonella* infections. Besides the medical relevance, there are also practical reasons why *Salmonella* species are widely used to study principles of host-pathogen interaction mechanisms. *Salmonella* bacteria can easily be cultured and genetically manipulated, and several tissue culture and animal models for *Salmonella* infections are available, including the mouse and the zebrafish (van der Sar et al., 2003; Watson & Holden, 2010).

The work in this thesis is focused on the cellular process of autophagy as an innate host defense mechanism against intracellular *Salmonella* bacteria. Autophagy (Greek word *auto*- self and *phagein*- eating) broadly refers to a number of cellular processes whereby cells degrade their cytoplasmic contents in lysosomes (Mizushima & Levine, 2010). The lysosome was discovered by Nobel laureate of 1974, Christian De Duve, who also introduced the term autophagy and provided the first biochemical proof of this process (de Duve, 2005; Klionsky, 2008). Autophagy became Nobel Prize winning research again in 2016, when Yoshinori Ohsumi was honored for his pioneering work in yeast that led to major advances in our understanding of the genes and molecular mechanisms involved in autophagy. Autophagy has become an intensive field of study because defects in this process have been found to underlie many diseases, from cancer to neu-

rodegenerative disorders (Levine & Klionsky, 2017). The strong association between autophagy and disease is not surprising, considering that autophagy is responsible for maintaining cellular homeostasis by continuous elimination of misfolded and oxidized aggregates of proteins and by clearing damaged organelles (Ohsumi, 2014). Furthermore, autophagy maintains energy support to cells, particularly during starvation. A fast growing body of evidence supports that autophagy also plays multiple roles in the immune system, ranging from the direct elimination of intracellular pathogens to indirect functions in the regulation of the immune response and effector mechanisms (Deretic et al., 2013).

Anti-*Salmonella* autophagy has been widely studied *in vitro*, providing evidence that engagement of the autophagy machinery is an essential element of the innate immune defense against this pathogen (Huang & Brumell, 2014). In our work we aimed to extend the study of anti-*Salmonella* autophagy to the whole organismal level. This objective could be achieved by exploiting the zebrafish-*Salmonella* infection model. Non-invasive imaging in zebrafish embryos expressing a fluorescently tagged version of the autophagy marker Lc3 (microtubule-associated protein light chain 3) made it possible to visualize the autophagy response during systemic Salmonellosis, where cells of the innate immune system, particularly the macrophages, are the main carriers of the infection. This thesis reports on a detailed characterization of the *in vivo* dynamics of anti-*Salmonella* autophagy and on results of functional studies of different host and pathogen factors affecting this innate immune response. First, in this chapter, a brief overview of *Salmonella* pathogenesis and the zebrafish-*Salmonella* model is given, and the current knowledge of the autophagy response to *Salmonella* infection is discussed.

Pathogenicity of *Salmonella*

Salmonellae are Gram-negative bacteria that are a major cause of enteric infections around the globe. These bacteria infect not only humans but also other vertebrates and are a common cause of zoonotic disease. The strains causing these infections are different serovars of the species *Salmonella enterica*. *S. enterica* serovar Typhi (*S.* Typhi) is the causative agent of typhoid fever, a life-threatening systemic infection characterized by nausea, fever, abdominal pain, and enlargement of the spleen and liver. *S.*

enterica serovar Paratyphi (*S.* Paratyphi) causes a similar but a less severe illness, called paratyphoid fever. *S.* Typhi and *S.* Paratyphi are endemic in regions of the world where drinking water quality and sewage treatment facilities are poor (House et al., 2001). In the Western world, typhoid and paratyphoid fever are rare diseases, but non-typhoid *Salmonella* infections, caused by *S. enterica* serovar Enteriditis (*S.* Enteriditis) or *S. enterica* serovar Typhimurium (*S.* Typhimurium) are common. In experimental research, *S.* Typhimurium is often used as a surrogate model for *S.* Typhi, since it causes a systemic typhoid-like disease in mouse. However, in humans, *S.* Typhimurium and *S.* Enteriditis infections usually are limited to mild cases of gastroenteritis (food poisoning). Nevertheless, these infections can be dangerous to elderly people, young children, HIV patients, or other immunocompromised people. Invasive *Salmonella* infections can usually be treated by antibiotic therapy, but the increasing frequency of resistant subtypes poses a serious global health concern (Kingsley et al., 2009; Wain et al., 1999).

Salmonellae are orally acquired by ingesting contaminated food or water. After entering the small intestine, Salmonellae traverse the inner mucus layer and evade killing by digestive enzymes, bile salts, and antimicrobial peptides. Subsequently, they can invade non-phagocytic enterocytes by bacteria-mediated endocytosis (Francis et al., 1992). In addition, Salmonellae can cross the epithelial barrier by entering M cells, which are specialized epithelial cells involved in sampling of intestinal antigens and transporting these to the lymphoid nodules of the gut, known as Peyer's patches (Haraga et al., 2008; LaRock et al., 2015). Moreover, *Salmonella* infections can disrupt epithelial integrity by breaching tight junctions (Finlay & Brumell, 2000; Jepson & Clark, 2001). Once the epithelial cell barrier is breached or following the translocation by M cells, Salmonellae may be phagocytosed by macrophages or actively invade these cells. Following internalization by macrophages, Salmonellae activate various virulence mechanisms in order to survive the microbicidal environment (Agbor & McCormick, 2011; Haraga et al., 2008; LaRock et al., 2015)

As soon as *Salmonella* cells gains entry into a host cell it is restricted in a phagosome-derived compartment called the *Salmonella*-containing vacuole (SCV). *Salmonella* controls the maturation of the SCV via secretion of effector proteins and the bacteria can replicate inside this compartment.

However, *Salmonella* can also escape into the cytoplasm shortly after its invasion and can replicate there. The success of Salmonellae as virulent intracellular survivors depends on their abilities to manipulate host cells via their specialized type III secretion systems (T3SS), which are needle-like structures that inject effector proteins into the host cell cytosol (Finlay & Brumell, 2000; Kubori et al., 1998; Ramos-Morales, 2012; Ruby et al., 2012). These effectors can alter host cellular functions, such as cytoskeletal architecture, membrane trafficking, signal transduction and cytokine gene expression, in order to promote bacterial survival and intracellular growth. Salmonellae encode two distinct virulence-associated T3SSs within *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2) that function at different times during infection (Hansen-Wester & Hensel, 2001). The SPI1-encoded T3SS is activated upon contact with the host cell and translocates bacterial proteins across the plasma membrane, whereas the SPI2 T3SS is expressed within the phagosome and translocates effectors across the vacuolar membrane. The SPI1 system has been shown to be required for colonization of the intestine, invasion of non-phagocytic cells, and induction of intestinal inflammatory responses and diarrhea (Deiwick et al., 1998; Hensel et al., 1997). The SPI2 T3SS, by contrast, has an important role in bacterial survival in macrophages and establishment of systemic disease (Figueira & Holden, 2012; Giacomodonato et al., 2007; Hensel, 2000).

The Zebrafish-*Salmonella* model

Zebrafish has rapidly gained ground as a versatile model for studying mechanisms of human diseases, including host-pathogen interactions (Meijer & Spaink, 2011). There is a striking similarity between the human and zebrafish genome and both have comparable immune cell types (Howe et al., 2013; Renshaw & Trede, 2012; Tobin et al., 2012). Because the cells of the innate immune system (macrophages and neutrophils) develop earlier than cells of the adaptive immune system (T and B cells), zebrafish embryos are frequently used as a laboratory animal model to study the role of innate immune responses during bacterial infections (Torraca et al., 2014) **(Chapter 2)**. There are many practical advantages in using zebrafish embryos as a model system, such as the ease of obtaining them in large numbers, their small size and accessibility due to external development, and their optical transparency that enables non-invasive

microscopic imaging. Furthermore, a wide range of genetic and pharmacological tools and extensive mutant resources are available for zebrafish research (Auer at al., 2014; Hwang et al., 2014; Jao et al., 2013). A large collection of transgenic reporter lines, including macrophage, neutrophil and autophagy marker lines, add to the usefulness of the zebrafish model for exploring human infection diseases (Torraca et al., 2014). Various zebrafish embryo infection models for clinically relevant pathogenic bacteria, including *Salmonella*, have been successfully established over the recent years (Torraca et al., 2014; van der Sar et al., 2003). The advantages of the zebrafish model are exploited in this thesis to gain insight into the role of autophagy in innate immune defense against *Salmonella* infection **(Chapter 3)**.

The study establishing the zebrafish-*Salmonella* model was one of the first demonstrating the usefulness of zebrafish embryos for imaging pathogen-macrophage interactions in a living host (van der Sar et al., 2003). Intravenous infection of one-day-old embryos with a low inoculum of wild type *S.* Typhimurium was found to result in a lethal infection. Bacterial cells were observed replicating intracellularly in macrophages and extracellularly attached to blood vessel epithelial cells. In contrast, heat-killed bacteria were completely lysed inside the blood circulation of the embryos, even before they were phagocytosed. Lipopolysaccharide (LPS) mutants of *S.* Typhimurium proved to be non-pathogenic in this model, in agreement with studies in murine models (van der Sar et al., 2003). Extending this work, we investigated a number of other virulence factors of *S.* Typhimurium, using mutant strains including Δ*ssrB* (mutation in one of the SPI2 regulators, SsrB), Δ*sipB* (mutation in a translocator of SPI1 effectors, SipB), Δ*phoP* (mutation in PhoP/Q, the two component sensor for host anti-bacterial cations and master transcriptional regulator of virulence), Δ*flhD* (mutation in flagellar transcription regulator FlhD) and Δ*purA* (mutation in adenylsuccinate synthetase required for purine metabolism). We found that the Δ*phoP* and Δ*purA* mutants showed attenuated virulence in the zebrafish embryo model, whereas the Δ*flhD* mutant caused more severe infection, suggesting that the presence of flagella on wild type bacteria elicits a protective innate immune response in the zebrafish embryo **(Chapter 4)**.

Previous work from our laboratory showed that the innate immune response to *Salmonella* flagellin is dependent on Toll-like receptor 5 (Tlr5a/b) and the adaptor molecule Myeloid differentiation factor 88 (Myd88) (Stockhammer et al., 2009). Morpholino knockdown or mutation of the *myd88* gene increased the susceptibility of zebrafish embryos to *S.* Typhimurium wild type or LPS mutant strains (van der Sar, 2006; van der Vaart et al., 2013). Furthermore, deficiency in Myd88 strongly reduced the *Salmonella*-mediated induction of genes encoding transcriptional regulators of the immune response (such as NFkB and AP-1) and genes encoding proinflammatory cytokines (such Il1b and Tnfa) (van der Vaart et al., 2013). TNF receptor associated factor 6 (Traf6), which functions downstream of Myd88, was also shown to be required for the innate immune response to *S.* Typhimurium infection (Stockhammer et al., 2010).

The immune response of zebrafish embryos to *S.* Typhimurium has been characterized in detail by our group using microarrays and RNA sequencing (Ordas et al., 2011; Stockhammer et al., 2010; Stockhammer et al., 2009; van der Vaart et al., 2013). These studies led to the identification of a large set of novel immune response genes that had not been previously implicated in host defense against *S.* Typhimurium infections. Notably present among a large pool of upregulated genes in *S.* Typhimurium-infected embryos was an autophagy related gene, known as DNA-damage regulated autophagy modulator 1 (*dram1*) (Stockhammer et al., 2010). Our group showed that this gene is regulated by Myd88-dependent innate immune signaling and plays a host protective role during *Mycobacterium marinum* infection in zebrafish (van der Vaart et al., 2014). This inspired us to investigate the role of Dram1 during *Salmonella* infection in this thesis **(Chapter 5)**.

Functions of autophagy in homeostasis and immune defense

Autophagy is conserved throughout eukaryotic life forms and functions as a housekeeping mechanism for disposal of intracellular organelles and protein aggregates, maintaining both the quantity and quality of cellular components (Levine et al., 2011). Basal levels of autophagy are required to maintain cellular homeostasis. However, different stressors, ranging from deprivation of nutrients to immune signaling due to disease and invading microbes, can induce autophagy at a transcriptional level and/or

by means of post-translational modifications. In response to starvation, eukaryotic cells use autophagy to recover nutrients. During infections, autophagy plays a key role as an immune effector, contributing both directly and indirectly to pathogen clearance (Deretic et al., 2013; Levine et al., 2011). More specifically, the four major immune related responses that intersect with autophagy have been categorized as (i) direct elimination of microorganisms, (ii) control of inflammation via pro-inflammatory signaling, (iii) control of antigen presentation and maintaining lymphocyte homeostasis (adaptive immunity) and (iv) secretion of immune mediators (Deretic et al., 2013).

Macroautophagy is the predominant form of autophagy, and when the general term autophagy is used, it usually refers to this process (also in this thesis). Macroautophagy is the process whereby cytoplasmic material (the cargo) is entrapped in a double membrane structure, the isolation membrane or phagophore, which eventually closes to form a double membrane vesicle, the autophagosome (usually 0.5-1 μm in diameter). The maturation of autophagosomes, resulting in fusion with lysosomes and degradation of the content by lysosomal hydrolases, is described as autophagic flux. Autophagosomes can also fuse with endosomes or multivesicular bodies (mvb) and major histocompatibility complex MHC-class II loading compartments (Schmid et al., 2007). Macroautophagy can be a bulk process, capturing the cargo in a non-specific manner, or it can be a selective process mediated by receptors that recognize cargo marked for degradation by signals such as ubiquitin. For example, the selective autophagy of protein aggregates is called aggrephagy, whereas mitophagy refers to selective degradation of mitochondria, and the selective targeting of microbial invaders is called xenophagy.

During infection, autophagy can be induced as a response to deficiencies in intracellular nutrients arising due to competition between the host cell and the microbial invader (Shelly et al., 2009; Tattoli et al., 2012a). However, autophagy induction is also a direct element of the host innate immune response controlled by pattern recognition receptor (PRR) signaling. Several classes of PRRs have been shown to induce autophagy, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs), all of which are specialized in recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular

patterns (DAMPs) (Delgado & Deretic, 2009; Levine et al., 2011; Xu et al., 2007). In addition, other immune signaling pathways, such as interferon gamma (IFNγ) and 1,25-dihydroxyvitamin D3 signaling, may promote expression and early delivery of anti-microbial peptides to the bacteria-containing (autophagic) compartments (Alonso et al., 2007; Harris et al., 2007; Kim et al., 2011; Ponpuak et al., 2010).

Direct anti-bacterial autophagy or xenophagy was first demonstrated for *Streptococcus pyogenes*, also known as group A *Streptococcus* (GAS). In this study GAS were reported to be enclosed in vesicles that were up to ten times larger in diameter (5-10 μm) than the previously reported size of autophagosomes (Nakagawa et al., 2004). Simultaneously, similar studies showed that induction of autophagy suppresses intracellular survival of *Mycobacterium tuberculosis* (Gutierrez et al., 2004). Since then autophagy has been shown to interact with a growing and diverse number of bacterial pathogens, including *S.* Typhimurium (Birmingham et al., 2006), *Listeria monocytogenes* (Rich et al.,2003), and *Shigella flexneri* (Ogawa et al., 2005). Xenophagy is also directed against intracellular parasites and viruses, for example *Toxoplasma gondi* (Andrade et al., 2006) and Sindbis virus (Orvedahl et al., 2010).

Components of the autophagy machinery

The complete process of autophagy can be dissected into the following steps: (i) signal induction, (ii) membrane nucleation, (iii) cargo targeting, (iv) membrane elongation, (v) autophagosome formation, (vi) fusion with lysosomes, and (vii) cargo degradation and nutrient recycling (Parzych & Klionsky, 2014) **(Figure 1)**. This process depends on an ensemble of autophagy related proteins (ATG proteins) and a number of regulatory pathways. So far more than 36 members of the ATG family have been identified, among which some have additional roles in cell physiology independent of autophagy. Nutrient starvation can induce autophagy through inhibition of mammalian target of rapamycin (mTOR), resulting in translocation of the ULK1 complex from the cytosol to certain domains of the endoplasmic reticulum (ER). This protein complex consists of Unc-51-like Kinase 1 (ULK1 or Atg1 in yeast), autophagy related protein 13 (ATG13), FAK family kinase-interacting protein of 200kDa (FIP200), and autophagy related protein 101 (ATG101) (also known as C12orf44)

(Itakura et al., 2010; Mizushima et al., 2011). The activation of the ULK1 complex results in recruitment of among others Beclin1 (BECN1, Atg6 in yeast) and ATG14L, which mediate phagophore nucleation together with the class III phosphatidylinositol 3-kinase (PI3KC3) VPS34, resulting in the production of phosphatidylinositol 3-phosphate (PI3P) and recruitment of effectors of the autophagy pathway like double FYVE containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) family proteins. DFCP1 is diffusely presented on the ER and Golgi complex but translocated to phagophore assembly sites (PAS) in a PI3P-dependent manner to generate ER-associated Ω-like structures called omegasomes, which serve as intermediates for the formation of phagophores (Axe et al., 2008). WIPI2 is the major effector required downstream of DFCP1 for promoting phagophore genesis (Polson et al., 2010). While the ER is thought to be the major site of phagophore formation, there is also evidence that autophagy membranes can originate from other sources such as mitochondria and the plasma membrane (Hailey et al., 2010; Rubinsztein et al., 2012).

The subsequent elongation of the phagophore and completion of the enclosure requires two ubiquitin-like conjugates. The first is the ATG12–ATG5 conjugate, which is produced by the ATG7 (E1-like) and ATG10 (E2-like) enzymes, and functions as a dimeric complex together with ATG16L1 (Fujita et al., 2013). The second ubiquitin-like conjugate comprises the homologues of yeast ATG8, which are the members of the LC3 (MAP1LC3) family (LC3A, LC3B, and LC3C) and the γ-aminobutyric acid receptor associated protein (GABARAP) family (GABARAP, GABARAPL1, and GABARAPL2). LC3 is activated by the ATG7 and ATG3 (E2-like) enzymes and conjugated to the lipid phosphatidylethanolamine (PE) at the target membrane through the action of the ATG12-ATG5-ATG16L1 complex and the cysteine protease ATG4B, which exposes a glycine residue at the carboxyl terminus of LC3 that is attached to PE (Nakatogawa et al., 2008; Weidberg, 2010). ATG4 is also required for recycling of LC3 as it can deconjugate LC3-PE complexes when autophagosome formation is completed. LC3 marks all the stages of autophagy, from phagophore formation until the fusion with lysosomes. Therefore, fluorescent proteins fused N-terminally to LC3 (usually LC3B) are widely used to monitor autophagy, resulting in the appearance of characteristic fluorescent punctae when LC3 is conjugated to PE (Klionsky et al., 2016).

Finally, the maturation of autophagosomes and fusion with lysosomes requires proteins of the small GTPase family (RAB7), the SNARE family (syntaxin 17 on autophagosomes and VAMP8 on lysosomes), and lysosomal membrane proteins like lysosomal-associated membrane glycoprotein 1/2 (LAMP1/2). The lysosomal association with autophagosomes can be monitored using fluorescently tagged LAMP1/2, or tracking reagents of acidified compartments like Lysotracker (He & klionsky, 2010; Hubert et al., 2016).

Figure 1: Schematic representation of the autophagy pathway. *(figure on next page).* Autophagy is induced upon starvation, aging of cellular organelles, or microbial invasion of a eukaryotic cell. Autophagy is initiated via the activation of the ULK1 complex, which downstream activates the Beclin1-VPS34-Atg14L complex, resulting in the formation of isolation membranes (phagophores) from membrane sources such as endoplasmic reticulum, Golgi apparatus or plasma membrane. Cargo is enclosed by the elongating phagophore in a non-specific manner or in a targeted manner mediated by selective autophagy receptors (not shown). Map1 LC3b (LC3) is incorporated in the growing phagophore and autophagosomes as it conjugates to lipid phosphatidylethanolamine at the membranes, dependent on the action of the ATG12-ATG5-ATG16L complex. Completion of membrane closure results in a double membrane compartment, the autophagosome, enclosing the cargo. Autophagosome maturation is positively regulated by the Beclin1-VPS34-UVRAG complex and inhibited by interaction of this complex with Rubicon. Fusion of lysosomes to autophagosomes converts these into autolysosomes, where acid hydrolases degrade the enclosed cargo. The resulting cargo components are recycled to be used as a source of energy during starvation or as building blocks for anabolic processes. Alternatively, direct elimination of invading microbes can also be achieved by the autophagy process. LC3 is a faithful marker of the autophagic process as it remains attached to autophagy structures throughout the whole process of their formation until subjected to degradation by acid hydrolases of lysosomes.

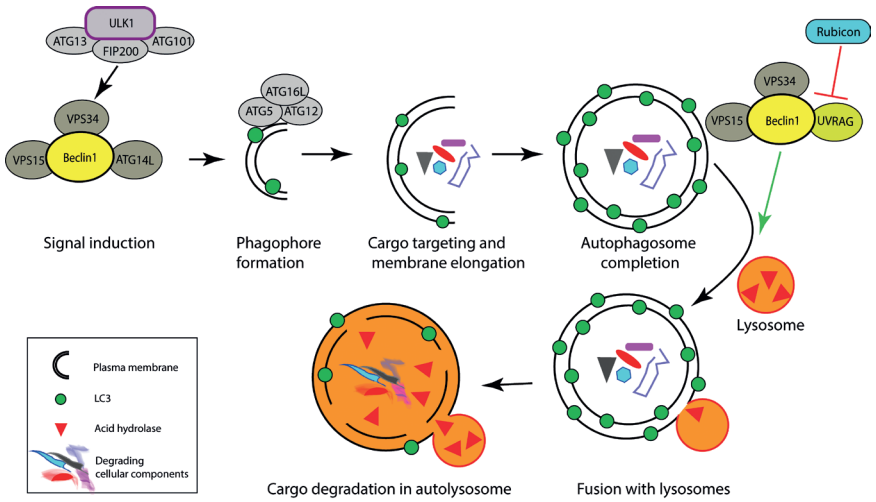

Figure 1: Schematic representation of the autophagy pathway. *(figure legend on previous page).*

Different types of autophagy

Besides macroautophagy, several other types of autophagy exist, including microautophagy, chaperon-mediated autophagy, and different non-canonical forms of autophagy. Microautophagy refers to a process whereby small portions (less than 0.5 µm) of cytoplasm are taken in directly by lysosomes. In chaperon-mediated autophagy, the cargo for degradation is recognized by the Hsc70 chaperon protein, which binds to LAMP-2A at the lysosomal surface, leading to translocation of the substrate across the membrane into the lysosomal lumen for degradation.

Non-canonical autophagy is macroautophagy that occurs independently of one or more members of the autophagy machinery (ATG system) (Deretic et al., 2013). Several types of non-canonical autophagy have been reported, such as ULK1 and ULK2-independent autophagy in cells after long term glucose starvation (Cheong et al., 2011), ATG5-independent autophagy in mouse embryonic fibroblasts (Nishida et al., 2009), and Beclin1-independent autophagy in cancer cells induced by apoptotic stimuli (Grishchuk et al., 2011; Scarlatti et al., 2008).

Another type of recently identified non-canonical autophagy is endosome-mediated autophagy (ENMA) (Kondylis et al., 2013). ENMA has been described as the major type of autophagy in dendritic cells, activated by TLR signaling upon binding of ligands such as LPS. During ENMA autophagosomes are derived from late endosomal MHC class II compartments (MIICs) and therefore these autophagosomes might function in processing and presentation of cytosolic antigens. LC3 and ATG16L1 were found to associate with these MIIC-derived autophagosomes in dendritic cells, but ENMA was shown to be independent of ATG4B and Lc3 lipidation (Kondylis et al., 2013).

Another autophagy-related process that plays an important role in host immunity is LC3-associated phagocytosis (LAP). LAP is a shared pathway involving components of the autophagy machinery and conventional phagocytosis, as further described in the next section. Canonical autophagy and LAP have been identified as two arms of autophagic innate immunity, and are studied in this thesis in the context of *Salmonella* infections **(Chapter 3)**.

LC3-associated phagocytosis (LAP)

The phagocytic activity of professional phagocytes, macrophages and neutrophils, plays a vital role in innate immunity (Kantari et al., 2008; Silva, 2010). Phagocytosis results in engulfment of microbes into an intracellular vacuole with a single membrane, termed the phagosome. During LAP, LC3 is recruited to phagosomes enclosing the invading microbes **(Figure 2)**. LAP was first reported in a study with *Escherichia coli* infection in RAW 264.7 macrophages expressing GFP-LC3, where this marker was recruited to phagosomes as early as 5-10 minutes post internalization of *E.coli* cells (Sanjuan et al., 2007). Other examples indicative of LAP in response to bacterial infections were observed during *S.* Typhimurium infection of murine neutrophils and Henle-407 cells (Huang et al., 2009), and in RAW 264.7 macrophages infected with *M. marinum* (Lerena & Colombo, 2011), *Burkholderia pseudomallei* (Gong et al., 2011), *Listeria monocytogenes* (Lam et al., 2013), and *Legionella dumoffii* (Hubber et al., 2017).

The exact mechanisms responsible for triggering LAP are not clear and require more investigation. However, LC3 recruitment to phagosomes is de-

pendent on microbial recognition by TLRs, as proteomic studies revealed that in absence of TLR signaling, LC3 fails to get recruited to phagosomes (Sanjuan et al., 2007; Shui et al., 2008). Additionally, activation of nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2 /NADPH oxidase 2) by TLRs or FcϒR receptors (FcϒR) to produce microbicidal reactive oxygen species (ROS) was shown to be required for LC3 recruitment to the phagosome membrane (Huang et al., 2009). Activation of the NADPH oxidase complex is critical for establishing the potent anti-microbial environment of the LC3-associated phagosome. This multi-protein oxidase complex, composed of the membrane-spanning factors NOX2 and p22phox as well as a number of soluble proteins, generates superoxide inside the phagosome. Superoxide is quickly consumed by several reactions, including those converting it into other reactive oxygen species (ROS) (Boyle & Randow, 2015). In addition to the role of TLRs and FcϒR in activating NOX2, a cell surface receptor on macrophages named signaling lymphocyte-activation molecule (SLAM) was shown to be involved in regulation of autophagy-associated proteins of the Beclin1-VPS34 complex (Berger et al., 2010).

LAP can also be induced by Nod-like receptors (NLRs) upon invasion of phagocytic and non-phagocytic cells by bacteria. The NLRs are a group of cytosolic PRRs responsible for recognizing intracellular bacteria and initiating a proinflammatory response (Ramjeet et al., 2010). The NLRs NOD1 and NOD2 recruit Atg16L to the site of bacterial invasion at the plasma membrane, potentially facilitating LC3 recruitment to the phagosomal membrane (Travassos et al., 2010). Of note, ATG16L1 and NOD2 polymorphisms are associated to Crohn's disease, suggesting that this type of chronic inflammatory bowel disease is associated with an inappropriate response to gut microbes (Brest et al., 2010).

An important difference in molecular requirement between autophagy and LAP is that the ULK1 complex initiation is not required for LAP but it is essential for autophagy (Martinez et al., 2011). Furthermore, it was recently found that the RUN and Cysteine rich domain containing Beclin1 interacting protein (Rubicon) is the molecular switch between autophagy and LAP, as it represses autophagy and activates LAP (Martinez et al., 2015); **(Figure 2)**. Rubicon was found to activate and stabilize the NOX2 complex and induce LAP in the immune reaction against *Aspergillus fumigatus*, eventually resulting in killing of the fungus. Rubicon had already been identified as a

negative regulator of the Beclin1-VPS34 complex in macrophages (Matsunaga et al., 2009). In agreement, macrophages from Rubicon-deficient mice showed increased autophagosome biogenesis under starvation conditions or upon rapamycin treatment, but failed to generate a LAP response when stimulated with the TLR2 ligand zymosan or with *A. fumigatus* (Martinez et al., 2015). Furthermore, it was identified that UV radiation resistance-associated gene (UVRAG), a component of the Beclin1-VPS34 complex, is essential for recruitment of LC3 to the phagosome (LAPosome). During LAP this complex lacks the canonical autophagy components ATG14L and AMBRA1. The role of Rubicon in the activation of NADPH oxidase is twofold: first, Rubicon activates VPS34 on the phagosome to generate the PI3P required for the recruitment of the soluble oxidase component p40phox; second, Rubicon binds p22phox to stabilize the oxidase complex directly (Yang et al., 2012; Martinez et al., 2015). Underscoring its importance in the activation of the NADPH oxidase, Rubicon-deficient macrophages fail to mount a ROS response upon phagocytosing the yeast cell wall extract zymosan (Boyle & Randow, 2015; Martinez et al., 2015).

In addition to its role in microbial defense, LAP has recently also been implicated in the process of efferocytosis, which is the clearance of dying or dead cells by macrophages (Green et al., 2016; Lai & Devenish, 2012; Martinez et al., 2011). LAP-deficient mice showed an acute proinflammatory response to injection of dying cells and developed a lupus-like autoinflammatory disease upon repeated injection, indicating that the engagement of the autophagy machinery through LAP is important to limit inflammation (Martinez et al., 2016).

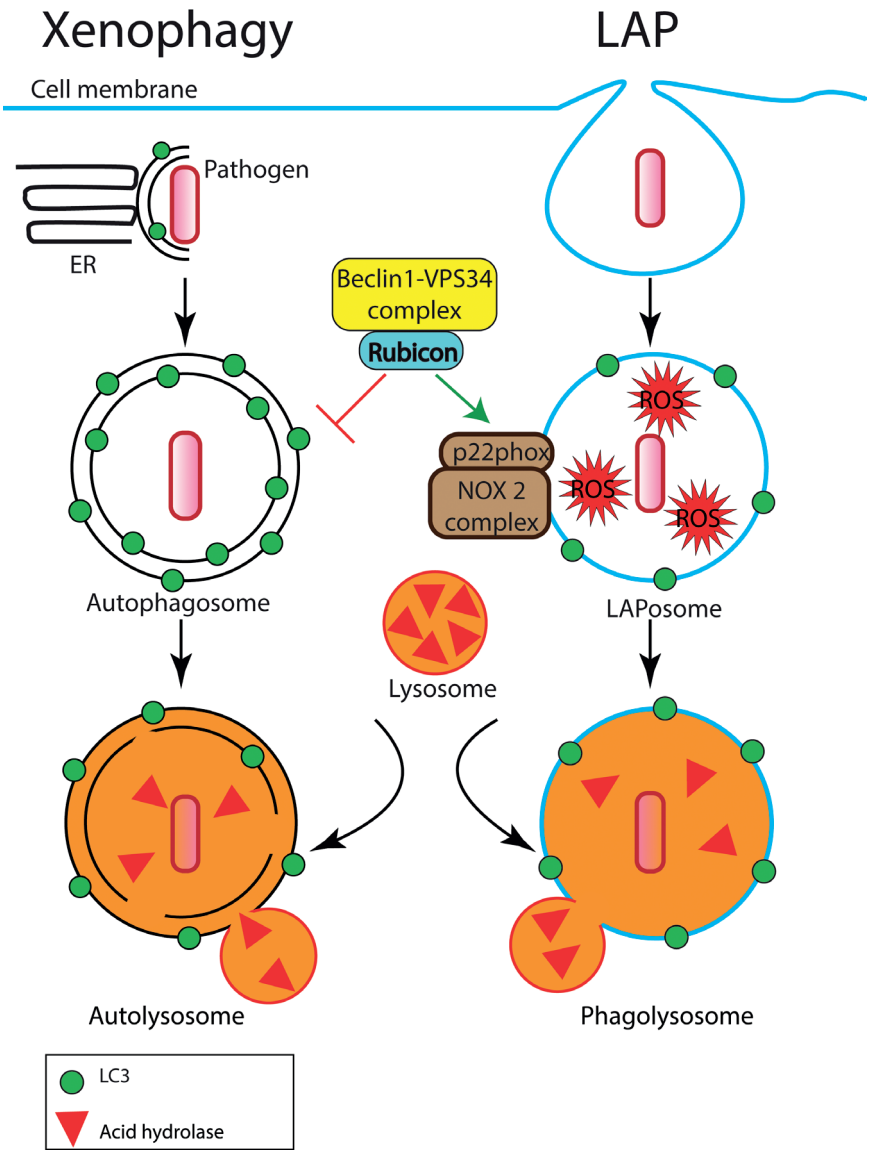

Figure 2: Xenophagy and LC3-associated phagocytosis (LAP).
(figure legend on next page).

Figure 2: Xenophagy and LC3-associated phagocytosis (LAP). *(figure on previous page).* Xenophagy and LAP play important roles in immunity and both depend upon the autophagy machinery. Xenophagy is a canonical form of autophagy that targets cytoplasmic microbial invaders, or detects damage to intracellular compartments where microbial invaders reside. This process depends strictly on all autophagy proteins shown in **Figure 1**. In contrast, LAP is an autophagy-related process that does not require the ULK1 complex for its induction. Rubicon plays contrasting roles in canonical autophagy and LAP. Rubicon blocks the Beclin1-VPS34-UVRAG complex and suppresses canonical autophagy, whereas it is required for LAP. During LAP, Rubicon stabilizes p22phox to activate the phagosomal NADPH oxidase (NOX2) complex and facilitates ROS generation. The LC3 protein is involved in both xenophagy and LAP, but autophagosomes are double membrane structures orginating from isolation membranes as shown in **Figure 1**, whereas LAPosomes possess a single membrane, whose source is the plasma membrane. Unless the process is inhibited by virulence factors of pathogens, autophagosomes and LAPosomes eventually fuse with lysosomes, resulting in degradation and direct elimination of the microbial invaders contained within these structures.

Selective autophagy

Pathogenic bacteria can evade the microbicidal function of macrophages by arresting phagosome maturation or by escaping into the cytoplasm. Escape is mediated by virulence factors, such as the T3SS of Salmonellae and the ESX1 secretion system of Mycobacteria, which induce damage to the phagosomal membrane. Damaged phagosomes and cytoplasmic bacteria are marked by molecular tags like ubiquitin, galectins, and membrane phospholipid modifications. A group of specialized autophagy adaptors, known as Sequestosome 1-like receptors (SLRs), recognize these molecular tags and thereby can target cytoplasmic microorganisms to autophagosomes (Johansen & Lamark, 2011; Rogov et al., 2014). SLRs have one or more cargo recognition domains (CRDs) that recognize ubiquitin-tags (Mostowy et al., 2011; Wild et al., 2011) or galectin tags (Li et al., 2013; Thurston et al., 2012). Since SLRs also have an LC3 interacting region motif (LIR) they can function as a bridge between cargo and phagophore.

The SLR family is named after its first described member, Sequestosome-1 (SQSTM1), better known as p62, which functions as ubiquitin receptor in autophagic targeting of protein aggregates, organelles, and microorgan-

isms (Bjorkoy et al., 2005; Geisler et al., 2010; Zheng et al., 2009). Several other SLR members have been implicated in xenophagy, including nuclear dot protein 52 (NDP52 or CALCOCO2), NDP52-like receptor TAX1-binding protein (TAX1BP1 or CALCOCO3), optineurin (OPTN), and neighbor of BRCA gene 1 (NBR1) (Dupont, 2009; Mostowy et al., 2013; Thurston et al., 2009; Wild et al., 2011). While all these SLRs contain motifs for binding mono- or polyubiquitinated cargo, NDP52 can also recognize galectin-8, which binds to β-galactose-containing glycans exposed on the membranes of damaged phagosomes or other vesicles (Boyle & Randow, 2013; Thurston et al., 2012). Furthermore, NDP52, OPTN, and TAX1BP1 bind to the actin-associated motor protein MYOSIN VI on endosomes, facilitating autophagosome maturation and autophagosome-lysosome fusion (Tumbarello et al., 2016; Tumbarello et al., 2012).

Tank binding kinase (TBK1) plays an important role in the regulation of selective autophagy. TBK1 is recruited by NDP52 and has been reported to phosphorylate OPTN and p62, which enhances their affinity for LC3B and ubiquitin chains (Heo et al., 2015; Pilli et al., 2012; Richter et al., 2016; Thurston et al., 2009; Wild et al., 2011). TBK1 is a central serine/threonine kinase in the stimulator of interferon gene/TBK1/interferon response factor 3 (STING/TBK1/IRF3) signaling axis that leads to production of type I interferon in response to infection (Manzanillo et al., 2012). The autophagy activating function of TBK1 is also dependent on STING, which is activated when extracellular bacterial DNA is exposed to the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS), following disruption of the phagosomal membrane. (Collins, 2015; Dey et al., 2015; Liang et al., 2014; Watson et al., 2012). In **Chapter 5** we address the role of autophagy modulation by Dram1 in *S.* Typhimurium infection of zebrafish embryos. In previous work Dram1 induction via the Myd88 pathway has been shown to promote zebrafish host defense against *M. marinum* by a mechanism dependent on STING and p62 (van der Vaart et al., 2014).

Anti-*Salmonella* autophagy

The role of autophagy as a defense response against *Salmonella* infection was first demonstrated by Brumell and coworkers, who showed that within 1 hour after infection approximately 20% of intracellular *S*. Typhimurium co-localized with GFP-LC3 in human epithelial cells (HeLa) or

mouse embryonic fibroblasts (MEFs) (Birmingham et al., 2006). Induction of the autophagy response was dependent on the SPI1 T3SS and GFP-LC3 positive autophagosomes containing bacteria often co-localized with polyubiquitin staining and with SCV markers. Together, these observations suggested that the autophagy machinery targets *Salmonella* bacteria in damaged SCVs. Increased cytosolic replication of *S.* Typhimurium was observed in ATG5-deficient MEFs, supporting the view of autophagy as a host protective response that counteracts SCV damage and prevents colonization of the cytosol (Birmingham et al., 2006). Furthermore, an NADPH oxidase dependent autophagy mechanism was found to restrict intracellular replication of *S*. Typhimurium in neutrophils and Henle-407 epithelial cells (Huang et al., 2009). Although one study reported contrary results in HeLa cells (Yu et al., 2014), many other studies in this cell type support the host protective function of autophagy in defense against *S.* Typhimurium (Cemma et al., 2011; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). Furthermore, knockdown of autophagy genes in *Caenorhabditis elegans* (homologs of Atg6 and Atg8) and *Dictyostelium discoideum* (homologs of Atg1, Atg6 and Atg7) and mutations of ATG16L1 and OPTN in mice resulted in increased replication of *S.* Typhimurium, providing also *in vivo* support for the anti-*Salmonella* function of autophagy (Conway et al., 2013; Jia et al., 2009; Riquelme et al., 2016).

The view of autophagy as a host defense mechanism does not exclude the possibility that Salmonellae could also have some benefit from the autophagy response. When *S.* Typhimurium invades non-phagocytic cells by means of the SPI1 T3SS, its entry is inevitably associated with membrane damage. Autophagy-mediated repair of this membrane damage could help the pathogen to establish its intracellular niche in the SCV and promote the subsequent expression of SPI2 virulence factors. Thus, by facilitating SCV maturation, autophagy can be beneficial to the pathogen during early stages of infection, whereas it is a host-beneficial response at later stages by restricting SCV rupture and cytosolic replication (Kreibich et al., 2015). In addition, *S.* Typhimurium has been found to co-opt chaperone-mediated autophagy for its intracellular growth (Singh et al., 2016).

S. Typhimurium can invade a variety of cell types, such as epithelial cells, fibroblasts, dendritic cells, macrophages, and neutrophils, and this patho-

gen employs different strategies for survival in each of these target cells. Therefore, it is not surprising that autophagy responses to *Salmonella* have also been found to differ between cell types. Most of the autophagy studies have been performed on cultured epithelial cells, where *S*. Typhimurium replicates at high rates and resides inside SCVs that form tubular networks consisting of *Salmonella*-induced filaments (SIFs). Bacteria escaping from these SCVs as well as the membranes from damaged SCVs are targeted by xenophagy (Shahnazari et al.,2011; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). In contrast to the situation in epithelial cells, *S.* Typhimurium hardly replicates in fibroblasts. Furthermore, SIF formation is rare in fibroblasts, and cytosolic escape is not observed. In this cell type *S.* Typhimurium has been found to be surrounded by double membrane structures while still inside the SCV and to induce an aggrephagy response. During this process, some of the SCVs become entrapped in aggregates of endosomal and lysosomal membranes that are marked by LC3 and p62 but not by ubiquitin (Kageyama et al., 2011; Lopez-Montero et al., 2016). While bacteria in such aggresomes are eventually digested, *S.* Typhimurium persists in other SCVs that escape this selective destruction process (Lopez-Montero et al., 2016). The formation of double membrane structures around SCVs in fibroblasts was found to require proteins involved in the early steps of autophagy initiation (ATG9L1 and FIP200), but recruitment of LC3 and ATG16L was shown to be independent from these proteins (Kageyama et al., 2011).

Studies of autophagy in macrophages are limited, despite that these cells are the main site of *S.* Typhimurium replication during systemic infection (Watson & Holden, 2010). In a study of bone marrow-derived murine macrophages, *S.* Typhimurium has been shown to cause autophagy-mediated programmed cell death by disrupting mitochondria (Hernandez et al., 2003). A host protective effect of autophagy has been observed in RAW 264.7 macrophages treated with a celecoxib derivative, AR-12. This compound increases LC3 association with *S*. Typhimurium and restricts replication within the first two hours after infection through a Beclin1 and ATG7 dependent mechanism. At later stages of infection AR-12 has an inhibitory effect that is independent from autophagy (Chiu et al., 2009). In this thesis we provide evidence that macrophages restrict *S.* Typhimurium growth during systemic infection of zebrafish by the LAP pathway, which

is dependent on Rubicon and NADPH-oxidase and independent from the ULK1 complex component ATG13 **(Chapter 3)**.

The molecular mechanisms for elimination of *Salmonella* from infected cells via xenophagy can be broadly categorized into two types on the basis of the molecular tags involved (Huang & Brumell, 2014). These mechanisms are either ubiquitin-dependent or ubiquitin independent. During ubiquitin-dependent autophagy in HeLa cells, the LRR-containing RING E3 ligase L1 specifically recognizes *S.* Typhimurium but is not required for ubiquitination of protein aggregates (Huett et al., 2012). Another E3 ligase, Parkin (PARK2), ubiquitinates mitochondria and *M. tuberculosis*, but might also contribute to anti-*Salmonella* autophagy because human polymorphisms are linked with susceptibility to *S.* Typhi and Parkin-deficient flies are more susceptible to *S.* Typhimurium (Manzanillo et al., 2013).

Four members of the SLR family have been implicated in ubiquitin-dependent xenophagy of *S*. Typhimurium, including p62 (Zheng et al., 2009), NDP52 (Thurston et al., 2009), OPTN (Wild et al., 2011), and TAXBP1 (Tumbarello et al., 2016). All four receptors are recruited independently to *Salmonella* and for p62 and NDP52 it has clearly been shown that they interact with different microdomains of the bacteria (Cemma et al., 2011; Tumbarello et al., 2016; Wild et al., 2011). The p62 microdomains can also be decorated with the ubiquitin-like modifier FAT10, which has been shown to contribute to *S.* Typhimurium defense in mice (Spinnenhirn et al., 2014). P62, NDP52, OPTN and TAX1BP1 have non-redundant functions in xenophagy, since depletion of any of these receptors leads to hyperproliferation of *S.* Typhimurium (Thurston et al., 2009; Tumbarello et al., 2012; Wild et al., 2011; Zheng et al., 2009). The ubiquitin-dependent recognition of *S.* Typhimurium by NDP52 is preceded by its transient recruitment to damaged SCVs via galectin-8 (Thurston et al., 2012). Another mechanism that could be responsible for selective autophagy of ubiquitin-decorated SCVs is through direct interaction with ATG16L1 upstream of LC3 (Fujita et al., 2013). Furthermore, WIPI2b binding to ATG16L1 and the SCV was shown to be required for initiating LC3 conjugation (Dooley et al., 2014). NDP52 interacts specifically with LC3 to target *S.* Typhimurium to xenophagy, but the subsequent maturation of autophagosomes relies on its interaction with other LC3 family members (LC3A and LC3B and/or GABARAPL2) and the molecular motor MYOSIN VI (Verlhac et al., 2015;

von Muhlinen et al., 2012). MYOSIN VI-mediated xenophagy of *S.* Typhimurium also requires the function of TAX1BP1 (Tumbarello et al., 2016).

A xenophagy pathway independent from ubiquitin requires the lipid second messenger diacylglycerol (DAG), which co-localizes with the SCV. The initiation of this process is not understood, but the damaged SCV could drive DAG production and initiate autophagy via protein kinase C activation and downstream NADPH oxidase and Jun N- terminal kinase (JNK) pathways (Shahnazari et al., 2010). Interestingly, ubiquitin-dependent autophagy and DAG-dependent autophagy are independent of each other, as the majority of LC3-positive *S.* Typhimurium bacteria were found to co-localize with DAG but were negative for ubiquitin and *vice versa* (Shahnazari et al., 2010).

Given the substantive experimental support for the host-protective function of autophagy, Salmonellae must have been under selective pressure to develop strategies to evade or block autophagy. From studies on other intracellular pathogens, especially cytosolic invaders such as *Listeria* and *Shigella*, there is evidence for many autophagy inhibitory mechanisms, which involve the targeting of Beclin1, inhibition of autophagosomal maturation, proteolytic cleavage of LC3, and masking of epitopes or tags which are recognized by SLRs (Huang & Brumell, 2014). The mechanisms by which Salmonellae inhibit autophagy are not fully understood. *S.* Typhimurium infection of HeLa cells leads to an acute amino acid starvation response in the cytosol leading to inhibition of mTOR, thus activating autophagy signaling. However, this is a transient, SPI1-dependent response that is rapidly restored by mTOR reactivation at the SCV surface (Tattoli et al., 2012b; Tattoli et al., 2012a). This proposed autophagy evasion mechanism is independent of SPI2 but requires host amino acid transporters (Tattoli et al., 2012b). Another autophagy inhibition mechanism does require an SPI2 effector, namely the SseL deubiquitinase, which removes ubiquitin tags from cytosolic protein aggregates that form during *Salmonella* infection and are thought to contribute to host defense (Mesquita et al., 2012). Furthermore, a SPI2-induced mechanism has been shown to recruit the non-receptor tyrosine kinase focal adhesion kinase (FAK) to the surface of the SCV in macrophages, which results in autophagy inhibition through mTOR activation and simultaneously prevents the induction of a protective immune response mediated by IFNβ and IFNγ (Owen et al.,

2014). Recent studies indicate that one of the *Salmonella* plasmid virulence genes, *spvB*, inhibits autophagic defense against *S.* Typhimurium in Hela cells and murine J774A.1 macrophages as well as in zebrafish and mouse models (Chu et al., 2016; Li et al., 2016; Wu et al., 2016). Finally, the *S.* Typhi plasmid pR(ST98), which is linked with virulence and multidrug resistance, mediates autophagy inhibition in fibroblasts, dendritic cells, and macrophages (Chu et al., 2014; Lv et al., 2012; Wu et al., 2014; Wu et al., 2010).

Contribution of this thesis: a brief outline

The aim of the work in this thesis was to utilize the zebrafish-*Salmonella* infection model to study the dynamics of anti-*Salmonella* autophagy and the functions of different components of the host autophagy machinery and pathogen virulence factors. The use of optically transparent embryos of the zebrafish enabled high resolution *in vivo* imaging of the interaction of *S.* Typhimurium with phagocytes and the autophagy marker Lc3. The results provide evidence for the host-protective function of Lc3-mediated defenses during systemic infection. Furthermore, we identified the host factors critical for targeting of *S.* Typhimurium to Lc3-decorated compartments and investigated the effects of *Salmonella* virulence factors on this defense response. The studies led to the conclusion that Lc3-associated phagocytosis (LAP), a process whereby Lc3 is recruited directly to pathogen-containing phagosomes, plays a major role in the host defense against *Salmonella*.

This introductory **Chapter 1** has provided background on the molecular mechanisms underlying autophagy and LAP and summarized the current knowledge of anti-*Salmonella* autophagic responses.

Chapter 2 is a detailed review highlighting recent contributions of zebrafish systems for studying innate immunity and modelling human infections.

Chapter 3 elaborates on the *in vivo* dynamics of GFP-Lc3 recruitment during infection of zebrafish embryos with *Salmonella* and shows that macrophages are the main players in defense against this pathogen, which

requires the host factors Rubicon and NADPH oxidase that are essential for LAP.

Chapter 4 describes the *Salmonella* virulence factors that are required for pathogenicity in the zebrafish model and provides further evidence for the host-protective function of LAP, showing that virulence of different *Salmonella* mutants is increased when LAP is inhibited by depletion of Rubicon.

Chapter 5 shows that the autophagy modulator Dram1 promotes LAP and is required for an effective defense of the zebrafish host to *Salmonella* infection.

Chapter 6 summarizes and contextualizes the findings of this thesis in relation to the current scientific literature.

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Chapter 2

Modelling infectious diseases in the context of a developing immune system

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Abstract

Zebrafish has been used for over a decade to study the mechanisms of a wide variety of inflammatory disorders and infections, with models ranging from bacterial, viral, to fungal pathogens. Zebrafish has been especially relevant to study the differentiation, specialization and polarization of the two main innate immune cell types, the macrophages and neutrophils. The optical accessibility and the early appearance of myeloid cells that can be tracked with fluorescent labels in zebrafish embryos and the ability to use genetics to selectively ablate or expand immune cell populations have permitted studying the interaction between infection, development and metabolism. Additionally, zebrafish embryos are readily colonized by a commensal flora, which facilitated studies that emphasize the requirement for immune training by the natural microbiota to properly respond to pathogens. The remarkable conservation of core mechanisms required for the recognition of microbial and danger signals and for the activation of the immune defenses illustrates the high potential of the zebrafish model for biomedical research. This review will highlight recent insight that the developing zebrafish has contributed to our understanding of host responses to invading microbes and the involvement of the microbiome in several physiological processes. These studies are providing a mechanistic basis for developing novel therapeutic approaches to control infectious diseases.

Introduction

Infectious diseases remain a major global health problem, with tuberculosis (TB) and HIV/AIDS as the biggest killers, each responsible for over a million deaths annually according to reports of the World Health Organization (www.who.int). The increasing occurrence of multidrug-resistant strains of *Mycobacterium tuberculosis*, the bacterial pathogen causing TB, indicates that current antibiotic treatment regimens are ineffective. Antibiotic resistances represent a serious problem also in hospital settings, with methicillin-resistant *Staphylococcus aureus* as a notable example of a pathogen causing opportunistic infections in immunocompromised patients. Despite intense research efforts, there are no effective vaccines against some of the major human bacterial pathogens, including *M. tuberculosis* and *S. aureus*. Furthermore, vaccines are not yet available for newly emerging viral diseases, which can spread rapidly due to transmission by insect vectors, as exemplified by the recent Zika virus outbreak. Development of novel therapeutic approaches for the treatment of infectious diseases requires detailed understanding of the mechanisms by which pathogens subvert the immune system of the infected host. As we discuss in this review, the zebrafish is a valuable addition to the range of animal models used for preclinical research into infectious disease biology.

The immune system of vertebrates functions by cooperative mechanisms of innate and adaptive immunity. During infection, innate immunity is activated by the recognition of microbial molecules and danger signals released by damaged host cells. Across species, innate immunity is mediated primarily by phagocytic cells, including macrophages, neutrophils and dendritic cells. Activated innate immune cells represent an important line of defense against a large spectrum of pathogens as they provide an immediate response to invading microbes. Additionally, cells of the innate immune system, by functioning as antigen presenting cells and by providing stimulatory signals, are essential to alert the adaptive immune system to mount a more specific immune response mediated by antibody-producing B-lymphocytes and cytotoxic T-lymphocytes. These cells collaborate to target, isolate or kill infected cells to prevent infection spreading throughout the organism.

Developing organisms rely more heavily on innate immunity, because the adaptive immune system takes longer to mature. For instance, it is well known that human neonates depend on maternal antibodies for adequate protection against infectious diseases. In zebrafish larvae, the first immature T-cell precursors are the first signs of an adaptive immunity, detected by 3 days post fertilization (dpf) (Langenau et al., 2004), however, functional phagocytes are present earlier, at 1 dpf **(Figure 1)** (Herbomel et al., 1999). B cells emerge from the pronephros of juvenile zebrafish only at 19 dpf and (Langenau et al., 2004) and antibody production does not occur until at least 21 dpf (Page et al., 2013). As a result, the zebrafish embryo and early larval stages have become widely used as an in vivo model to study innate immunity in separation from adaptive immunity (Harvie & Huttenlocher, 2015; Levraud et al., 2014; Meijer & Spaink, 2011; Ramakrishnan, 2013; Renshaw & Trede, 2012).

Figure 1: Development of zebrafish immune system. *(figure on next page).* In zebrafish, immune cells are generated via a primitive, intermediate and definitive wave of hematopoiesis, which are active in the indicated tissues in the developmental windows reported on the timeline. The figure also indicates the key transcriptional regulators controlling the differentiation fate and the distinctive markers expressed by each cell type (described in more detail in the main text). Abbreviations: Anterior lateral mesoderm (ALM), Posterior lateral mesoderm (PLM), Rostral blood island (RBI), Intermediate cell mass (ICM), Posterior blood island (PBI), Aorta-gonad-mesonephros (AGM), Ventral wall of dorsal aorta (VDA), Caudal hematopoietic tissue (CHT), Head kidney (HK), Myeloid progenitor cell(MPC), Erythromyeloid progenitor (EMP), Hematopoietic stem cells (HSC), Common myeloid progenitor (CMP).

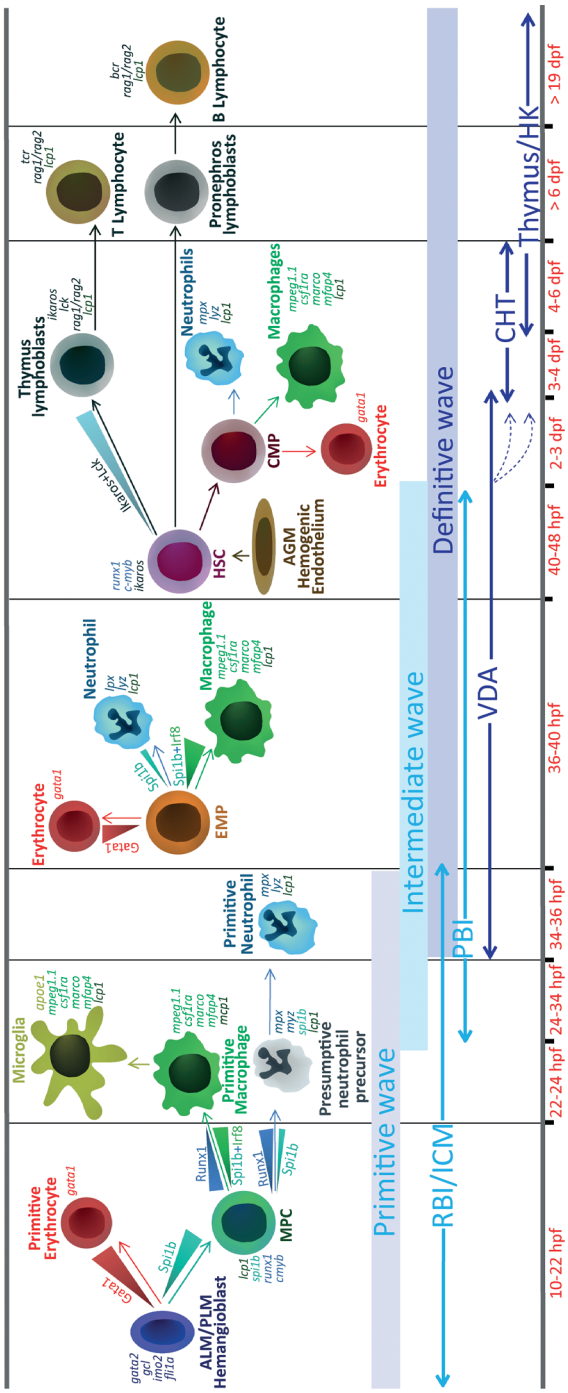

Figure 1: Development of zebrafish immune system. *(figure legend on previous page).*

The different cell types of the immune system are generated by hematopoiesis, defined as the differentiation of multipotent, self-renewing stem cells into all cellular components of the blood (Davidson & Zon, 2004; Jagannathan-Bogdan & Zon, 2013). In all vertebrates, hematopoiesis is a highly conserved process that involves successive waves of primitive, intermediate, and definitive generation of hematopoietic progenitor cells during ontogeny **(Figure 1)** (Bertrand et al., 2007; Galloway & Zon, 2003). Hematopoiesis can be further differentiated into erythropoiesis (the development of red blood cells), myelopoiesis (the development of leukocytes mediating innate immunity), and lymphopoiesis (the generation of the leukocytes (lymphocytes) of the adaptive immune system). Myeloid cells consist of two main categories based on cellular contents: (i) granulocytes and (ii) agranulated cells. Granulocytes (including neutrophils, eosinophils, basophils, and mast cells) display characteristic secretory granules in the cytoplasm containing antimicrobial molecules and inflammatory mediators. Furthermore, granulocytes can be recognized by a polymorphic nucleus, while agranulated cells, including monocytes and macrophages, are mononuclear.

In zebrafish embryos and early larval stages, all mononuclear cells are commonly referred to as (primitive) macrophages, irrespective of whether these cells are circulating in the blood or have invaded tissues (Herbomel et al., 1999; Herbomel et al., 2001). The specialized macrophages resident in the brain (microglia) are also already present in the early life stages of zebrafish and their progenitors can be distinguished as early as 1 dpf **(Figure 1)**. Neutrophils are the main granulocyte cell type in embryos and larvae (Lieschke et al., 2002). Mast cells can also be distinguished, but eosinophils are only described in adult zebrafish and basophils have not been identified (Balla et al., 2010; Dobson et al., 2008).

In this review, we describe how innate immune cell types arise during the normal course of zebrafish embryo and larval development, and how the production, differentiation and function of these cells can be affected by infection, inflammation and the presence of the gut microbiota. We discuss recent studies that show how innate immune responses are intricately linked with the regulation of energy metabolism and homeostasis, in which autophagy plays a major role. Furthermore, we review work that contributed to develop zebrafish infection models **(Table 1)**, which has

been particularly helpful to dissect the specific implications of different innate immune cell types in infectious disease pathologies. To illustrate this, we highlight recent studies of bacterial infections, including causative agents of human infectious diseases or opportunistic infections, such as Mycobacteria, Listeria, Shigella, Staphylococci and a range of viral, and fungal pathogens. These studies are providing new insight into host-pathogen interaction mechanisms that hold promise for translation into novel therapeutic strategies for human infectious diseases.

Table 1: Human infection diseases modelled in zebrafish.

Infectious agents	Human disease	Zebrafish infection model	First description
Bacteria	Tuberculosis	*Mycobacterium marinum* surrogate model for *Mycobacterium tuberculosis*	Davis et al., 2002
	Salmonellosis	*Salmonella enterica* serovar Typhimurium	van der Sar et al., 2003
	Shigellosis	*Shigella flexneri*	Mostowy et al., 2013
	Listeriosis	*Listeria monocytogenes*	Levraud et al., 2009
	Opportunistic infections	*Burkholderia cenocepacia*	Vergunst et al., 2010
		Pseudomonas aeruginosa	Clatworthy et al., 2009
		Staphylococcus aureus	Prajsnar et al., 2008
Viruses	Influenza	Influenza A virus	Gabor et al., 2014
	Herpes Simplex	Herpes simplex virus type 1	Burgos et al., 2008
	Chikungunya fever	Chikungunya virus	Palha et al., 2013
Fungi	Candidiasis	*Candida albicans*	Chao et al., 2010
	Aspergillosis	*Aspergillus fumigatus*	Knox et al., 2014

Infectious agents	Human disease	Zebrafish infection model	First description
	Mucormycosis	*Mucor circinelloides*	Voelz et al., 2015
	Cryptococcosis	*Cryptococus neoformes*	Tenor et al., 2015

Development of the cell types of the innate immune system

To understand how the immune system works, we must first understand how the cells in the innate immune system form, and zebrafish have provided an outstanding system for such studies. This is covered in depth elsewhere (Kawan & Trista, 2017). Here, we review the developmental aspects of innate immunity that are relevant to understanding the response to infection.

Generation of primitive myeloid cells

The development of the zebrafish immune system mirrors processes observed in other vertebrates, including mammals, but at an accelerated scale **(Figure 1)**. The first innate immune cells of the zebrafish embryo are generated during primitive hematopoiesis, which occurs in two locations of the zebrafish embryo: the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM). As the development proceeds, the ALM and PLM differentiate into the rostral blood island (RBI) and intermediate cell mass (ICM), respectively (Bertrand et al., 2007). The primitive myeloid cells develop from the RBI, while primitive erythrocytes originate from the ICM. By the 6-somite stage, expression of *spi1b* (*pu.1*) is detected, which encodes Pu.1, a master transcriptional regulator of myelopoiesis (Lieschke et al., 2002; Rhodes et al., 2005). By 16 hours post fertilization (hpf), Pu.1 positive myeloid progenitors originating from the RBI start to migrate over the yolk sac **(Figure 1)** (Bennett et al., 2001; Lieschke et al., 2002). This process requires granulocyte colony-stimulating factor receptor (Gcsfr) signaling (Liongue et al., 2009). During migration, these myeloid progenitors turn on the pan-leukocyte marker L-plastin (*lcp1*) (Bennett et al., 2001; Herbomel et al., 1999; Herbomel at al., 2001; Liu & Wen, 2002). Morphologically distinguishable macrophages are observed as

early as 22 hpf on the yolk sac and enter the blood circulation by 26 hpf. Some macrophages migrate into the cephalic mesenchyme from 22 hpf onwards in a *csf1ra* dependent manner and can eventually develop into microglia (Herbomel et al., 2001; Peri & Nusslein-Volhard, 2008). These macrophages are functional, and are capable of phagocytosing apoptotic debris, senescent red blood cells and experimentally injected bacteria (Herbomel et al., 1999). Thus, as early as 1 dpf, zebrafish embryos can be used to study the response to infection.

The genes *csf1ra*, *mpeg1.1*, *marco*, and *mfap4* are marker genes that are predominantly expressed in macrophages in comparison with other leukocytes (Benard et al., 2014; Ellett et al., 2011; Walton et al., 2015; Zakrzewska et al., 2010). Several of these markers have been used to generate transgenic reporter lines that are frequently used in infectious disease research **(Table 2)** (Ellett et al., 2011; Gray et al., 2011; Walton et al., 2015).

Morphologically distinguishable neutrophils appear later than macrophages (Le Guyader et al., 2008). Using an *in vivo* photoactivatable cell tracer, it has been demonstrated that primitive neutrophils originate from the RBI-derived hemangioblasts, the same lineage as the primitive macrophages, after the dispersal of the progenitors into the tissues **(Figure 1)** (Le Guyader et al., 2008). At 34 hpf, differentiated neutrophils are detectable by electron microscopy (Willett et al., 1999). In agreement, granules are observed under video-enhanced differential interference contrast microscopy around 35 hpf, a time when neutrophils can also be detected by staining with Sudan Black, a lipid marker for granules (Le Guyader et al., 2008). Sudan Black-positive neutrophils also stain positive for myeloperoxidase (Mpx) enzyme activity (Le Guyader et al., 2008; Lieschke et al., 2001) as early as 24 hpf, along with expression of the other neutrophil marker lysosome C (*lyz*) (Le Guyader et al., 2008; Meijer et al., 2008). Transgenic reporter lines for the *mpx* and *lyz* marker genes are widely used to study neutrophil behavior **(Table 2)**, (Hall et al., 2007; Renshaw et al., 2006). The *mpx*/*lyz*-positive phagocytes first appear as migrating cells on the yolk sac, and these are most likely progenitors of the neutrophils that can be detected in tissues of older embryos using Sudan Black staining (Harvie & Huttenlocher, 2015; Le Guyader et al., 2008)

Table 2: Markers for cell types of the zebrafish innate immune system.

Cell type	Transgenic marker[1]	Gene marker	Antibody/Cell staining	Functional assay
Pan-leukocytic	-	*lcp1*	anti-L-plastin	Morphological and functional characterization of macrophages and neutrophils.
	Tg(coro1a:EGFP)	*coro1a*	-	
Myeloid cell precursors	*Tg(-5.3spi1b:EGFP)* *Tg(-9.0spi1b:EGFP)* *Tg(-4spi1b:Gal4)* *Tg(-4spi1:LY-EGFP)*	*spi1b/pu.1*	-	Marker of macrophage and neutrophil precursors
Macrophages	*Tg(mpeg1:EGFP)* *Tg(mpeg1:Gal4-VP16)* *Tg(mpeg1:mCherry-F)* *Tg(mpeg1:Dendra2)*	*mpeg1.1*	-	Specific marker of macrophages, but down-regulated by several infections; also labels microglia
	TgBAC(csf1ra:Gal4-VP16)	*csf1ra/fms*	-	Specific marker of macrophages; also labels non-motile pigment cells (xanthophores)
	Tg(mfap4:dLanYFP-CAAX) *Tg(mfap4:mTurquoise)*	*mfap4*	-	Specific marker of macrophages; less sensitive to infection down-regulation than *mpeg1.1*
Neutrophils	*TgBAC(mpx:EGFP)* *Tg(mpx:GFP)* *Tg(mpx:mCherry)* *Tg(mpx:EGFP-F)* *Tg(mpx:DsRed-F)* *Tg(mpx:Dendra2)*	*mpx*	anti-Mpx/Mpx enzyme activity staining	Specific marker of neutrophils

Cell type	Transgenic marker[1]	Gene marker	Antibody/Cell staining	Functional assay
	Tg(lyz:EGFP) *Tg(lyz:DsRed2)* *Tg(lyz:Gal4-VP16)*	*lyz/lysc*	-	Specific marker of neutrophils; some overlap with macrophages at early developmental stages
	-	-	Sudan black	Staining of neutrophil granules
Activated macrophages/ neutrophils	*Tg(il1b:GFP-F)*	*il1b*	anti-Il1b	Reporter to distinguish inflammatory phenotypes of macrophages (M1) and neutrophils
	Tg(tnfa:eGFP-F)	*tnfa*	-	Marker for activated macrophages (M1)
	Tg(irg1:EGFP)	*irg1*	-	Marker for activated macrophages (M1)
	Tg(CMV:EGFP-map1lc3b)	*map1lc3b*	-	Marker for autophagy activation

Cell type	Transgenic marker[1]	Gene marker	Anti-body/Cell staining	Functional assay
	Tg(Myd88:EGFP) *Tg(Myd88:Dsred2)*	*myd88*	-	Marker for TLR signaling potential
	Tg(NFκB:EGFP)	*nfκB*	-	Marker for transcriptional induction of innate immune response
Microglia	*Tg(apoeb:lynEGFP)*	*apoeb*	-	Specifically marker of microglia
	-	-	Neutral red	Efficient staining of microglia; partially effective staining of macrophages
Mast cells	-	*cpa5*	-	Marks a subpopulation of L-plastin positive myeloid cells by in situ hybridization

[1] Only the most frequently used transgenic lines are indicated; for additional lines and references we refer to the Zebrafish Model Organism Database (http://zfin.org/).

An important study in zebrafish has revealed previously underappreciated differences in phagocytic behavior between macrophages and neutrophils that are very relevant for the design of infection models (Colucci-Guyon et al., 2011). This study showed that, in contrast to macrophages, neutrophils possess limited ability to phagocytose fluid-borne bacteria, but can quickly migrate to wounded or infected tissues and efficiently re-

move surface-associated bacteria (Colucci-Guyon et al., 2011). A previous study describes a similar "surface phagocytosis" behavior for mammalian neutrophils (Wood, 1960). This property is likely to be relevant for human infectious disease, since the first encounter of microbes with phagocytes is critical for the outcome of infection (Colucci-Guyon et al., 2011). In zebrafish embryos and larvae, phagocytosis by macrophages is favored when microbes are injected into the blood or into a body cavity such as the hindbrain ventricle, whereas sub-cutaneous, muscle or tail fin injections will provide the conditions for efficient engagement of neutrophils (Colucci-Guyon et al., 2011). The technical options allowed by using zebrafish, where the initial infection site can be varied to investigate how macrophages and neutrophils respond differently, is a strength of zebrafish infection models.

In addition to neutrophil and macrophage lineages, also mast cells are thought to be generated from the RBI (Dobson et al., 2008). The activation of mast cells at sites of infection can have direct effector functions or contribute to the regulation of innate and adaptive immune responses (Prykhozhij & Berman, 2014). As the gene encoding carboxypeptidase A5 (*cpa5*), a marker for mast cells, is expressed as early as 24 hpf (Dobson et al., 2008), zebrafish embryos could become a valuable model to study the function of mast cells in context of infection. However, to date, studies in zebrafish infection models have concentrated on macrophage and neutrophil functions, where work has uncovered novel insights into how these cells respond to infection, and into the genes required for mounting an immune response, as further discussed below.

Generation of myeloid cells by the intermediate and definitive waves of hematopoiesis

As in all vertebrates, hematopoiesis in zebrafish occurs in waves (Jagannathan-Bogdan & Zon, 2013). The second wave of hematopoiesis is identified as an intermediate wave **(Figure 1)**, occurring at the posterior blood island (PBI) at the most posterior part of the ICM. The PBI is a temporary location of hematopoiesis in zebrafish (24-48 hpf), analogous with the mammalian fetal liver. The intermediate wave of hematopoiesis generates the first committed erythromyeloid progenitors (EMPs) which are capable of giving rise to both erythroid and myeloid lineage cells (Ber-

trand et al., 2007), including macrophages, neutrophils and mast cells **(Figure 1)** (Bertrand et al., 2007). The primitive and intermediate waves cannot sustain hematopoiesis for a long time. Only the final wave that occurs during embryogenesis, namely definitive hematopoiesis, is able to produce hematopoietic stem cells (HSCs) that can generate all types of hematopoietic cells for the whole life span. The development of HSCs is dependent on transcription factor Runx1 (Lam et al., 2009). In zebrafish, HSCs are generated from about 1 dpf to 2.5 dpf in the ventral wall of the dorsal aorta (VDA) **(Figure 1)**. This hematopoietic site derives from the aorta-gonad-mesonephros (AGM), which is also the origin of HSC in mammals. HSCs emerging from the VDA migrate to and colonize the three sites of definitive hematopoiesis: the caudal hematopoietic tissue (CHT) the thymus and the anterior part of the kidney (pronephros). From 3 to 6 dpf, the CHT is the main hematopoietic tissue of the larvae. However, the CHT does not produce lymphoid progenitors and is readily exhausted. From approximately 4 dpf, the thymus and the pronephros (which will later develop into the adult head kidney) start to contribute to hematopoiesis and only these organs will maintain erythroid, myeloid and lymphoid hematopoiesis throughout the life span of the fish (Jin et al., 2007; Kissa et al., 2008; Murayama et al., 2006; Willett et al., 1999).

In the VDA, HSCs are shown to originate from hemogenic endothelial cells via a developmental process termed endothelial hematopoietic transition (EHT) (Bertrand et al., 2010; Kissa & Herbomel, 2010). The hemogenic cells are bipotential precursors that can differentiate into both hematopoietic and endothelial cells (Vogeli et al., 2006). These HSCs undergo limited divisions to either maintain the stem cell pool throughout the life of the host, or give rise to multipotent and lineage-committed hematopoietic progenitor cells (HSCs) that generate all mature blood cell lineages (Takizawa et al., 2012). Macrophages originating from the primitive and the intermediate wave play a decisive role in the expansion and specification of definitive HSCs. They colonize the AGM during the HSCs emergence stage, start patrolling between the dorsal aorta and the posterior caudal vein, and intimately interact with the HSCs. Genetic or chemical depletion of macrophages derived from the non-definitive waves impairs the accumulation of the definitive HSCs in the AGM and their colonization of the CHT (Travnickova et al., 2015). Furthermore, it has been shown that the mobilization of HSCs and the intravasation and colonization of tis-

sues is dependent on the function of matrix metalloproteinases (MMPs), in particular Mmp9, which can be produced by myeloid and surrounding tissue cells (Travnickova et al., 2015). Mmp9 is known as a strongly inducible component of the pro-inflammatory response to infections, facilitating leukocyte migration and cytokine processing (Stockhammer et al., 2009; Van Lint & Libert, 2007; Volkman et al., 2010). Therefore, the role of Mmp9 in HSC mobilization is likely to be significant also under conditions of infection, which demand enhanced hematopoiesis.

Functional diversification of myeloid subtypes

It is not precisely known to what extent the zebrafish macrophages or neutrophils generated by primitive, intermediate, or definitive hematopoiesis have different functional competencies when dealing with infections. It is clear, however, that zebrafish embryos are less competent to combat infections at 1 dpf than at later stages, which likely can be attributed for a major part to the fact that neutrophils are still undergoing differentiation between 1 and 2 dpf **(Figure 1)** (Clatworthy et al., 2009). Indeed, these early neutrophils have been shown to phagocytose less well than neutrophils at later developmental stages (Le Guyader et al., 2008). Nevertheless, zebrafish embryos infected at 1 dpf are already capable of inducing a robust innate immune response with expression of genes for cytokines, complement factors, proteases, and other mediators of pathogen defense (Stockhammer et al., 2009; Van der Vaart et al., 2012).

A pioneering study using zebrafish showed, for the first time in a living vertebrate, that macrophages undergo polarization to develop into functional M1 (classically activated) and M2-like (alternatively activated) subtypes (Nguyen-Chi et al., 2015). M1 macrophages promote inflammation, while M2 macrophages are involved in the resolution of inflammation and wound healing. Therefore, in many diseases, the persistence of M1 macrophages signifies an inflammatory state that can promote a range of negative outcomes, including inflammatory disorders (Mills, 2012). On the other hand, tumor-associated macrophages often display an M2 phenotype linked with properties that stimulate tumor growth, angiogenesis, tissue invasion, and metastasis (Noy & Pollard, 2014). Nguyen-Chi et al. used live imaging of a zebrafish fluorescent reporter line for tumor necrosis factor alpha (Tnfα), a distinctive proinflammatory marker for M1 mac-

rophages. They showed that a subset of macrophages start to express the *tnfa* reporter in response to wounding, or in response to a tissue infection with *E. coli*. Moreover, these *tnfa* positive macrophages revert back to an M2-like phenotype when the inflammation is resolving (Nguyen-Chi et al., 2015). By separating *tnfa*-expressing and *tnfa*-negative macrophages using fluorescent cell sorting, it was found that *tnfa* positive cells express other typical M1 markers, such as interleukin 1β and 6 (*il1b* and *il*6), while negative cells express M2 markers, such as tumor growth factor β *(tgfb)*, CC-motif chemokine receptor 2 *(ccr2)* and CXC-motif chemokine receptor 4b (*cxcr4b*).

Macrophage activation has also been demonstrated using a fluorescent reporter fish line **(Table 2)** for immunoresponsive gene 1 (*irg1*), which is strongly induced by injection of bacterial lipopolysaccharide (LPS) (Sanderson et al., 2015). Arginase-2 (*arg2*) is considered to be a reliable M2 marker for teleost fish and a reporter line for this gene would thus be a valuable addition to further study M1/M2 polarization in zebrafish (Wiegertjes et al., 2016).

There is increasing interest also in neutrophil subtypes, which by analogy with macrophage subtypes are referred to as N1 and N2 (Mantovani, 2009). With new transgenic lines being generated by several labs **(Table 2)**, zebrafish embryos and larvae provide a unique opportunity to carry out live imaging of such possible neutrophil polarization and of neutrophil-specific defense mechanisms, like the formation of neutrophil extracellular traps (NETs) (Palic et al., 2007). The release of NETs coincides with a specific type of neutrophil cell death, named NETosis, resulting in an extracellular network of chromatin and granular proteins that can entrap and kill microbes. Besides this direct antimicrobial function, NETosis is thought to deliver danger signals that alert the innate immune system, and, if not properly controlled, NETosis may contribute to inflammatory and autoimmune diseases (Brinkmann & Zychlinsky, 2012). A newly established zebrafish notochord infection model is very useful to address neutrophil-specific defenses (Nguyen-Chi et al., 2014). The notochord is the developmental precursor of the vertebral column and this structure is inaccessible to phagocytes. However, injection of *E. coli* bacteria into this tissue induces massive macrophage and neutrophil accumulation in the surrounding area. The accumulating neutrophils are polarized to express

high levels of *il1b* and a significant proportion of them show release of the Mpx-containing granules. This response results in rapid elimination of the bacterial infection, but the inflammatory reaction is persistent and has long term consequences leading to notochord damage and vertebral column malformations (Nguyen-Chi et al., 2014). This study provided the first *in vivo* evidence that neutrophils can degranulate without making direct contact with a pathogen. Furthermore, the zebrafish notochord model developed in this study provides a new tool to study human inflammatory and infectious diseases of cartilage and bone, such as osteomyelitis and septic arthritis.

Genetic control and experimental manipulation of the zebrafish innate immune system

Development and differentiation of innate immune cells

Primitive myelopoiesis in zebrafish is genetically controlled by two parallel pathways, the *cloche-estrp-scl* pathway and the *bmp*/*alk*8 pathway (Hogan et al., 2006; Liao et al., 1998). Cloche is required very early for development of normal hemangioblasts as *cloche* mutants have defects in both endothelial and hematopoietic (erythroid and myeloid) lineages . The *estrp* and *scl* genes act downstream of *cloche* to regulate hematopoietic and endothelial development (Liao et al., 1998; Liu & Patient, 2008; Sumanas et al., 2008; Sumanas & Lin, 2006). The Bmp receptor Alk8 specifically regulates primitive myelopoiesis in the RBI but is not required for erythropoiesis. In agreement with an instructive role of the *bmp/alk8* pathway in myelopoiesis, the expression of *pu.*1 is lost in the absence of *alk8* while constitutively expressed *alk8* can increase *pu.1* expression (Hogan et al., 2006). The differentiation of EMPs is controlled by the orchestrated expression of transcription factors, where Pu.1 is the master regulator of the myelopoiesis and Gata1 is the key regulator of the erythroid cell lineage. Pu.1 and Gata1 negatively regulate each other and an interplay between these two transcription factors determines myeloid versus erythroid cell fate **(Figure 1)** (Galloway et al., 2005; Rhodes et al., 2005).

Myeloid progenitors need additional factors to differentiate into any of the innate immune cell type populations. Some of these factors are required for pan-myeloid development, while some are required for a specific lineage development. The *spi1l* gene encodes an ETS transcription factor, closely related to Pu.1. It functions downstream of Pu.1 and promotes myeloid development (Bukrinsky et al., 2009). Extrinsic factors like granulocyte-colony stimulating factor (Gcsf) also play a critical role in myeloid cell development (Liongue et al., 2009). Pu.1, Runx1, and Irf8 are important for the cell fate determination between macrophages and neutrophils. High levels of Pu.1 promote macrophage fate whereas low levels promote neutrophil fate during primitive myelopoiesis (Jin et al., 2012; Su et al., 2007). Increased levels of Runx1 promote the expansion of the neutrophil population, whereas low levels of Runx1 result in more macrophages at the expense of the neutrophil progeny (Jin et al., 2012). In contrast to Runx1, Irf8 is necessary for macrophage fate determination. Suppressing *irf8* leads to reduced macrophage and increased neutrophil numbers, while increased *irf8* expression has the opposite effect (Li et al., 2011). The regulation of mast cell fate is less well understood, but it has recently been shown to be influenced by Gata2, which functions downstream of the Notch pathway. Pu.1 is also required for mast cell development, independent from Gata2 and the Notch pathway (Da'as et al., 2012). As discussed below, the knowledge of the genetic pathways that control myeloid development can be exploited in infection studies to determine the specific roles of macrophages and neutrophils in host defense and pathology.

Genetic and chemical approaches to manipulating the zebrafish innate immune system

The transcription factor, Pu.1 is essential for development of both macrophages and neutrophils. A low dose of a *pu.1* morpholino can block macrophage development up to 3 dpf, and can also block neutrophil development when injected at a higher dose (Su et al., 2007). *pu.1* morphants are more susceptible to various pathogens such as *Mycobacterium marinum*, *Salmonella enterica* Typhimurium, *Staphylococcus aureus*, and Chikungunya virus (CHIKV), indicating that macrophages are essential for defense against these. Additionally, similar experiments demonstrated that mac-

rophages are critical vectors for dissemination of *M. marinum* (Clay et al., 2007; Palha et al., 2013; Prajsnar et al., 2012; van der Vaart et al., 2012).

Not only macrophages, but also neutrophils are critical for the defense against *M. marinum*, which has been shown using a transgenic zebrafish line which mimics the WHIM (Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis) syndrome. In the WHIM zebrafish line, the neutrophil specific *mpx* promoter is used to overexpress a constitutively active form of *cxcr4b*, which is an important retention factor for myeloid progenitors that permits their maintenance in the hematopoietic tissues. As a result, mature neutrophils are retained in the hematopoietic tissues that express Cxcl12a, the chemotactic ligand of Cxcr4b. Thus, neutrophils are unable to reach the tissue infection sites, resulting in increased growth of *M. marinum* (Yang et al., 2012). However, neutrophils cannot control *M. marinum* infection in the absence of macrophages, as shown by using *irf8* morpholino to expand neutrophils at the expense of macrophages (Elks et al., 2015; Pagan et al., 2015). In contrast, the essential role for neutrophils in controlling viral infection was shown by knockdown of *csf3r* (*gcsfr*) which mostly depletes the neutrophil population and renders embryos more susceptible to CHIKV infection ((Liongue et al., 2009; Palha et al., 2013). The selective depletion of neutrophils can also be achieved with *cebp1* morpholino, an approach used in a study demonstrating the importance of neutrophils as a source for inflammatory cytokines promoting hematopoiesis (He et al., 2015).

Alternative to examples of genetic manipulation of macrophage/neutrophil ratios, transgenic drug-inducible cell ablation systems have been applied in zebrafish infection studies. For example, selective ablation of macrophages demonstrated that these cells are less important than neutrophils in defense against CHIKV (Palha et al., 2013). The same approach showed that both macrophages and neutrophils are required for defense against *S. aureus*, but that neutrophils also function as a potential reservoir where the pathogen find a protected niche that enables it to subsequently cause a disseminated and fatal infection (Prajsnar et al., 2012). Finally, macrophages have been selectively depleted using clodronate-containing liposomes, showing their essential role in control of *Mycobacterium abscessus* and *Cryptococcus neoformans* infections (Bernut et al., 2014; Bojarczuk et al., 2016). Together, these examples demon-

strate the advantage of zebrafish infection models for in vivo dissection of innate immune cell functions, due to the ease of genetic and chemical manipulation of macrophage versus neutrophil ratios in this model.

Pathways required for pathogen recognition and activation of the innate immune response

Cells composing the innate immune system can recognize invading microbes by expressing a series of pattern recognition receptors (PRRs). PRRs were evolved to sense and respond to recurrent molecular patterns that are found in microbes (e.g. LPS, peptidoglycan, lipoprotein, flagellin, exogenous nucleic acids) or that are derived from the host as a consequence of the infection (e.g. heat shock proteins and aberrantly processed, exposed or localized cell components). These signals are collectively referred to as Pathogen/Damage Associated Molecular Patterns, P/DAMPs) (Akira et al., 2006). PRRs belong to different families, which comprise membrane proteins on the cell surface or endosomal compartments, cytosolic proteins as well as secreted proteins. PPRs are not only essential for innate immune responses, but also for the activation of adaptive immunity, and defects or polymorphisms in these receptors have been linked to numerous immune-related diseases in human (Caruso et al., 2014; Netea et al., 2012). The major families of PRRs are well conserved between mammals and zebrafish. However, as reviewed below, the current knowledge of PRRs and downstream signaling in zebrafish is still relatively limited.

Families of PRRs

Scavenger receptors

Scavenger receptors represent a heterogeneous group of surface PRRs receptors, able to recognize a broad spectrum of molecules from bacterial/fungal wall, viral capsid parasite glycocalyx as well as host derived ligands. The interaction of these receptors with their ligands can directly mediate phagocytosis of the pathogen or can contribute as co-stimulatory signal for the activation of downstream signaling pathways, such as cytokine responses mediated by NFκB signaling (Bowdish et al., 2009). The zebrafish homologs of human macrophage receptor with collagen structure (Mar-

co) and Cd36 were recently characterized (Benard et al., 2014; Fink et al., 2015). Marco expression by macrophages is important for rapid phagocytosis of *M. marinum* and mediates an initial transient proinflammatory response to this pathogen (Benard et al., 2014). Consequently, knockdown of this receptor impairs bacterial growth control. Although not highly expressed by macrophage and neutrophils, also the knockdown of Cd36 in zebrafish larvae led to higher bacterial burden upon *M. marinum* infection (Fink et al., 2015).

C-type lectin receptors

The mammalian C-type lectin receptors (CLRs) include cell surface as well as secreted proteins (collectins) that are able to bind to different surface carbohydrate moieties from viruses, bacteria, fungi or eukaryotic parasites and similarly to scavenger receptors, they can guide phagocytosis of non-opsonized bacteria, and their destruction in acidified phagolysosomes. Several homologs of CLRs have been detected in zebrafish, but a real functional characterization of this class of receptors in zebrafish is still missing. Only recently the zebrafish mannose receptor was cloned and found to be highly induced upon infection with *Aereomonas sobria* (Fink et al., 2015). In addition to this cell surface receptor for mannose-rich glycans, mannose recognition is also mediated extracellularly by the mannose binding lectin (MBL).

Zebrafish embryos express a homolog of mammalian MBL and this molecule can opsonize both Gram-negative and Gram-positive bacteria, promoting their phagocytosis by macrophages, like its mammalian counterpart (Yang et al., 2014). Neutralization of this molecule could also increase mortality of embryos infected with *Aereomonas hydrophila*, while injection of the recombinant protein promotes resistance to this pathogen. This study also suggests that the lectin pathway may be already functional in the early embryos in zebrafish before their cell-mediated innate immunity is fully matured, and largely contributes to the protection of the developing embryos.

Toll-like receptors

Toll-like receptors (TLRs) are a family of PRRs located on the plasma membrane or on the endosome/phagosome membranes that can sense a wide variety of PAMPs and DAMPs. Their extracellular ligand binding domain contains conserved leucine-rich repeat motifs and their cytoplasmic signaling domain consists of a TIR (Toll-Interleukin-1 Receptor) homology domain. TLRs are known to essentially signal as hetero- or homo-dimers, via coupling with downstream adaptor molecules (Akira et al., 2006). In mammals, five adaptors have been identified, namely MYD88 (myeloid differentiation factor 88), TIRAP, TRIF, TRAM and SARM1 (Akira et al., 2006). Among these, MYD88 represents the most central mediator, since most of the TLRs rely heavily on MYD88 to activate their downstream signaling pathway. This consists mostly of modulation of gene expression via activation and translocation of transcription factors such as NFκB, ATFs, IRFs, AP-1 and STATs (Akira et al., 2006). Stimulation of these factors triggers profound modification of gene expression, especially upregulation of an array of proinflammatory effector molecules, including cytokines, chemokines, antimicrobials and activators of adaptive immunity (Kanwal et al., 2014).

Orthologs of TLR1-2-3-4-5-7-8-9 and of their adaptor intermediates (Myd88, Tirap, Trif and Sarm1) and other downstream signaling intermediates (e.g. Traf6) have been identified and studied in zebrafish too (Kanwal et al., 2014). However, for some of them it is still unclear what ligands they respond to. The zebrafish Tlr2-3-5-9 maintain ligand-specificity consistent with their mammalian counterparts, yet the closest orthologs to mammalian TLR4 in zebrafish are unable to respond to LPS, its ligand in mammals (Kanwal et al., 2014). Several functional and fish-specific Tlrs also exist, such a Tlr21 and Tlr22, which can respond to dsRNA and CpG-oligodeoxynucleotides respectively (Kanwal et al., 2014). Another fish specific Tlr cluster is represented by Tlr20, which phylogenetically seems related to mammalian Tlr11-12 (Kanwal et al., 2014). In agreement with studies in mammalian models, transcriptional analysis of the responses to bacterial infections has demonstrated that activation of downstream transcription factors and proinflammatory immune response genes is largely dependent on the function of the Myd88, which serves as an adaptor in

both Tlr and Interleukin 1 receptor signaling (Gay et al., 2011; van der vaart et al., 2013).

A reporter zebrafish line **(Table 2)** containing promoter elements of the zebrafish *myd88* gene (Hall et al., 2009) has helped to define that the innate immune cells, have the highest potential for MyD88-dependent/ TLR-mediated signaling. *Myd88*:GFP labelled cells include a set of myeloid leukocytes which not only are highly responsive to wounding and infections, but also express a full battery of Tlrs and other Tlr-downstream adaptors together with *myd88*.

Application of the zebrafish model has recently also contributed to define common and specific downstream signaling targets controlled by several Tlrs. While a large part of well-defined inflammatory markers such as *il1 b, tnfa, mmp9* and *Cxcl18b/Cxcl-c1c* were inducible by either Tlr2 and Tlr5 stimulation at a similar extents, other infection-responsive genes, especially transcription factors (e.g. *fosb*, *egr3*, *cebpb*, *hnf4a*) but also some effector molecules, including *il6* and *il10* were found to rely more heavily on one or the other signaling system. Comparative studies of Tlr signaling in zebrafish with other teleost and mammalian species have been more comprehensively reviewed in (Kanwal et al., 2014) and these studies, in summary, demonstrate how zebrafish genetics can be used to dissect the specific molecules that contribute to a robust immune response.

Nod-like receptors

Differently from scavenger receptors and TLRs, Nucleotide-binding-oligomerization-domain (NOD) like receptors (NLRs) are soluble receptors and can detect PAMPs and DAMPs in the cytosol, such as those deriving from pathogens escaping from phagosomes (Akira et al., 2006). NOD1 and NOD2 have been implicated in the recognition of bacterial cell wall, although several studies suggest a broader range of ligands for these NLRs, since they seemed implicated also into recognition of intracellular eukaryotic parasites (Silva et al., 2010). Other NLR include IPAF, NALP1, and NALP3, which can assemble in the inflammasome, a cytosolic multicomponent complex which is involved in the activation of procaspase 1 to caspase 1 (Martinon et al., 2002). The active form of caspase 1, in turn, can process pro-IL1β and pro-IL18 into IL1β and IL18 (Martinon et

al., 2002). Most of NLRs are conserved in zebrafish in addition to another large teleost-specific subfamily of NLRs (Stein et al., 2007). The functional conservation of NOD1-2 was demonstrated by depletion of these genes during *S. enterica* Typhimuruim infection, which resulted in increased burden, and decreased host survival (Oehlers et al., 2011). Investigation of the NLR-dependent inflammasome activation and Il1β processing still requires a more detailed characterization in this species (Ogryzko et al., 2014; Varela et al., 2014).

RIG-I-like receptors

RIG-I-like receptors (RLRs) are another family of cytosolic PRRs that activate the inflammasome (Kell & Gale, 2015). RLRs can detect the presence of RNA from a broad range of viruses. The downstream signaling cascade is cooperative with Tlr signaling and induces activation of transcription factors like IRF3, IRF7 and NFκB, leading to high production of interferons (IFN) and interferon-stimulated genes (ISGs) (Kell & Gale, 2015). Both type I and type II interferons exist in zebrafish, and like in humans, these molecules are key for the antiviral response. However, direct homologies with the mammalian systems cannot be definitively traced. Zebrafish Ifnγ1 and Ifnγ2 are the type II homologs, while Ifnϕ1 and Ifnϕ2, members of a large Ifnϕ family in zebrafish, represent a fish-specific type of interferons that more closely resemble the mammalian type I interferon molecules (Aggad et al., 2009; Langevin et al., 2013).The zebrafish homologs for RIG-I and other members of RLRs are predicted in the zebrafish genome but functional characterization in zebrafish is still incomplete. The RLRs were shown to be involved in IFN gene induction in zebrafish by overexpression of the key RLR-adaptor IPS-1/MAVS. This led to massive induction of ISGs, similar to what was found in mammalian models (Biacchesi et al., 2009). This role in IFN induction places RLRs as a central factor in containing viral infections. Studies in zebrafish suggest that they might also have a significant function in defense against bacterial infections (Zou et al., 2013).

Inflammatory signaling initiated by PRRs

The downstream mediators activated by most PRR signaling include pro- and anti-inflammatory protein and lipid molecules secreted at the infection site. Cytokines are small secreted proteins exerting central modula-

tory activities in both adaptive and innate immunity. This heterogeneous group of peptides includes TNF, interleukins, and chemokines (CCLs, CXCLs, CX3CLs and XCLs). All these classes exist in zebrafish and other teleosts. However, expansions and diversifications have occurred (Nomiyama et al., 2008).

Similarly to mammalian models, a large number of these mediators is transcriptionally modulated by infection with different pathogens (Stockhammer et al., 2009; Veneman et al., 2013), or cleaved to their mature/active form. In zebrafish, functional similarities are proven for the Tnf, Il1β, Il8/Cxcl8, Cxcl11, Il6, and Il10 (Roca & Ramakrishnan, 2013). Knockdowns or full knockouts of several of these molecules or their cognate receptors led to significant aberrancies in the containment of infections (Roca & Ramakrishnan, 2013). For example, knockdown of the Tnfa receptor *tnfrsf1a* in mycobacterial infection revealed a key function of this axis to control the host inflammatory status (Roca & Ramakrishnan, 2013). The chemokines Il8/Cxcl8 and Cxcl11, like in mammalian species, were found to recruit neutrophils (via Cxcr2) and macrophages (via Cxcr3.2), respectively and impacted on the mobilization and response of phagocytes to infection.

The mechanisms for lipid inflammatory/anti-inflammatory mediators, including prostaglandins, leukotrienes and lypoxins are highly conserved from zebrafish to human. The importance and functional conservation of these molecules are exemplified by the results of a zebrafish genetic screen for genes causing hypersusceptibility to *M. marinum ,* which uncovered the gene encoding Lta4h (leukotriene A4 hydrolase) (Tobin et al., 2010). Lta4h catalyzes the final step of synthesis of the lipid mediator leukotriene B4 (LTB4) and its deficiency in zebrafish impairs the balance between anti-inflammatory and proinflammatory lipid mediators (Tobin et al., 2010). Similarly, polymorphisms in the human *LTA4H* locus have been reported to associate with susceptibility to *M. tuberculosis* (Tobin et al., 2010). LTB4 synergizes with Tnfα in order to maintain a balanced level of inflammation. Via its cognate receptor (Tnfr), Tnfα mediates activation of Rip1/2 kinases and release of reactive oxygen species (ROS) by increasing mitochondrion permeability (Roca & Ramakrishnan, 2013). ROS act as a double edged-sword, by both exerting a microbicidal function and mediating activation of necroptosis of the host cell. Therefore, impaired (too

high or too low) inflammatory statuses lead to increased susceptibility to mycobacterial infection in zebrafish (Roca & Ramakrishnan, 2013). A tight control of the inflammatory status is critically important also in human tuberculosis and other infectious diseases (Dorhoi & Kaufmann, 2014).

Complement system

In addition to the PRR-mediated cellular responses of the innate immune system, zebrafish embryos highly upregulate components of the complement system upon challenge with a variety of pathogens, indicating that soluble complement factors and complement receptors may be critical for opsonization, recognition and lysis of pathogens in this developmental window. In early zebrafish embryos, extracellular *S. enterica* Typhimurium LPS mutant and heat-killed bacteria are rapidly lysed, a phenomenon that was suggested to be complement-mediated, since LPS-mutants were found to be highly susceptible to complement killing in other models (van der Sar et al., 2003). Bacteriolytic mechanisms ascribed to complement are also proposed to contribute to the antibacterial activity in zebrafish egg cytosol (Wang & Zhang, 2010). Mostly complement components are known to derive from the liver. However, complement components are infection-inducible in the early embryos long before hepatic development (Wang et al., 2008). In line with these observations, we have found by transcriptional profiling of sorted phagocytes during infections that these cells can be a relevant source of extrahepatic production of complement components (unpublished results). Additionally, many of the complement factors in zebrafish can be transferred from mothers to eggs at either protein or mRNA level (Hu et al., 2010). Maternal immunization with *A. hydrophila* also resulted in increased protein transfer of complement factors to their offspring (Wang et al., 2009) and contributed to immunoprotection of the early embryo against this pathogen (Wang et al., 2008).

Effects of commensal microbes on development of the immune system

The impact of the gut microbiota on development of the mammalian immune system is well known (Kaplan et al., 2011). Following a large body of work in rodents, methods for growing zebrafish in a germ-free envi-

ronment or in the presence of defined microbial communities (gnotobiotic) are now well established (Pham et al., 2008). Comparison of studies in germ-free and gnotobiotic zebrafish and rodent models has revealed strong similarities among vertebrates in how microbes shape the development of the gut epithelium and the mucosal immune system, and influence the expression of genes involved in processes such as cell proliferation, metabolism, and inflammation (Cheesman & Guillemin, 2007; Rawls et al., 2004).

Inside the chorion, the zebrafish embryo develops in an axenic environment, but the intestine of larvae hatching around 3 dpf is rapidly colonized by microbes (Kanther & Rawls, 2010). Zebrafish larvae reared in germ-free water were shown to express lower levels of the pro-inflammatory cytokine gene *il1b* compared to larvae reared under conventional conditions (Galindo-Villegas et al., 2012). This microbiota-induced *il1b* expression is mediated by the TLR/MyD88 signaling pathway described in section 4 (Galindo-Villegas et al., 2012). This microbial recognition pathway can also be activated before hatching under conditions of experimental infection with bacterial pathogens (Van der Vaart et al., 2013). Microbial colonization leads to activation of a reporter for NFκB **(Table 2)**, a master transcriptional regulator of the immune response downstream of Tlr/Myd88 signaling (Kanther et al., 2011). Furthermore, the presence of a microbiota has been shown to result in increased numbers of neutrophils and systemic alterations in neutrophil localization and migratory behavior, which were found to be dependent on the microbiota-induced acute phase protein serum amyloid A (Kanther et al., 2014). In another study, commensal microbes were not found to promote a higher rate of myelopoiesis, but did affect neutrophil activity in response to injury (Galindo-Villegas et al., 2012). In addition, this study showed that the presence of commensal microbes primes the innate immune system of zebrafish larvae resulting in an increased resistance to experimental infections.

Independent from the effect of commensal microbes, the expression of proinflammatory genes appears to be controlled by epigenetic mechanisms that likely serve to protect of zebrafish larvae against infectious agents before adaptive immunity has developed and prevent pathologies associated with excessive inflammation during development (Galindo-Villegas et al., 2012). This is corroborated by a recent study showing that

mutation in the epigenetic regulator *uhrf1* leads to a strong induction of the proinflammatory cytokine gene *tnfa* in zebrafish larvae (Marjoram et al., 2015). The *tnfa* induction in these *uhrf1* mutants is associated with severe damage of the intestinal epithelium and infiltration by neutrophils, mimicking the chronic inflammation seen in inflammatory bowel diseases (IBD). The development of zebrafish models for IBD provides new avenues to study the factors that contribute to the onset of these complex multifactorial diseases where inappropriate responses of the immune system to the intestinal microbiota are thought to play a major role (Marjoram & Bagnat, 2015).

Adaptation to infection and inflammation

In response to infection or inflammation, the hematopoietic system can mount an adaptive response that is known as demand-driven hematopoiesis or emergency hematopoiesis (Takizawa et al., 2012). This response serves in the first place to replenish neutrophils, which due to their short life span are rapidly consumed during infections. Both the expansion of HSCs and the skewing of myeloid cell specification into the direction of granulopoiesis play a role in demand driven adjustments of hematopoiesis in zebrafish larvae (Hall et al., 2016; Hall et al., 2012; Herbomel, 2012).

That zebrafish embryos can mount an emergency granulopoietic response was first recognized in as study showing that intravenous administration of LPS at 2 dpf led to a Gcsf/Gcsfr-dependent increase in the numbers of neutrophils within 8 hours (Liongue et al., 2009). A recent report shows that phagocyte numbers can be modulated by immune stimulation even at an earlier stage. In this case a host defense peptide, chicken cathelicidin-2, was injected into the yolk of embryos shortly after fertilization, resulting in a 30% increase of *lcp1* positive cells at 2 dpf and an increased resistance of embryos to bacterial infection (Schneider et al., 2016). We review recent work in zebrafish that has brought new insights into the molecular pathway underlying emergency hematopoiesis and has revealed roles for several proinflammatory mediators as well Tlr signaling in hematopoiesis.

Molecular mediators of emergency granulopoiesis

Embryos infected with *S. enterica* Typhimurium into the hindbrain at 2 dpf develop neutropenia within one day and counter this within 2 days by emergency granulopoiesis throughout the VDA/AGM and CHT regions (Hall et al., 2012). While this Gcsf/Gcsfr-dependent response is at the expense of lymphoid progenitors, it is not due only to an increased commitment of HSCs to myeloid rather than lymphoid fate but also due to increase in the number of Gcsfr-expressing HSCs (Hall et al., 2012). The zebrafish orthologue of CCAAT-enhancer binding protein (Cebpb), a well-known transcriptional regulator of emergency granulopoiesis in mammals, is required for the expansion of the HSC compartment (Hall et al., 2012). Importantly, the study in zebrafish revealed that inducible nitric oxide synthase (iNOS, Nos2a) functions downstream of Cebpb in the emergency granulopoiesis pathway (Hall et al., 2012). Knockdown of *nos2a* to block the infection-induced expansion of neutrophils was subsequently shown to be associated with increased viral replication and mortality of embryos during CHIKV infection (Palha et al., 2013). It is currently unknown if the role of nitric oxide in emergency hematopoiesis is conserved across species, but this is to be expected in view of the fact that nitric oxide is involved steady state hematopoiesis in both zebrafish and mouse (Hall et al., 2012). The newly discovered roles of Cebpb and nitric oxide therefore prompt further investigations into the possibilities of therapeutic targeting of these factors in human disease (Hall et al., 2012).

Through work in zebrafish, a highly conserved myeloid-specific microRNA, *miR-142a-3p*, has recently been linked with Gcsf/Gcsfr and nitric oxide (NO) dependent signaling (Lu et al., 2013). Depletion of *miR-142a-3p* was found to reduce the numbers of HSCs in the VDA/AGM and CHT, associated with reduced expression of *gcsfr* as well as decreased production of NO (Lu et al., 2013). The inflammatory transcription factor Interferon regulatory factor 7 (Irf7) is a potential target of this microRNA, suggesting that this pathway might also be relevant not only to steady state but also to infection-induced hematopoiesis. When *irf7* and *miR-142a-3p* were depleted simultaneously *gcsfr* expression and NO production could be restored, suggesting that Irf7 acts as a repressor of Gcsfr/NO signaling and that in turn *miR-142a-3p* can repress Irf7 function to promote HSC development (Lu et al., 2013). This mechanism is conserved in mouse

and therefore also of potential interest for therapeutic targeting (Lu et al., 2013).

Implication of cytokines and interferons in hematopoiesis

Macrophages are thought to be the source of Gcsf that promotes emergency granulopoiesis (Hall et al., 2012). It has recently been shown that also several proinflammatory cytokines that are produced by macrophages and neutrophils can influence the production of HSCs in the embryo. These cytokines include Tnfα, Ifng1-1, Ifng1-2 and Il1β (Espin-Palazon et al., 2014; He at al., 2015; Li et al., 2014). Tnfα in zebrafish is encoded by two genes, *tnfa* and *tnfb*, and the expression of both genes is inducible by infections (van der Vaart et al., 2013). Tnfα is expressed as a transmembrane protein functional on the cell surface and signals through two receptors, Tnfr1 (Tnfrsf1a) and Tnfr2 (Tnfrsf1b). Signaling through Tnfr1 is important for resistance to mycobacterial infection as it prevents necrosis of infected macrophages (Clay et al., 2008), whereas Tnfr2 is the receptor that has been implicated in hematopoiesis (Espin-Palazon et al., 2014). Primitive neutrophils were found to be the primary source of Tnfα, which was found to promote the specification and emergence of HSCs through Tnfr2 and the Notch and NFκB signaling pathways (Espin-Palazon et al., 2014).

Similar to Tnfα, interferon gamma (IFNγ) is another important activator of macrophages that has been implicated in hematopoiesis. Overexpression of a zebrafish homolog of IFNγ, ifng1-2, increases HSC counts in embryos with an intact Notch signaling pathway (Sawamiphak et al., 2014). Ifng1-2 specifically controls the endothelial to HSC transition by activating Signal transducer and activator of transcription 3 (Stat3) (Sawamiphak et al., 2014). In agreement, knockdown of Interferon regulatory factor 2 (Irf2) increases HSC production in zebrafish (Li et al., 2014). The other zebrafish homolog of IFNγ, Ifng1-1, and fish specific type I interferons (*ifnphi1-2-3* and *4*) also contribute to HSC development and expansion (Li et al., 2014). Thus, Ifns are key regulators of HSC behavior and this suggests that HSCs are a prime response to an infection that stimulates Ifns.

Role of Tlr signaling in hematopoiesis

The primary pathway of pathogen recognition, namely Tlr4-MyD88-NFκB signaling, has recently been linked to HSC development (He et al., 2015). Expression of *runx1* in the VDA/AGM at 1 dpf and *cmyb* in the CHT at 2 dpf is significantly reduced in *tlr4bb* or *myd88* deficient embryos when compared to controls (He et al., 2015). However, *myd88* mutant larvae at 3 dpf show no significant alterations in macrophage or neutrophil numbers (van der Vaart et al., 2013), suggesting that the defect in HSC development is compensated for by Myd88-independent mechanisms. Embryos deficient in *tlr4bb* or *myd88* show a reduction in the expression of Notch target genes, and overexpression of the intracellular domain of Notch in endothelial cells can rescue *runx1* expression in *tlr4bb* and *myd88* morphants (He et al., 2015). As discussed above, Notch signaling can regulate NFκB, and therefore it is likely that the Tlr4-MyD88-NFκB and Notch-NFκB signaling routes function cooperatively in HSC development (Espin-Palazon et al., 2014; He et al., 2015). The HSC defect in *tlr4bb* and *myd88* morphants can also be rescued by overexpression of the gene for Il1β, adding also this cytokine to the list of proinflammatory mediators that modulate hematopoiesis and the production of innate immune cells (He et al., 2015). Studies in *tlr4*$^{-/-}$ knockout mice confirmed that TLR-mediated inflammatory signaling plays an evolutionary conserved role in HSC development (He et al., 2015). In conclusion, a number of recent studies in zebrafish and mouse models support a previously unrecognized link between inflammatory signaling and hematopoiesis that might be translated into new approaches for treatment of immune-related diseases or to improve the success of HSC transplantations (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014).

The interface of immunity and metabolism

During the first five days of development the zebrafish embryo/larva derives all its nutrients from the yolk and it has to adapt its metabolism to switch to external feeding when yolk proteins become limiting. How this metabolic adaptation might affect the immune system is currently unknown and worthy of exploration, especially considering new links between immunity and metabolism that have recently been revealed in

zebrafish (Hall et al., 2013; Marin-juez et al., 2014; van der Vaart et al., 2013). The relevance of immunometabolism for human disease is emerging strongly from recent studies that have revealed extensive metabolic reprogramming of human macrophages and dendritic cells in response to environmental conditions and during activation of innate and adaptive immune responses (O'Neill & Pearce, 2016).

Lipid and glucose metabolism as fuels for fighting infection

Fatty acid metabolism has been shown to fuel the production of mitochondrial ROS in zebrafish macrophages following infection of embryos with *S. enterica* Typhimurium (Hall et al., 2013). Immunoresponsive gene 1 (*irg1*), an infection-inducible and macrophage-specific gene encoding a homolog of bacterial 2-methylcitrate dehydratase, was found to be required for the utilization of fatty acids during this response, and knockdown of this gene increased the susceptibility to infection (Hall et al., 2013). This study showed that also murine macrophages require fatty acid β-oxidation for infection-induced mitochondrial ROS production and bactericidal activity. ROS production is also dependent on glucose metabolism and overproduction of ROS, which can have tissue damaging effects, has been associated with diabetes(Coughlan & Sharma, 2016). Studies in a zebrafish model for hyperinsulinemia suggest that the metabolic switch between insulin-sensitive and insulin-resistant states is mediated by protein tyrosine phosphatase non-receptor type 6 (Ptpn6), which is well known as a negative regulator of the innate immune response (Kanwal et al., 2013; Marin-juez et al., 2014). The dual role of this phosphatase in the regulation of glucose metabolism and immunity is particularly interesting in the light of the emerging co-epidemic of tuberculosis and diabetes (Pizzol et al., 2016). There are many ongoing efforts to develop zebrafish models for metabolic diseases, including diabetic complications, providing new opportunities to study the relation with infectious diseases (Schlegel & Gut, 2015).

Autophagy

The process of autophagy might be considered as the most important link between metabolism and immune function. Autophagy is a cellular process of self-degradation that functions to regulate energy metabolism

and it can be activated by nutrient stress, such as the depletion of the yolk during zebrafish larval development (Varga et al., 2015). During autophagy (or strictly macroautophagy), the cytosolic material is entrapped in double membrane structures (autophagosomes) and delivered to lysosomes for degradation. Autophagy has an important housekeeping function in removing and recycling aggregates of misfolded proteins and damaged organelles (Levine et al., 2011). The same machinery can also target intracellular microbes to lysosomal degradation and therefore several pathogens are thought to have evolved mechanisms to counteract the autophagic defenses (Huang & Brumell, 2014). Autophagy also controls inflammation, cytokine secretion, antigen presentation, and the regulation of innate and adaptive immune responses (Deretic et al., 2013).

Some recent work showing that autophagy is important in infection has used knockdown of the autophagy receptor p62 (Sqstm1) which mediates selective autophagy of ubiquitinated cargo. Sqstm1 morphants have an impaired defense against *Shigella* and *Mycobacterium* infections (Mostowy et al., 2013; van der Vaart et al., 2014). These bacterial pathogens have the ability to escape from phagosomes into the cytosol, where they can be tagged by ubiquitin (Ub) ligation and subsequently targeted to autophagy by p62 (Huang & Brumell, 2014). The susceptibility of p62-deficient zebrafish larvae to these pathogens clearly shows that autophagy is an essential cellular process for effective immunity against some deadly bacteria. Similarly, many studies in human cells have shown increased replication of *M. tuberculosis* under conditions of autophagy inhibition. In contrast, loss of p62 and other essential autophagy genes did not correlate with susceptibility to *M. tuberculosis* in mice (Kimmey et al., 2015). This suggests that, in the context of full adaptive immunity, *M. tuberculosis* might be less subject to phagosomal escape and autophagic targeting and that this pathogen is capable of effectively inhibiting the anti-bacterial function of the autophagy process.

The microtubule-associated light chain 3 protein (Lc3) is widely used as a marker of autophagosomes and the generation of a zebrafish reporter line **(Table 2)** expressing a GFP-Lc3 fusion protein *Tg(CMV:GFP-Lc3)* allows to monitor the process of autophagy *in vivo* (He et al., 2009). The zebrafish GFP-Lc3 reporter is activated by autophagy-inducing drugs (such as rapamycin), in different tissues of the developing embryo (for example

the heart), and in response to infections with *Shigella* and *Mycobacterium* (He et al., 2009; Hosseini et al., 2014; Lee et al., 2014; Mostowy et al., 2013; van der Vaart et al., 2014). The autophagic morphology of *M. marinum*-containing GFP-Lc3 positive vesicles in zebrafish has been confirmed by correlative light and electron microscopy (Hosseini et al., 2014). Furthermore, small GFP-Lc3 vesicles are frequently seen to accumulate around mycobacterial aggregates in infected zebrafish hosts (Hosseini et al., 2014; van der Vaart et al., 2014). These autophagosomes might serve to deliver neo-antimicrobial peptides and enhance the bactericidal properties of the autolysosomal compartment (Ponpuak et al., 2010).

From studies in human and mammalian cells, autophagy is known to be induced downstream of pathogen recognition by TLR signaling (Deretic et al., 2013). The DNA-damage regulated autophagy modulator 1 (Dram1) was discovered in zebrafish as a novel mechanistic link between autophagy induction and the TLR/Il1R-MydD88-NFκB innate immune sensing pathway (van der Vaart et al., 2014). Dram1 overexpression in the zebrafish host promotes the formation of autophagosomes and the p62-dependent selective autophagy targeting of *M. marinum*. Although the molecular mechanism remains to be elucidated, this host protective role of Dram1 might be exploited as a therapeutic strategy for treatment of mycobacterial disease in humans (van der Vaart et al., 2014). In further support of autophagy modulation as a therapeutic approach, a clinically approved anticonvulsant drug, carbamazepine, was recently shown to trigger autophagy in zebrafish embryos and protect against *M. marinum* infection (Schiebler et al., 2015). This drug was also shown to be effective against *M. tuberculosis* within primary human macrophages and in a mouse model of TB. Therefore, despite recent findings that deficiency in essential autophagy genes did not correlate with *M. tuberculosis* deficiency in mice, pharmacological activation of autophagy still remains a promising therapeutic strategy to be further explored (Kimmey et al., 2015).

Recent insights from modeling infectious diseases in developing embryos and larvae

Bacterial infections

Zebrafish infection models have been established for a wide variety of bacterial pathogens that are the causative agents of human infectious diseases or opportunistic infections, including species of the Mycobacteria, *Listeria*, *Shigella*, *Salmonella*, Streptococci, *Burkholderia* and other genera **(Table 1**). Since most of these models have been reviewed elsewhere (Cronan & Tobin, 2014; Meijer, 2016; Ramakrishnan, 2013; Saralahti & Ramet, 2015; Torraca et al., 2014; Vergunst et al., 2010), we focus here on some examples of recent work showing how these models are contributing to a better understanding of macrophage and neutrophil functions in the containment or the promotion of specific disease features.

Listeria and *Shigella* infections

Listeria monocytogenes and *Shigella flexneri* are two human pathogens that can cause serious gastrointestinal infections (food poisoning), especially in infants, the elderly, and immunocompromised patients. These bacteria share the capability to extensively manipulate the host cytoskeleton. Despite not being natural fish pathogens, these species were seen to escape into the cytosol after phagocytosis and to induce in the heterologous host the same cytoskeleton rearrangements, including actin tails and septin cages (Levraud et al., 2009; Mostowy et al., 2013). Mechanistically, *Shigella* and *Listeria* models in zebrafish mimic the main disease-causing feature of human shigellosis and listeriosis. Shigella bacteria are phagocytized by both neutrophils and macrophages, but while well contained by the first cell type, they rapidly induce cell death in the second. Both Shigella and Listeria, in human and in zebrafish tissue can largely exploit host actin polymerization to be propelled from the infected cell and invade new cells. These findings emphasize how these mechanisms of pathogenicity are shared across distant bacterial species and across vertebrates.

Similarly to *Shigella*, *Salmonella* (*S. enterica*) is an enterobacterial species that does not generally infect ectothermic animals. However, injection of

S. enterica Typhimurium establishes severe infection in zebrafish, which cannot be contained in most of the infected embryos and goes together with profound transcriptional induction of inflammatory genes (cytokine storm), a response that is largely dependent on Myd88-dependent signaling and negatively regulated by Ptpn6 phosphatase (Kanwal et al., 2013; van der Vaart et al., 2013). Deficiency in either of these signaling factors is detrimental to the resistance of zebrafish embryos to Salmonella infection (Kanwal et al., 2013; van der Vaart et al., 2013), indicating that the inflammatory response is protective when properly controlled but leads to lethality when inhibitory mechanisms are lost.

Staphylococcal infections

Staphylococcus aureus causes a range of serious infections in human and mammalian models, including skin ulceration, osteomyelitis, pneumonia and septicemia. Injections of large inoculums of this bacterium in zebrafish embryos also provoke septicemic death (Prajsnar et al., 2013; van der Vaart et al., 2013). Histologically, in zebrafish like in mammals, *S. aureus* determines formation of necrotizing lesions, the abscesses. Interestingly, the zebrafish model revealed that the bacteria forming the individual abscesses derive from clonal expansion of persistent bacteria. While the vast majority of the injected Staphylococci are cleared by macrophages and neutrophils, some bacteria gain the capability to avoid intracellular killing and will secondarily expand, forming the localized lesion (Prajsnar et al., 2012). This study identified the neutrophils as the main niche necessary to establish this immunological bottleneck and to determine the emergence of clonal infection foci.

Experimental *S. aureus* infection in zebrafish has also been instrumental in a recent study that revealed an unexpected role of the Spaetzle-like nerve growth factor β (NGFβ) in pathogen-specific host immunity to Staphylococcal infection (Hepburn et al., 2014). Spaetzle is a key mediator of the immune response to Gram-positive bacteria in *Drosophila*, and is required for the activation of the Toll signaling pathway (Lemaitre & Hoffmann, 2007) . While Spaetzle had always been thought to lack a vertebrate equivalent, chordate NGFβ in fact shares remarkable structure similarities to this mediator. It was found that Staphylococcal infection triggers release of NGFβ in human macrophages, a mechanism that de-

pends on recognition of pathogen exoproducts and on activation of the immune response via NOD-like receptor signaling. Subsequently, knockdown in zebrafish of tropomyosin-related kinase receptor A (TrkA), the corresponding receptor of NGFβ, was found to impair neutrophil recruitment and to increase susceptibility to *S. aureus* infection (Hepburn et al., 2014). This study supports an evolutionary conserved role for NGFβ acting as an alarm signal in the inflammatory response to *S. aureus* infection. Moreover, this work suggests that variation between individuals in secretion of NGFβ by macrophages might determine susceptibility to Staphylococcal disease.

Tuberculosis

One third of the world population carries *M. tuberculosis* and more than 10 million people fell ill with TB in 2015 (www.who.int). The zebrafish model for TB is by far the best studied zebrafish infection model, and has made great contributions to our understanding of TB pathology **(Figure 2)** (Cronan & Tobin, 2014; Meijer, 2016; Ramakrishnan, 2013). *M. marinum is* a natural pathogen of zebrafish and is phylogenetically very close to *M. tuberculosis,* the causative agent of human TB. The establishment of *M. marinum* pathogenesis in the zebrafish host is strikingly similar to human TB **(Figure 2)**. The disease hallmark in both host-pathogen systems consists of granulomas, essentially consisting of organized collections of immune cells that engulf and confine the bacteria.

Granulomas were previously considered relatively static structures generated by the host as a protective mechanism to restrict bacterial spread. Use of the zebrafish- *M. marinum* infection model demonstrated that these structures are instead highly dynamic **(Figure 2)** (Ramakrishnan, 2012). Non-invasive imaging in live zebrafish has shown that granulomas are characterized by a continuous trafficking of innate immune cells and that the pathogen takes advantage of infected macrophages to dissemi-

nate secondary lesions (Clay et al., 2007; Oehlers et al., 2015; Torraca et al., 2015). This model also helped to reconsider the contribution of the innate and the adaptive branches of the immune system in initiating the formation of granulomas. Imaging the earliest stages of granuloma formation in zebrafish embryos has shown that this process is initially driven by macrophages and occurs before lymphocyte differentiation, demonstrating that cells of the adaptive immune system are not required for granuloma formation (Davis et al., 2002).

Establishing TB infection in the form of granulomas depends on both pathogen and host factors, including mycobacterial virulence components, macrophage chemoattractants and inflammatory mediators. The Region of Difference 1 (*RD1*) is a virulence-associated locus covering the ESX-1 bacterial secretion system, and is notably shared between pathogenic mycobacteria, including *M. marinum*. ESX-1 is crucial for the establishment of granulomas and the zebrafish model helped to understand that the ESAT-6 virulence released via ESX-1 mediates macrophage aggregation in the early granulomas by stimulating production of Mmp9 in the epithelium surrounding the infection focus. By digesting the extracellular matrix, Mmp9 in turn facilitates infiltration of macrophages and establishment of chronic intracellular parasitosis **(Figure 2)** (Ramakrishnan, 2013). MMP9 is highly expressed in human TB and other inflammatory conditions; therefore, the observation that Mmp9 depletion confers resistance to mycobacterial infection in zebrafish highlights MMP9 as a potential therapeutic target (Volkman et al., 2010).

In addition to bacteria-driven mechanisms of granuloma expansion, chemokine signaling affecting macrophage recruitment is important to establish mycobacterial infection and to sustain granuloma expansion and secondary dissemination. Using the zebrafish model it was shown that deficiency in Ccr2/Ccl2 signaling reduces the chances of successful establishment of infection and that abrogation of Cxcr3-Cxcl11 signaling delays granuloma formation and attenuates seeding of the pathogen throughout the host **(Figure 2)** (Cambier et al., 2014; Torraca et al., 2015). However, the equilibrium controlling macrophage supply to the granuloma is very delicate, and while slight perturbations lead to host-beneficial effects, more drastic alterations can promote bacterial growth.

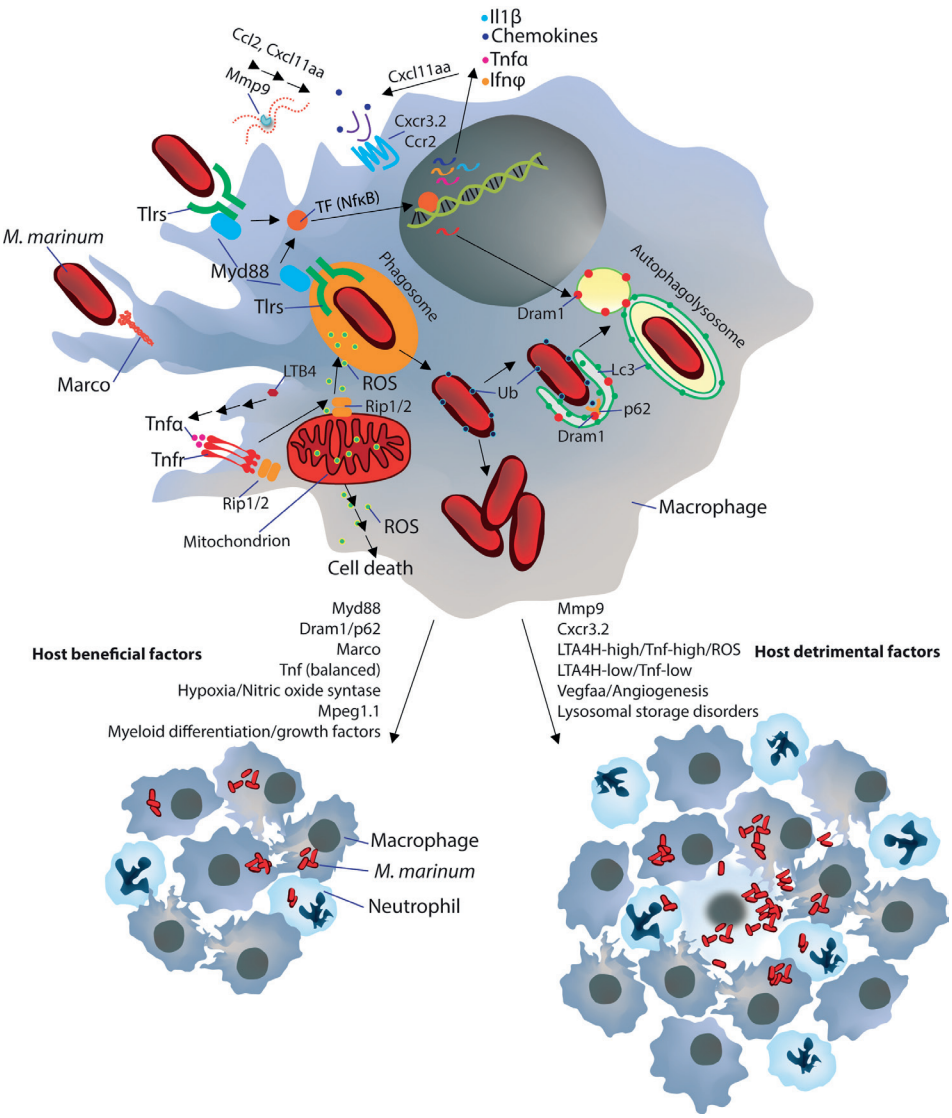

Figure 2: Mechanistic insight into mycobacterial pathogenesis provided by the zebrafish-*M. marinum* infection model. The host factors implicated in *M. marinum* infection of macrophages in the zebrafish host are summarized in this figure. The factors limiting (host beneficial) or promoting (host detrimental) the early expansion of granulomas are indicated below the schematic drawing of the macrophage. Macrophage-recruitment and tissue-inflammation mediators (such as Ccl2 and Mmp9) are also produced by neighboring cells as indicated by the arrows above the macrophage. Genes, pathways, and molecular functions depicted in

the figure: Marco, scavenger receptor important for efficient phagocytosis and immune recognition; Tlr/Myd88/NFκB signaling pathway, leading to induction of inflammatory cytokines (e.g. Tnfα, Il1β), interferons (e.g. Ifnϕ), chemokines (e.g. Cxcl11aa) and autophagy modulators (e.g. Dram 1); Mmp9, matrix metalloproteinase 9 facilitating macrophage migration; Ccl2/Ccr2 and Cxcl11aa/Cxcr3.2, chemokine ligand/receptor signaling axes implicated in macrophage migration; LTB4, lipid mediator of inflammation; Tnfr-Rip1/2 pathway, mediating release of reactive oxygen species (ROS) from mitochondria; Dram1, lysosomal/autophagosomal membrane protein stimulating autophagic flux; p62, pattern recognition receptor targeting ubiquitin-tagged (Ub) mycobacteria (escaped from the phagosomal compartment into the cytosol) to autophagy; Lc3, marker for autophagic activity. Vegfaa, angiogenesis promoting factor. See text for further details.

Macrophages that are engorged with undigested contents, such as in lysosomal storage disorder (LSD) patients and in smokers, display severe migratory aberrations, which can be mimicked in the zebrafish model by knockdown of LSD-associated genes (*gba*, *arsa*, *hexa*), by filling macrophages with indigestible particles or by compromising the levels of lysosomal cathepsins. These paralyzed macrophages cannot sufficiently contain the infection and will permit extracellular growth of the pathogen (Berg et al., 2016). Similarly, blockade of key macrophage differentiation regulators, such as *spi1*, *csf1ra* or *irf8,* leads to severe depletion of macrophages, with the consequent massive non-cellular bacterial growth (Clay et al., 2007; Elks et al., 2015; Pagan et al., 2015). Conversely, drastic increase of macrophage supply, evoked by overexpression of myeloid growth factors, can encourage resistance to mycobacterial infection, by preventing granuloma necroptosis (Pagan et al., 2015; Ramakrishnan, 2012). Taken together, recent findings from the zebrafish model are helping to critically dissect the highly debated dual role of macrophages in tuberculosis pathogenesis (Clay et al., 2007).

Human granulomas are amply vascularized, which suggested that, similarly to affecting tumor growth, curtailing vascularization might help to restrict granuloma formation. By injecting bacteria in the poorly vascularized zebrafish trunk tissue, the granuloma-driven promotion of angiogenesis could be mimicked in this model (Oehlers et al., 2015). Establishment of the intra-macrophage parasitosis, the production of RD1-encoded virulence factors, and the induction of local hypoxia is critical to mediate this response, which coincides with local induction of the angiogenic media-

tor *vegfaa*. In turn, depletion of Vegf signaling, which suppresses pathological angiogenesis, leads to contained granuloma expansion (Oehlers et al., 2015). Using the zebrafish-*Mm* model and genetic tools to control the function of Hif-1α/Hif-2α (the two main variants of hypoxia inducible factor alpha), it was found that hypoxia signaling not only controls angiogenesis, but also the production of nitric oxide (NO) by neutrophils, an important signaling mediator and antimicrobial factors (Elks et al., 2013). Interestingly, stabilization of Hif-1α stimulated activity of the nitric oxide synthase (Nos2a), while stabilization of the Hif-2α variant could antagonize NO production, with consequent opposing effects in inhibiting or promoting bacterial growth (Elks et al., 2013; Elks et al., 2015). These studies suggest angiogenic and hypoxia signaling pathways as possible targets for TB treatment. Several other host-directed therapeutic strategies have been proposed based on work in the zebrafish model and these are extensively covered in previous reviews. (Cronan & Tobin, 2014; Ramakrishnan, 2012; Torraca et al., 2014). In conclusion, the zebrafish *M. marinum* model has provided mechanistic insight into host factors that have been implicated either in protection against human TB or in the pathology of the disease, and provides a valuable anti-tubercular drug testing platform to develop novel therapeutic approaches.

Viral infections

Viral epidemics, with influenza and HIV/AIDS as prominent examples, have had devastating effects throughout human history and emerging viral diseases such as Dengue, Chikungunya and, most recently Zika, are a growing concern (Tilak et al., 2016) . While bacterial infections have been modeled in zebrafish for about 2 decades, the concept that the heterologous zebrafish model could be useful also to address viral infection with natural human pathogens, emerged relatively recently in the field (Goody et al., 2014; Levraud et al., 2014; Meijer & Spaink, 2011). In fact, while the zebrafish model proved immediately very useful to address economically relevant fish-specific viral infections, three main aspects represented a limitation into the use of zebrafish to model human viral disease. These include the tight and evolutionary rapid adaptation of viruses to their natural hosts, the large implication of a mature adaptive immunity during virus pathogenesis and the fact that the interferon-mediated signaling (the main pathway used by innate immune cells to counteract viral infections)

remains poorly characterized in (zebra)fish and diverges in some aspects from mammalian systems (Briolat et al., 2014; Levraud et al., 2014).

Despite these considerations, zebrafish models for several important human viral disease have now been established, including Chickungunya, Influenza and Herpes Simplex. (Antoine et al., 2014; Burgos et al., 2008; Gabor et al., 2014; Goody et al., 2014; Levraud et al., 2014; Palha et al., 2013).

Chikungunya Virus (CHIKV) is a mosquito-transmitted virus, causing serious and sometimes deadly illness in humans with acute fever, persistent rash, and debilitating muscle and joint pain. Infection of three-day-old zebrafish larvae with CHIKV showed that the pathogen can invade multiple host tissues such as muscles, liver, jaws and spinal cord cartilages, gills, fins, vascular endothelium and even eyes and brain (Palha et al., 2013). Thus, in some tissues, CHIKV infection in zebrafish mimics the pattern in humans. Interestingly, in zebrafish CHIKV infection persists persistent in the brain, while other tissues mostly clear the infection (Palha et al., 2013). Use of an *ifnφ1* fluorescent reporter line demonstrated that neutrophils are important to mediate an antiviral response to CHIKV infection via Ifn-signaling (Palha et al., 2013). The fact that CHIKV displays a remarkable brain tropism and persistence suggests that in humans too this pathogen might persist in this organ. The hypothesis of a brain reservoir in humans is in line with the fact that, in adults, some CHIKV symptoms can persist for years, even after the apparent eradication of the pathogen. Furthermore, CHIKV is known to cause encephalitis in newborns (Gerardin et al., 2016; Rajapakse et al., 2010). Therefore, further use of the zebrafish model could elucidate how CHIKV crosses the blood-brain barrier and persists in the central nervous system (CNS).

Influenza A virus (IAV) is the causative agent of annual epidemics of influenza. Similarly to CHIKV infection, IAV infection could be followed over time in zebrafish, using fluorescently-labelled viruses (Gabor et al., 2014). Strikingly, the viral kinetics and tissue tropisms in zebrafish recapitulate those observed in other models. Heart and skeletal muscles, blood endothelium and the mucosa-associated epithelium of the swim bladder accumulate the GFP-labelled virus, which is consistent with the fact that IAV preferentially infects human muscle, epithelial and endothelial cells *in*

vitro. The pathology evoked in zebrafish shows relevant parallels also at the molecular level, since the viremia coincides with upregulation of the antiviral transcripts of *ifnφ1* and Myxovirus influenza resistance a (*mxa*), the latter being a close fish ortholog of human MX1. The study also successfully proved that the zebrafish disease can be reverted by treatment with the known human anti-influenza drug Zanamivir, which indicates that zebrafish has a potential use as a screening platform for the discovery of novel antiviral compounds (Gabor et al., 2014).

Adult zebrafish have been used to study Herpes simplex virus type 1 (HSV-1) infection, a common cause of mucocutaneous orolabial, ocular and genital infections in humans (Antoine et al., 2014; Burgos et al., 2008). HSV-1 can also invade and damage the CNS, persist in nervous ganglia and lead to severe complications such as blindness and encephalitis. Following injection into the zebrafish abdominal cavity, the viral infection could spread to the midbody and ultimately reach the head, where it replicated abundantly in the CNS (Burgos et al., 2008). The current model of HSV-1 entry is that surface heparan sulfate derivatives mediate the initial viral adhesion, which in turn permits the fusion of the viral envelope with the host cell. These heparan sulfate moieties that act as viral receptors are remarkably conserved in zebrafish and are widely expressed in the CNS, like in mammals (Baldwin et al., 2013). The entry in the CNS causes the most severe HSV-1 complications and the penetration in nervous ganglia is a well-known mechanism by which this pathogen can establish latent infections. Therefore, the zebrafish model can be used to address the mechanisms responsible for HSV-1 CNS-invasion and provide new insight into how HSV-1 establishes latency and provokes repetitive episodes of disease reactivation.

Together, these studies have demonstrated that the possibility to longitudinally follow the infection course with fluorescently-labelled viruses in developing zebrafish embryos or adult fish is very attractive to model important aspects of human viral infections, such as the cellular and molecular bases of tissue and organ-specific viral tropisms. These successes indicate that it will be also be worth to explore the possibility of developing a zebrafish model for other problematic human viral infections, including Zika virus. Studying Zika infection in developing zebrafish embryos and larvae could be a valuable addition to mouse models that have only

recently been established and could provide new opportunities for studying the mechanistic basis of the association of this virus with microencephaly in newborns (Cugola et al., 2016; Li et al., 2016).

Fungal infections

A variety of fungi are present in the commensal flora of human mucosae and skin. Most of them represent opportunistic pathogens and can cause mycotic disease in immunocompromised individuals or when subjects are exposed to large doses. The increasing number of people following immunosuppressive regimens or that are HIV-positive have made fungal disease an important cause of illness, especially in hospitalized settings.

The zebrafish model has been used to study several fungal pathogens of global health interest, which include *Candida albicans*, *Aspergillus fumigatus*, *Mucor circinelloides* and *Cryptococcus neoformans*. All these studies have shown that an appropriate competency of the innate immunity is important to curtail fungal infections (Chao et al., 2010; Knox et al., 2014; Tenor et al., 2015; Voelz et al., 2015). However, the involvement and relevance of macrophage and neutrophils in the response to each of these pathogens (or at least to the particular strains used in these studies) shows interesting specificity. During *Mucor* and *Candida* infection, both macrophages and neutrophils are highly recruited to the infection site and both phagocytose the fungal spores (Chao et al., 2010; Voelz et al., 2015). In sharp contrast, it is observed that *Aspergillus* conidia (asexual fungal spores) and *Cryptococcus* cells are essentially engulfed by macrophages, with neutrophils playing only a marginal function in counteracting these pathogens (Knox et al., 2014; Tenor et al., 2015). *Aspergillus fumigatus* is a dimorphic fungus that grows in yeast and hyphal forms. Infected zebrafish showed that neutrophils did not engulf the fungal spores (conidia), but can tightly associate with the hyphal form of the fungus (Knox et al., 2014). This suggests differential specificity of macrophage and neutrophil responses to the vegetative and reproductive fungal forms (Knox et al., 2014).

Similarly to *A. fumigatus*, *Candida albicans* is an opportunistic dimorphic fungus and most of humans are healthy carries of this commensal. The most frequent *Candida* infections are those that remain localized to the

mucosal tissues, but life-threatening conditions can derive from systemic dissemination, especially in immunocompromised individuals (Brothers et al., 2013). Interestingly, when *Candida albicans* is injected locally in the zebrafish hindbrain, it readily causes disseminated infection and high mortality, which is associated to its germination from yeast to hypha. Both zebrafish macrophages and neutrophils can phagocytose *Candida* (Brothers et al., 2013). Uptake of the yeast form is important to contain the transition to the hyphal and more invasive form, indicating that immune cells are also crucial to counteract the yeast-to-hyphal transition of dimorphic fungi. While this model mimics human systemic candidiasis, injection of *Candida albicans* into the swimbladder of zebrafish larvae can be used to model mucosal Candida colonization and to study the distinctive immune mechanisms at play on the mucosal surfaces (Gratacap & Wheeler, 2014).

Recent use of the zebrafish model has been critical to better characterize the mechanism of virulence of *Cryptococcus neoformans*, which represents an emerging and often fatal human pathogen (Bojarczuk et al., 2016; Tenor et al., 2015). Cryptococcal infection in humans generally initiates in the lung. However, the pathogen displays a remarkable tropism for the CNS, which is the main life-threatening complication of this fungal disease. Live imaging in zebrafish demonstrated that the predisposition of this pathogen to colonize the brain is maintained in this host and that the capability of the pathogen to cross the blood brain barrier depends on the virulence gene FNX1 and on a known cryptococcal invasion-promoting pathway previously identified in a murine model (Tenor et al., 2015). Additionally, longitudinal studies in zebrafish showed that macrophages are important to counteract the acute infection with this pathogen (Bojarczuk et al., 2016). However, it was observed that cryptococci can still largely proliferate intracellularly in macrophages, and, within 24 hours, they can counteract macrophage phagocytosis by progressively increasing their capsule size until this reaches an extent that severely limits further phagocytosis. This study suggests that the early proinflammatory activation of macrophages can control cryptococcal infection in healthy individuals, while intracellular survival and modification of the cryptococcal capsule will lead to uncontrolled progression of infection in immunocompromised patients (Bojarczuk et al., 2016).

Concluding remarks

Modeling of infectious diseases using the early life stages of zebrafish is continuing to demonstrate striking similarities in the mechanism of action of the innate immune system across vertebrates, which not only is evolutionary relevant, but also adds a high biomedical value to the use of the zebrafish model. Notably, in many cases the zebrafish platform has served as a valuable springboard to more extended studies in mammals. In other cases, the zebrafish has worked well as a surrogate system to model certain disease features that have otherwise been difficult to reproduce or study in mammalian models. Considering the expanding genetic toolbox for zebrafish research and the advanced use for non-invasive intravital imaging, it is to be expected that the zebrafish model will attract an increasingly larger scientific audience and continue to enforce its position in translational research. With state-of-art genome editing techniques now being successfully applied in zebrafish, it will be possible to generate a collection of key immune gene knockouts that will help to better understand the core mechanisms of immune recognition and pathogen virulence and to generate knowledge that can be exploited for developing novel therapeutic strategies to combat infectious and inflammatory diseases.

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Chapter 3

Macrophages target *Salmonella* by Lc3-associated phagocytosis in a systemic infection model

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Abstract

Innate immune defense against intracellular pathogens, like *Salmonella*, relies heavily on the autophagy machinery of the host. This response is studied intensively in epithelial cells, the target of *Salmonella* during gastrointestinal infections. However, little is known of the role that autophagy plays in macrophages, the predominant carriers of this pathogen during systemic disease. Here we utilize a zebrafish embryo model to study the interaction of S. *enterica* serovar Typhimurium with the autophagy machinery of macrophages *in vivo*. We show that phagocytosis of live but not heat-killed *Salmonella* triggers recruitment of the autophagy marker GFP-Lc3 in a variety of patterns labelling tight or spacious bacteria-containing compartments, also revealed by electron microscopy. Neutrophils display similar GFP-Lc3 associations, but genetic modulation of the neutrophil/macrophage balance shows that macrophages are critical for the defense response. Deficiency of *atg5* reduces GFP-Lc3 recruitment and impairs host resistance, in contrast to *atg13* deficiency, indicating that association of Lc3 with *Salmonella* at this stage is independent of the ULK1-Atg13-FIP200 complex and that macrophages target *Salmonella* by Lc3-associated phagocytosis (LAP). In agreement, GFP-Lc3 recruitment and host resistance are impaired by deficiency of Rubicon, recently revealed as a negative regulator of canonical autophagy and a prerequisite for LAP. We also found strict dependency on NADPH oxidase, another essential factor for LAP. Both Rubicon and NADPH oxidase are required to activate a *Salmonella* biosensor for reactive oxygen species inside infected macrophages. These results identify LAP as the major host protective autophagy-related pathway responsible for macrophage defense against *Salmonella* during systemic infection.

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a common cause of self-limiting gastrointestinal infections in human hosts, but can provoke a systemic typhoid-like disease upon infection of mice (Fabrega & Vila, 2013). Therefore, this pathogen is widely used as surrogate model for *S. enterica* serovar Typhi (*S.* Typhi), the causative agent of typhoid fever, a life-threatening systemic human infectious disease. *Salmonella* can invade a variety of cell types owing to their ability to inject virulence effectors triggering phagocytosis by non-professional phagocytes, such as epithelial cells and fibroblasts (Finlay & Brumell, 2000). Following this self-induced entry, *Salmonella* begins to replicate inside a growing compartment called the *Salmonella*-containing vacuole (SCV). *Salmonella* can also replicate inside professional phagocytes, including macrophages, which are the main carriers of this pathogen when it causes systemic disease (Watson & Holden, 2010).

The invasion of host cells by *Salmonella* or other intracellular pathogens triggers autophagy, a cellular degradation pathway that delivers cytoplasmic content to lysosomes (Huang & Brumell, 2014). Many studies support that activation of the autophagy machinery functions to restrict cytosolic escape and intracellular replication of *Salmonella* (Birmingham et al.,2006; Cemma et al., 2011; Huang et al., 2009; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). Xenophagy is known as a selective autophagy process, wherein ubiquitin and galectin receptors target the membranes of damaged SCVs and bacteria that have escaped into the cytosol, and this is the main anti-*Salmonella* autophagy response in epithelial cells (Shahnazari et al., 2011; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). However, both the survival strategies of *Salmonella* and the host cell autophagy responses differ between cell types. For example, *S.* Typhimurium does not escape into the cytosol in fibroblasts, but in this cell type endosomal and lysosomal membranes accumulate around the SCV to form large aggregates that engage the autophagy machinery in an Atg9L1-independent manner (Kageyama et al., 2011; Lopez-Montero et al., 2016). Another process dependent on components of the autophagy machinery is known as LC3-associated phagocytosis (LAP) (Sanjuan et al., 2007). During LAP, the autophagy marker microtubule-associated protein

1A/1B-light chain 3 (MAP1LC3, LC3) is recruited to the single membrane of the phagosome, whereas it marks the double membranes of autophagosomes during canonical autophagy (Cunha & Martinez, 2017; Galluzzi et al., 2017). It is likely that LAP contributes to the response of neutrophils and epithelial cells to *Salmonella* infection (Huang et al., 2009). Studies of the autophagic response of macrophages to *Salmonella* infection are limited and point to roles in mediating programmed cell death as well as restricting bacterial replication (Hernandez et al., 2003).

The recruitment of LC3 to the phagosomal membrane of *Salmonella*-infected neutrophils and epithelial cells has been shown to depend on the function of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Huang et al.,2009). NADPH oxidase is required for the generation of reactive oxygen species (ROS) with potentially damaging properties against intracellular pathogens. NADPH oxidase is inactive in resting macrophages and neutrophils and gets activated when invading microbes trigger innate immunity signaling (Yang et al., 2012). Its importance in host defense is well exemplified by chronic granulomatous disease (CGD), a condition leading to life-threatening fungal and bacterial infections arising due to non-functional NADPH oxidase in phagocytes (Jones et al., 2008; Martire et al., 2008).

The RUN and cysteine rich domain containing Beclin1 interacting protein (Rubicon) has recently been shown to play an essential role in LAP and to be required for the activation of NADPH oxidase during this process (Martinez et al., 2015; Yang et al., 2012). Rubicon mediates the activation of NADPH oxidase in two ways, first by recruiting the p40phox component of NADPH oxidase via activating Class III phosphatidylinositol-2-OH kinase (PI3K) on phagosomes and generating phosphatidylinositol 3-phosphate (PI3P), and second by stabilizing p22phox to assemble the NADPH oxidase complex (Martinez et al., 2015; Yang et al., 2012). In contrast, Rubicon functions as a negative regulator of autophagosome formation, controlling the VPS34 lipid kinase complex and Rab7 GTPase activity (Matsunaga et al., 2009). Thus, Rubicon functions as a molecular switch between suppression of canonical autophagy and induction of LAP. Most components of the autophagy machinery are required for the formation of autophagosomes as well as LAPosomes. However, LAP is independent of the pre-initiation complex (ULK1 complex, including Unc-51-like kinases 1/2

(ULK1/2), FIP200, ATG13 and ATG101) (Martinez et al., 2011; Martinez et al., 2015), whereas this complex is essential for the induction of canonical autophagy (Kim et al., 2011). Several studies have provided evidence for a role of LAP in restricting the growth of intracellular pathogens (Gong et al., 2011; Hubber et al., 2017; Martinez et al., 2015; Yang et al., 2012), while other studies suggest that pathogens might exploit LAPosomes as replication niches (Lam et al., 2013; Ligeon et al., 2014). In addition, LAP has been proposed to promote MHC II class presentation of antigens (Ma et al., 2012; Romao et al., 2013). Furthermore, LAP can be activated during internalization of dead or dying cells (efferocytosis) or of live cells (entosis), which is thought to ensure that cells are cleared effectively without triggering pathological inflammatory responses (Florey et al., 2011; Martinez et al., 2011; Martinez et al., 2016).

It is currently unknown how the different autophagy-related mechanisms, such as xenophagy and LAP, are involved in the interaction of macrophages with *Salmonella* during systemic infection. To study the encounter of macrophages with this pathogen *in vivo*, we took advantage of a zebrafish embryo model of *S.* Typhimurium infection, wherein bacteria are delivered by microinjection into the blood circulation or sub-cutaneously (Benard et al., 2012; van der Sar et al., 2003). We have previously shown that zebrafish embryos respond to *Salmonella* infection by Toll-like receptor-mediated signaling inducing a strong proinflammatory gene expression signature similar as in mammalian hosts and human cells (Stockhammer et al., 2010; Stockhammer et al., 2009; van der Sar et al., 2006; van der Vaart et al., 2013). The zebrafish has become a widely utilized vertebrate model for human infection diseases (Tobin et al., 2012; Torraca et al., 2014) and has previously been used to study autophagic defense against *Shigella flexneri* and *Mycobacterium marinum* (Mostowy et al., 2013; van der Vaart et al., 2014). Here, by *in vivo* imaging of GFP-Lc3 transgenic zebrafish embryos we could dissect the role of macrophages and neutrophils in anti-*Salmonella* response and expose LAP as the major pathway responsible for the macrophage-mediated defense against this pathogen.

Results

GFP-Lc3 recruitment is a dynamic response limited to live *Salmonella* cells

LC3, the vertebrate ortholog of yeast Atg8, is a widely accepted marker for autophagy-related processes, which can be visualized by fusion with fluorescent proteins (Mizushima et al., 2010). We took this approach and used *Tg(CMV:GFP-map1lc3b)* transgenic zebrafish (He et al., 2009) to study the role of the autophagy machinery during *Salmonella* infection *in vivo*. To determine the host response to *S*. Typhimurium infection, embryos were systemically infected at 2 days post fertilization (dpf) via caudal vein microinjection with a low dose (200-400 colony forming units (CFU) of either live or heat-killed *S*. Typhimurium (SL1344). Both groups were scored for mortalities at 24 hour intervals up to 3 days post infection (dpi) **(Figure 1A)**. In contrast to heat-killed bacteria, live *S*. Typhimurium were able to kill approximately 50% of the infected zebrafish by 48 hours post infection (hpi), and the infection resulted in approximately 70% mortality at the endpoint of the survival experiment (72 hpi) **(Figure 1B)**. To assess bacterial growth kinetics, CFU counts were determined by retrieving *Salmonella* cells from surviving embryos at 24, 48 and 72 hpi. As expected, the heat-killed *S*. Typhimurium could not establish infection within the host as no bacterial growth on agar plates was observed (data not shown). Conversely, live *S*. Typhimurium showed exponential growth inside the host reaching approximately 10^5 CFU per larva **(Figure 1C)**. Of note, the above experiments were reproduced in the wild type zebrafish line AB/TL (data not shown); therefore the observed response to *S.* Typhimurium is not affected by the GFP-Lc3 transgene expression.

In order to visualize the dynamics of GFP-Lc3-associations to *Salmonella* cells, a high dose (2000-4000 CFU) of *mCherry*-expressing bacteria (either live or heat-killed) was injected and time-lapse confocal microscopy was performed. GFP-Lc3-*Salmonella* associations were found to be mostly limited to injection of live bacteria, where the bright GFP signal of Lc3 **(Figure 1D-G)** was observed associated to *S*. Typhimurium internalized by motile phagocytes **(see Supplementary Movies M1** and **M2**).

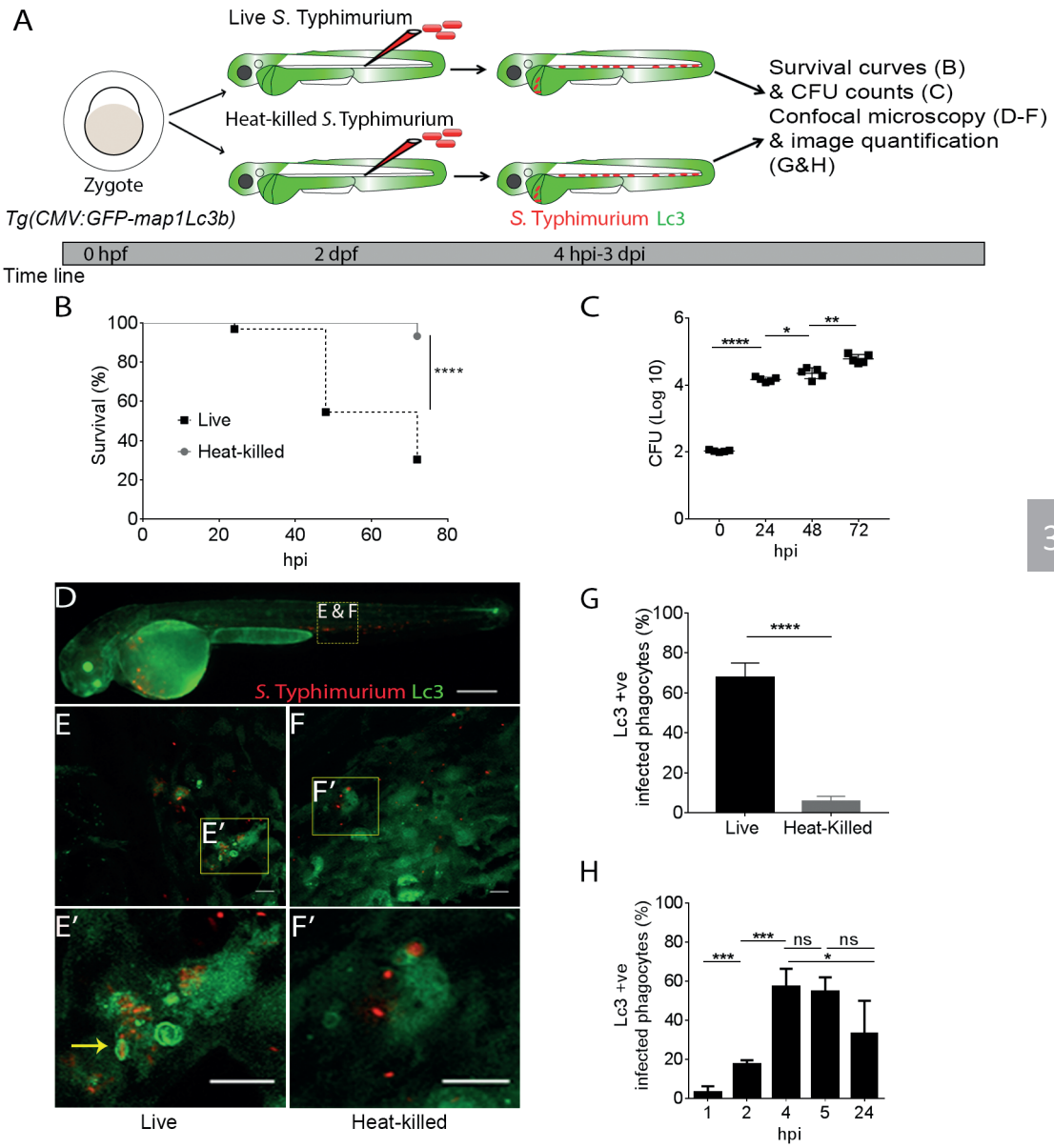

Figure 1: GFP-Lc3 recruitment to *Salmonella* during systemic infection of zebrafish. *(figure legend on next page).*

Figure 1: GFP-Lc3 recruitment to *Salmonella* during systemic infection of zebrafish. *(figure on previous page).* **A:** Work flow for experiments followed in B-H, displayed along the time line of zebrafish development. **B:** Survival curves of systemically infected larvae with either live or heat-killed *S*. Typhimurium. One representative of three replicates is shown (n= 50 embryos per group). **C:** CFU counts of the zebrafish infected with live *S*. Typhimurium. Five embryos/larvae per time point were used and the log-transformed CFU data are shown with the geometric mean per time point. One representative of three replicates is shown. **D:** Stereo fluorescence image of a 2 dpf *Tg(CMV:GFP-map1lc3b)* embryo, systemically infected with live *mCherry*-expressing *S*. Typhimurium SL1344. The yellow dotted box shows the area in the tail used for Z-stack confocal imaging in E & F. **E,F:** Representative confocal images at max projection taken at 4 hpi from time lapses recorded of embryos injected with live *S*. Typhimurium (E) or heat-killed *S*. Typhimurium (F) bacteria **(see Supplementary Movie S1 for live and S2 for heat-killed bacteria)**. Yellow boxes indicate the magnified regions shown in E' and F'. The arrow in E' indicates a ring-shaped structure of GFP-Lc3 signal enclosing an *mCherry*-expressing *S.* Typhimurium cell and several other GFP-Lc3-*Salmonella* associations can be observed in this image. **G:** Quantification of GFP-Lc3-*Salmonella* associations at 4 hpi. Embryos injected with live or heat-killed *S*. Typhimurium were imaged over the yolk sac circulation valley region and quantified for percentages of phagocytes positive for GFP-Lc3-*Salmonella* associations (Lc3+ve) over the total number of phagocytes with ingested bacteria. Error bars represent the SD. One representative of three replicates (each with n=5 embryos per group) is shown. **H:** Quantification of time course associations of GFP-Lc3-*Salmonella* during systemic infection with live *S*. Typhimurium. Five embryos per time point were imaged and quantified for percentages of phagocytes with GFP-Lc3-*Salmonella* associations as in G. Error bars represent the SD. Scale bars: D= 200 μm, E,F,E' & F'= 10 μm. ****P< 0.0001, ***P<0.001, **P< 0.01, *P< 0.05, ns= non-significant.

Approximately 60% of phagocytes that had ingested live bacteria were positive for GFP-Lc3-*Salmonella* associations at 4 hpi, whereas the proportion of GFP-Lc3 positive phagocytes was close to zero in response to injection of heat-killed bacteria **(Figure 1G)**. In addition, the dynamics of GFP-Lc3 associations to internalized *S.* Typhimurium was determined in a time course experiment where infected embryos with live *S*. Typhimurium were fixed at 1, 2, 4, 5 and 24 hpi and the proportion of infected phagocytes with Lc3 associations was quantified from confocal images. The highest percentage of GFP-Lc3-recruitment was observed at 4 hpi, which dropped to significantly lower levels at 24 hpi **(Figure 1H)**.

GFP-Lc3 associates to *Salmonella* in a number of patterns

To further describe the intracellular patterns of GFP-Lc3-*Salmonella* associations, we focused on the 4 hpi time point **(Figure 2A)**, where the highest observed Lc3 response occurred. Following intravenous infection, part of the *S.* Typhimurium bacteria contained by phagocytes residing in the yolk sac circulation valley could be classified as Lc3-positive associations **(Figure 2B$_{1-6}$)**, while another fraction of intraphagocyte *Salmonella* was observed as Lc3-negative **(Figure 2B$_{7-9}$)**. The Lc3-positive associations were observed in different patterns, categorized as (1) a single punctum associated with a single bacterial cell **(Figure 2B$_1$)**, (2) a spacious Lc3 ring around a single bacterial cell **(Figure 2B$_2$)**, (3) a tight Lc3 ring around a single bacterial cell **(Figure 2B$_3$)**, (4) a spacious Lc3 ring enclosing a cluster of loosely packed bacterial cells **(Figure 2B$_4$)**, (5) an Lc3 ring around a dense *mCherry* signal of bacteria where bacterial cell boundaries are not identifiable **(Figure 2B$_5$)**, and (6) multiple Lc3 puncta associating with clusters of bacteria **(Figure 2B$_6$)**. The Lc3-negative intracellular *Salmonella* were observed as single bacterial cells **(Figure 2B$_7$)**, and loosely **(Figure 2B$_8$)** or tightly packed **(Figure 2B$_9$)** clusters of bacteria that seemed contained inside a vesicular compartment.

In an attempt to visualize *S*. Typhimurium inside the classical double membrane autophagosome compartments, a hallmark of canonical autophagy (xenophagy), we used transmission electron microscopy (TEM). In order to facilitate the localization of infected cells in TEM sections, a localized infection was established by inoculating a high dose of *S*. Typhimurium subcutaneously into the 2 dpf embryos. In our limited number (n=80) of TEM

micrographs from multiple embryos (n=20), we were not able to identify any *S*. Typhimurium cells residing within a double membrane structure, while diverse other types of *Salmonella* containing vesicles were observed **(Figure 2C-H)**. For example, we observed a macrophage attached to the inner layer of the epidermis, where it centrally contains a phagosome with a large number of internalized *S.* Typhimurium **(Figure 2C**, also see **Supplementary Figure S1** showing the cell within the surrounding tissue). The membrane ruffles extending from the observed macrophage to extracellular bacteria in the sub-cutaneous space indicate that this phagocyte was involved in further phagocytosis of bacteria **(Figure 2C')**. Some of the phagocytosed bacteria showed normal *S.* Typhimurium morphology where bacterial envelopes and cytoplasm were intact. Other ingested bacteria were identified with unusual morphology where cytoplasm was found retracted away from the bacterial envelope. Additionally, the intraphagosome environment appeared cloudy and contained debris that could have resulted from dissociating bacteria. This large phagosome could be representative for one of the larger types of Lc3-positive **(Figure $2B_{4-6}$)** or Lc3-negative **(Figure $2B_{8-9}$)**, *Salmonella* containing vesicles with multiple bacteria inside.

Another type of intracellular compartment containing *S.* Typhimurium was observed where a spacious vesicle held two bacterial cells (S.T1 & 2 in **Figure 2D**) but also contained cytoplasmic (membranous) material that might have resulted from intraluminal vesicle formation or from fusion with autophagic vesicles. This structure is reminiscent of the GFP-Lc3 association event in **Figure $2B_4$**. In the same micrograph, another bacterial cell (S.T3) is observed inside a less expanded SCV, not containing cytoplasmic material, suggesting this structure to have originated from phagocytosis and not from xenophagy. Similar cases were observed in other micrographs (S.T2 in **Figures 2E and G**).

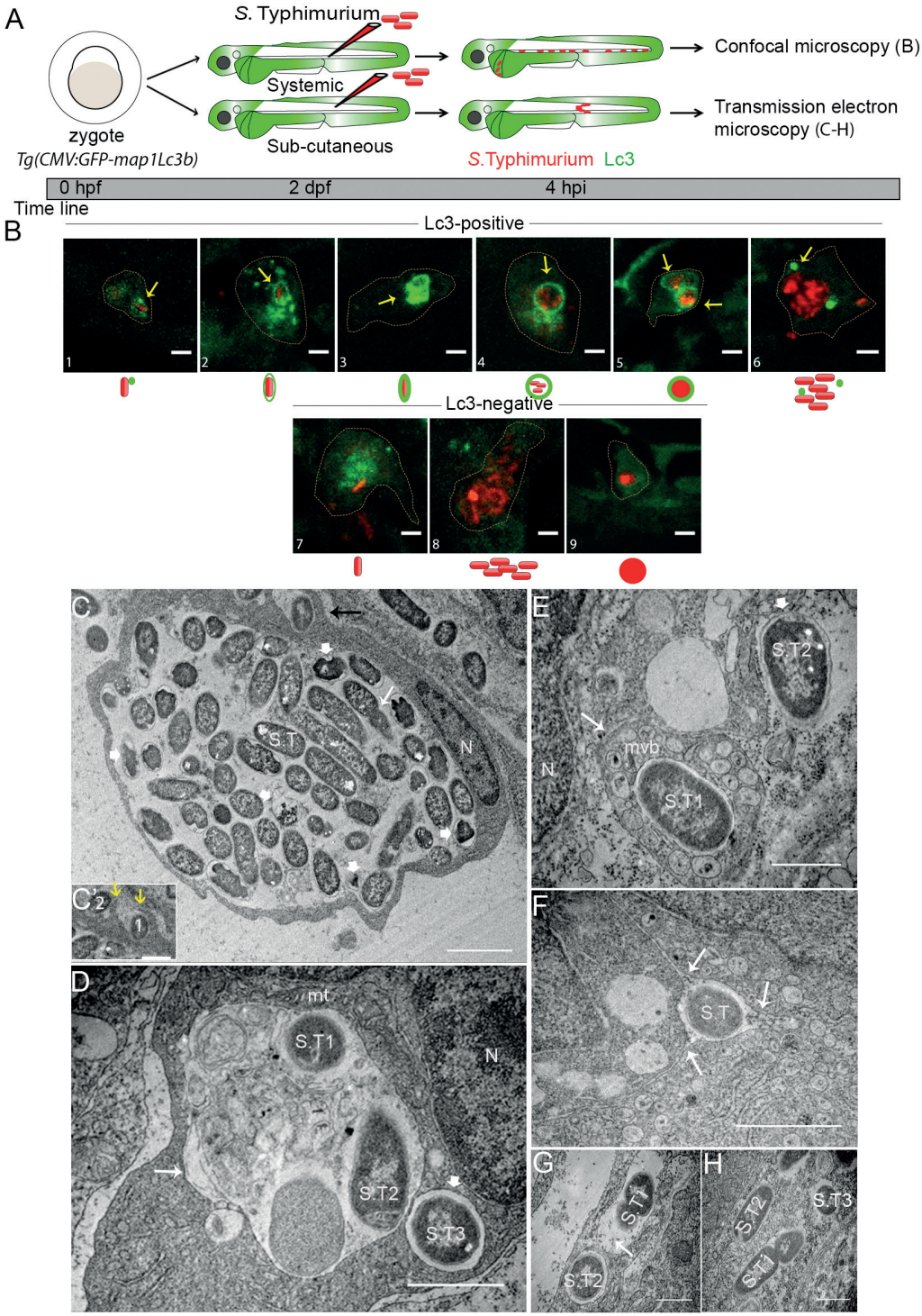

Figure 2: Types of *Salmonella*-containing vesicles in professional phagocytes. *(figure legend on next page).*

Figure 2: Types of *Salmonella*-containing vesicles in professional phagocytes. *(figure on previous page).* **A:** Work flow and time line of experiments in B-H. **B:** Representative confocal micrographs for 6 distinctive patterns of GFP-Lc3 association with *mCherry*-expressing *S*. Typhimurium (B_1-B_6) and 3 types of Lc3-negative cases (B_7-B_9) observed at 4 hpi in motile phagocytes in the tail and yolk sac regions. Under each image the symbolic representation of the pattern is shown. Dotted lines outline the cellular boundaries of the phagocytes and yellow arrows point towards GFP-Lc3 association with bacterial cells. **C:** Transmission electron micrograph (TEM) of a heavily infected macrophage with phagocytosed *S*. Typhimurium at 4 hpi, located in the lumen underneath the epidermis. Cytoplasm and nucleus (N) of the cell are pushed towards the cellular periphery and a large number of phagocytosed *S*. Typhimurium cells (S.T) are centrally contained in a large phagosomal compartment. The long white arrow indicates a dividing bacterial cell. The short thick white arrows indicate examples of dissociating *Salmonella* cells recognized by the retraction of the cytoplasm from the inner membrane**.** The black arrow indicates phagocytosing activity of the macrophage which is enlarged in C'. The yellow arrows in C' point to membrane ruffles of the macrophage formed to enclose extracellular *S*. Typhimurium (1 & 2). Supplementary Figure S1 shows a broader view of this macrophage in the tissue context. **D:** TEM micrograph showing two *S*. Typhimurium-containing compartments.The larger compartment (long arrow) contains cellular debris in addition to bacterial cells (S.T1 & S.T2). The lumen of the smaller vesicle (short thick arrow), which holds a single bacterium (S.T3), is clean. The nucleus (N) and a mitochondrion (mt) in close vicinity of the bacterial compartments are indicated. **E:** TEM micrograph of *S*. Typhimurium (S.T1) inside a multi-vesicular body (mvb, long arrow). The short thick arrow indicates a cytoplasmic *Salmonella* cell (S.T2) with intact outer and inner bacterial membrane. **F:** TEM of *S*. Typhimurium (S.T1) inside a vesicle that forms tubular extensions into the cytoplasm (arrows). **G:** TEM showing *S*. Typhimurium cells (S.T1 & S.T2) inside two single membrane-bound compartments. The presence of ribosomes (arrow) in the internal environment suggests membrane damage. **H:** TEM of three *S*. Typhimurium (S.T) cells in the cytoplasm, one of which (S.T1) is replicating. Scale bars: B= 5 µm, C-H= 2 µm, C'= 1 µm.

We also noticed an *S.* Typhimurium cell centrally placed inside a multi-vesicular body (MVB) (S.T1 in **Figure 2E**) and enclosed inside a single membrane compartment. We hypothesize that the intense and tight ring of Lc3 around a single bacterium in **Figure 2B$_3$** might correspond to such an MVB structure observed in the TEM analysis. A bacterial compartment forming tubular extensions into the cytoplasm was also observed **(Figure 2F)**, which is reminiscent of the formation of *Salmonella* induced filaments (SIFs), a process known to occur at later stages of SCV formation (Finlay & Brumell, 2000; Rajashekar et al., 2014). Finally, we spotted replicating *S.* Typhimurium cells inside vesicles (S.T1 in **Figure 2G**) as well as within the cytoplasm **(**S.T1 in **Figure 2H)**.

Macrophages show more Lc3-*Salmonella* associations than neutrophils

In order to determine which phagocyte cell types are involved in the GFP-Lc3 response to *S.* Typhimurium, infected embryos were stained with tyramide signal amplification (TSA) to visualize neutrophils and anti-L-plastin, which labels both neutrophils and macrophages **(Figure 3A)**. Lc3-*Salmonella* associations were not observed in L-plastin-negative cells, indicating that their presence is restricted to phagocytes at the time point of our analysis. It was observed that macrophages (L-plastin-positive/TSA-negative) show almost all types of Lc3-*Salmonella* associations recorded earlier **(Figure 3B$_{1\text{-}6}$** as compared with **Figure 2B$_{1\text{-}6}$)**. Neutrophils (L-plastin/TSA double positive) were also observed with Lc3-*Salmonella* associations **(Figure 3B$_{7\text{-}8}$)**, however not all types of Lc3-positive *Salmonella* containing vesicles were observed in these cells.

Macrophages are known for readily phagocytosing intravenously injected *E. coli* bacteria at higher rates than neutrophils, whilst neutrophils display a surface-associated phagocytic behavior and are reported to phagocytose bacteria more readily during a sub-cutaneous infection (Colucci-Guyon et al., 2011). Despite this difference in phagocytic behavior between the cell types, we observed that, in both types of infection routes, macrophages phagocytose *S.* Typhimurium in higher percentages when compared to neutrophils **(Figure 3C)**. Furthermore, irrespective of the infection route, a significantly higher percentage of infected macrophages were scored positive for the presence of GFP-Lc3-*Salmonella* associations as compared to the percentage of infected neutrophils **(Figure 3D).**

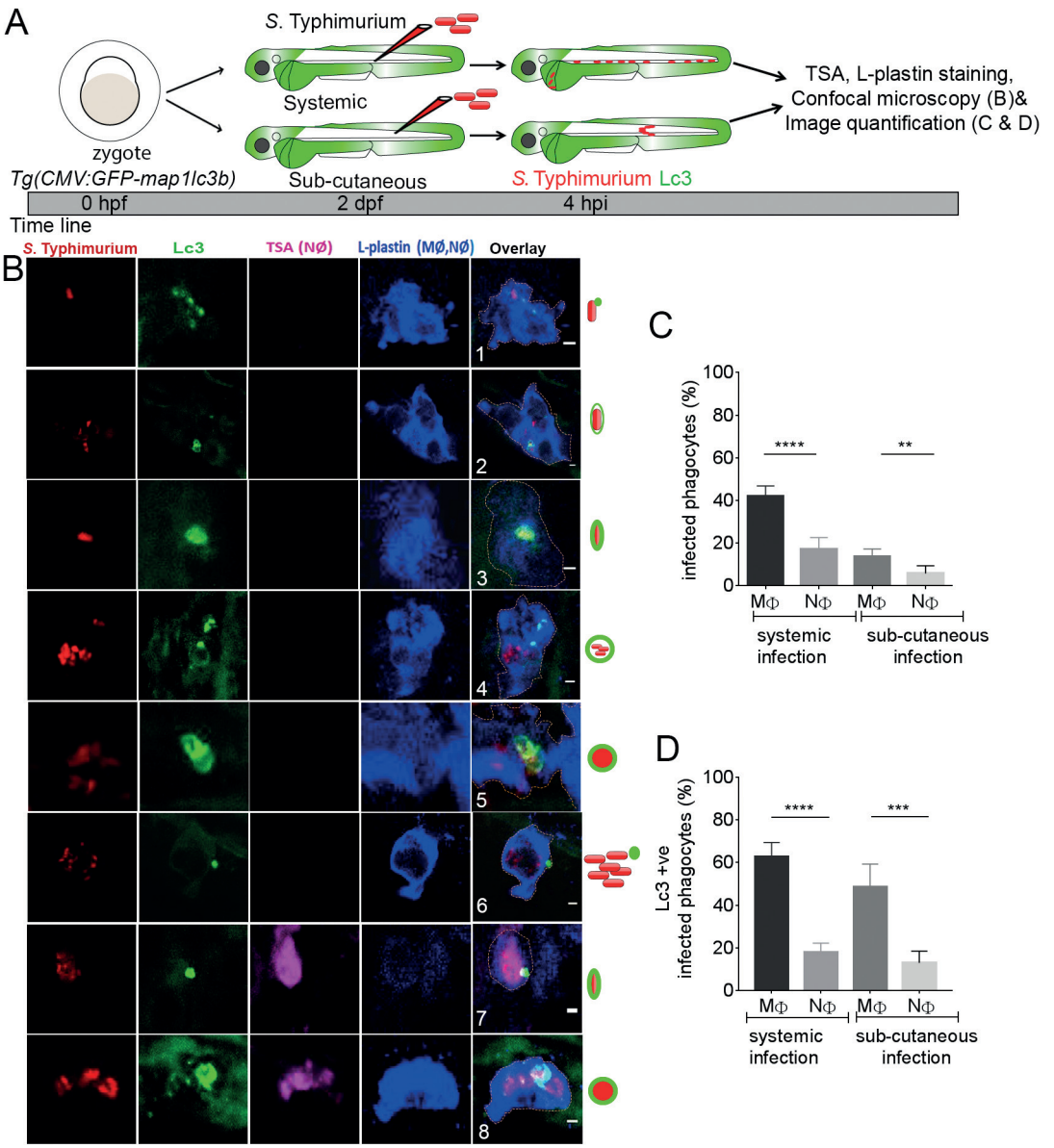

Figure 3: GFP-Lc3--*Salmonella* associations in specific phagocyte types.
(figure legend on next page).

Figure 3: GFP-Lc3--*Salmonella* associations in specific phagocyte types. *(figure on previous page).* **A:** Work flow and time line of experiments in B-D. **B:** Confocal micrographs in series (1-8) of distinctive patterns of GFP-Lc3 association with *mCherry*-expressing *S*. Typhimurium in macrophages (MΦ, TSA negative/L-plastin positive; 1-6) and neutrophils (NΦ, TSA positive/L-plastin positive; 7,8). The dotted lines in the overlay image of the separate channels indicate the cellular boundaries. The symbolic presentations shown next to the images refer to the different types of GFP-Lc3-*Salmonella* associations presented in Figure 2. **C:** Quantification of phagocytosing activity of macrophages and neutrophils in systemic and sub-cutaneous infections at 4 hpi. Numbers of infected and non-infected phagocytes were counted from confocal images and the percentages of infected over the total were averaged from five embryos per group. Macrophages (MΦ) and neutrophils (NΦ) were distinguished by L-plastin and TSA staining as in B. **D:** Quantification of GFP-Lc3-*Salmonella* associations in macrophages and neutrophils at 4 hpi. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive over the total were averaged from five embryos per group. Error bars represent the SD. Macrophages (MΦ) and neutrophils (NΦ) were distinguished by L-plastin and TSA staining as in B. Error bars represent the SD. Scale bars: B= 2 μm. ****P< 0.0001, ***P<0.001, **P< 0.01.

Macrophage depletion reduces Lc3-*Salmonella* associations and impairs host defense

The results showing that macrophages stand out as the predominant phagocyte cell type responsible for phagocytosing and Lc3 targeting of *S.* Typhimurium in our model, prompted us to investigate how the host would respond to *Salmonella* infection in a state without macrophages. We explored the impact of macrophage ablation on the host during *Salmonella* infection by an interferon regulatory factor-8 (*irf8*) knockdown approach **(Figure 4A)**, where zebrafish myelopoiesis is reoriented and no macrophages are produced, whilst almost doubling the number of neutrophils (Li et al., 2011). Indeed, the morpholino-mediated *irf8* knockdown resulted in embryos with almost no macrophages at 2 dpf, the time of *S.* Typhimurium infection, confirming the efficacy of this knockdown strategy **(Figure 4B,C & B',C')**. We found that macrophage-depleted embryos were hypersusceptible to *S.* Typhimurium infection as most of the *irf8* knockdown subjects died within 24 hpi regardless of the route of in-

fection **(Figure D)**. In addition, CFU counts revealed that in the absence of macrophages, the infection progresses faster as the bacterial numbers at 24 hpi were significantly higher in *irf8* knockdown compared to the controls **(Figure E)**. Furthermore, both infection routes led to higher percentages of infected phagocytes with Lc3-*Salmonella* associations in control embryos compared with the *irf8* knockdown group, supporting that the Lc3 response observed is mainly driven by macrophages **(Figure 4F)**.

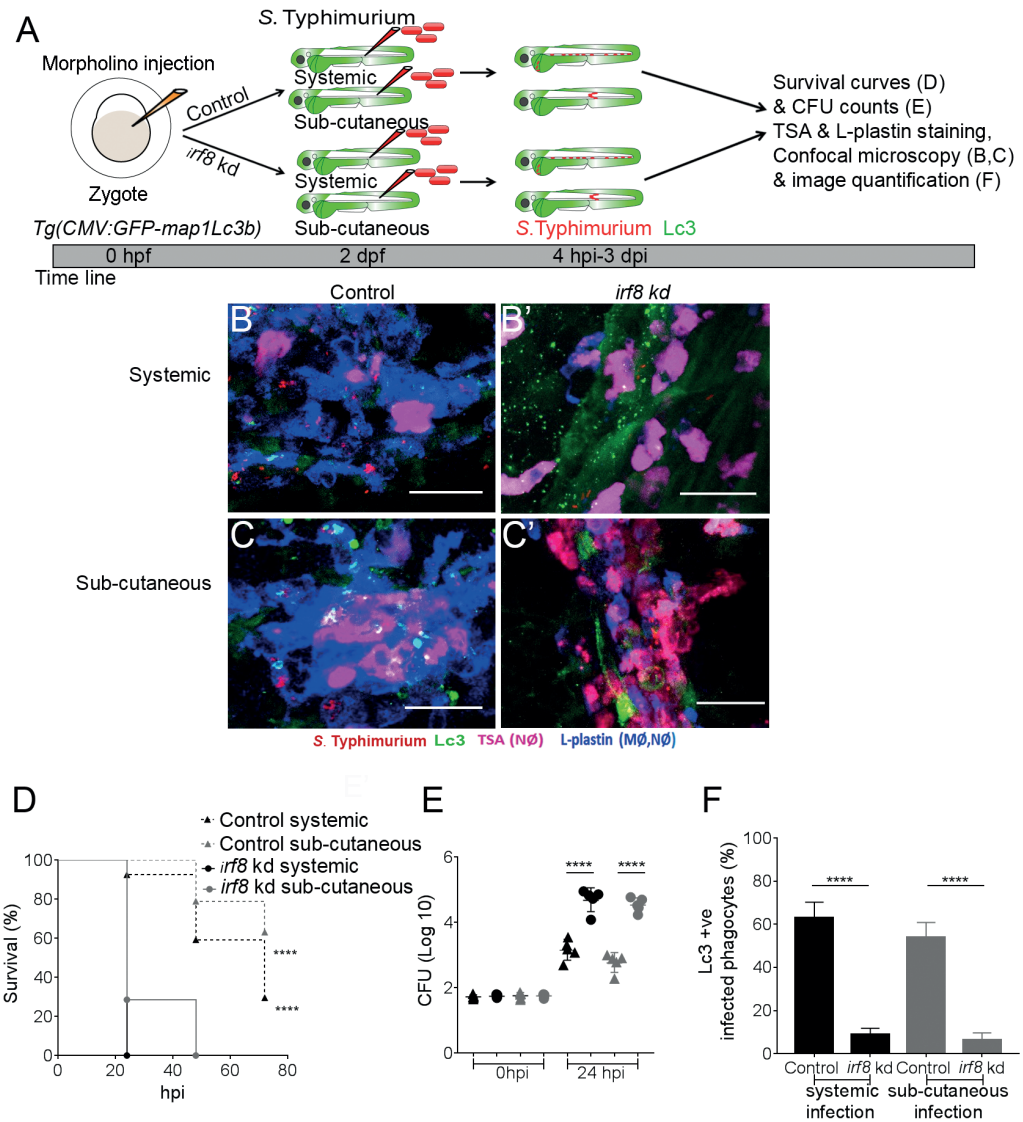

Figure 4 : Essential role of macrophages in host defense against systemic *Salmonella* infection. *(figure legend on next page).*

Figure 4 : Essential role of macrophages in host defense against systemic *Salmonella* infection. *(figure on previous page).* **A:** Work flow and time line of experiments in B-F. **B-C':** Confocal images of the tail region of systemically **(B&B')** and sub-cutaneously **(C&C')** infected GFP-Lc3-expressing embryos (4 hpi), stained to distinguish macrophages (MΦ, TSA negative/L-plastin positive) and neutrophils (NΦ, TSA positive/L-plastin positive) under control (B&C) and *irf8* knockdown (B'&C') conditions. **D:** Survival curves of control and *irf8* knockdown larvae in systemic and sub-cutaneous infection experiments. One representative of three replicates is shown. **E:** Representative CFU counts of control and *irf8* knockdown larvae in systemic and sub-cutaneous infection experiments. Five embryos/larvae per time point were used and the log transformed CFU data are shown with the geometric mean per time point. **F:** Quantification of GFP-Lc3-*Salmonella* associations during systemic and sub-cutaneous infection at 4 hpi. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive over the total were averaged from five embryos per group. Error bars represent the SD. Macrophages (MΦ) and neutrophils (NΦ) were distinguished by L-plastin and TSA staining as in B,C. Scale bars: 20 μm, ****P< 0.0001.

Lc3-*Salmonella* associations are Atg5-dependent and Atg13-independent

A number of ATG proteins are known to play essential roles during the process of autophagy. We wanted to determine the type of Lc3-*Salmonella* associations observed as evidence of either canonical autophagy or an autophagy-related process and therefore investigated the role of components of the ATG-machinery. ATG5 is known to form a complex with ATG12 and is required for the chain elongation process (Walczak & Martens, 2013). To determine the impact of zebrafish Atg5 in our *Salmonella* infection model, we took advantage of a previously validated *atg5* morpholino knockdown strategy (Hu et al., 2011). We concluded that Atg5 is required for host defense against *S.* Typhimurium as control embryos had significantly higher survival rates during *Salmonella* infection when compared to the *atg5* knockdown group **(Figure 5B)**. In agreement, control embryos were able to curtail bacterial infection better than *atg5* knockdown embryos as based on CFU counts **(Figure 5C)**. Quantification of the GFP-Lc3-*Salmonella* associations **(Figure 5D,D')** confirmed the importance of Atg5 for this response as controls showed a significantly higher percentage of GFP-Lc3-positive infected phagocytes when compared to

atg5 knockdown embryos **(Figure 5E)**.

Since Atg5 is required in both canonical autophagy (Mizushima et al., 1998) and autophagy-related processes (Huang et al., 2009; Kageyama et al., 2011; Sanjuan et al., 2007), the loss of this protein mediated by *atg5* knockdown did not reveal whether Lc3-*Salmonella* associations observed in our model are related to entrapment of *Salmonella* in autophagosomes (xenophagy) or could be due to Lc3 recruitment to phagosomes (LAP). Therefore, we knocked down *atg13,* a component of the ULK1 preinitiation complex, which is required only for canonical autophagy (Martinez et al., 2011). Efficacy of the *atg13* morpholino was validated with a functional analysis using bafilomycin to block autophagic flux, wherein *atg13* knockdown resulted in significantly lower GFP-Lc3 signal accumulation as compared to controls after bafilomycin treatment at 2 dpf **(supplementary Figure S2, A-C)**. Knockdown of Atg13 had no significant effect on survival rates during infection **(Figure 5F)**, and bacterial burdens within controls and *atg13* morphants did not differ from each other **(Figure 5G)**. These results indicate that the ULK1-Atg13-FIP200 complex is not required for host defense against *Salmonella*. Subsequent quantification of GFP-Lc3-*Salmonella* associations supported the non-essential role of the ULK1 complex as both controls and *atg13* morphants had similar numbers of Lc3-positive infected phagocytes **(Figure 5H-I)**. Collectively, these data support the hypothesis that Lc3-*Salmonella* association is mediated by the ULK1-independent LAP pathway in our model.

Rubicon plays a major role in the Lc3-mediated anti-*Salmonella* defense response

Rubicon is a Beclin-1 partner that negatively regulates canonical autophagy and has recently been identified as a molecular switch between autophagy and LAP (Martinez et al., 2015). Therefore, we investigated its importance in our model using a knockdown approach of the Rubicon gene (*rubcn*) with two different morpholinos, targeting either the translation start site or an intron-exon boundary. This study revealed a significant role for Rubicon in host defense as *Salmonella*-infected control larvae had significantly higher survival rates and lower bacterial burdens as compared to both *rubcn* knockdown groups **(Figure 5J,K&N,O)**. Furthermore, *rubcn* knockdown using either of the two morpholinos significantly

reduced the percentage of Lc3-positive infected phagocytes as compared to control embryos **(Figure 5L-M & PQ)**. The efficacy of *rubcn* knockdown with splice blocking morpholino was verified by RT-PCR **(supplementary Figure S3)**. Together, the Atg13 independency and the requirement of Rubicon point towards LAP as a host defense response towards *S.* Typhimurium infection.

Figure 5: Roles of autophagy-related proteins in the host response to *Salmonella* infection. *(figure on next page).* **A:** Work flow and time line of the knockdown (kd) experiments in B-Q. **B,F,J,N:** Survival curves of systemic *S*. Typhimurium infection experiments of *atg5* **(B)**, *atg13* **(F)**, *rubcn* kd(1) **(J)** and *rubcn* kd(2) **(N)** morpholino knockdown groups with their respective control morpholino-injected groups. Kd1 and kd2 refer to use of translation blocking (MO1-*rubcn*) and splice blocking (MO2-*rubcn*) morpholinos, respectively. For each comparison one representative of three replicates is shown. **C,G,K,O:** Representative CFU counts of the *S*. Typhimurium infections of *atg5* **(C)**, *atg13* **(G)**, *rubcn* kd(1) **(K)** and *rubcn* kd(2) **(O)** morpholino knockdown groups with their respective controls at 24 hpi. Five embryos/larvae per time point were used and the log transformed CFU data are shown with the geometric mean per time point. **D-P:** Representative confocal images of *atg5* **(D')**, *atg13* **(H')**, *rubcn* kd(1) **(L')** and *rubcn* kd(2)' **(P')** morpholino knockdown embryos with their respective controls **(D, H, L and P)** at 4 hpi. **E,I,M,Q:** Quantification of GFP-Lc3-*Salmonella* associations in *atg5* **(E)**, *atg13* **(I)**, *rubcn* kd(1) **(M)** and *rubcn* kd(2) **(Q)** morpholino knockdown groups with their respective controls at 4 hpi. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive over the total were averaged from five embryos per group. Error bars represent the SD. Scale bars: 10 µm. ****P< 0.0001, ***P<0.001, **P< 0.01, *P< 0.05, ns= non-significant.

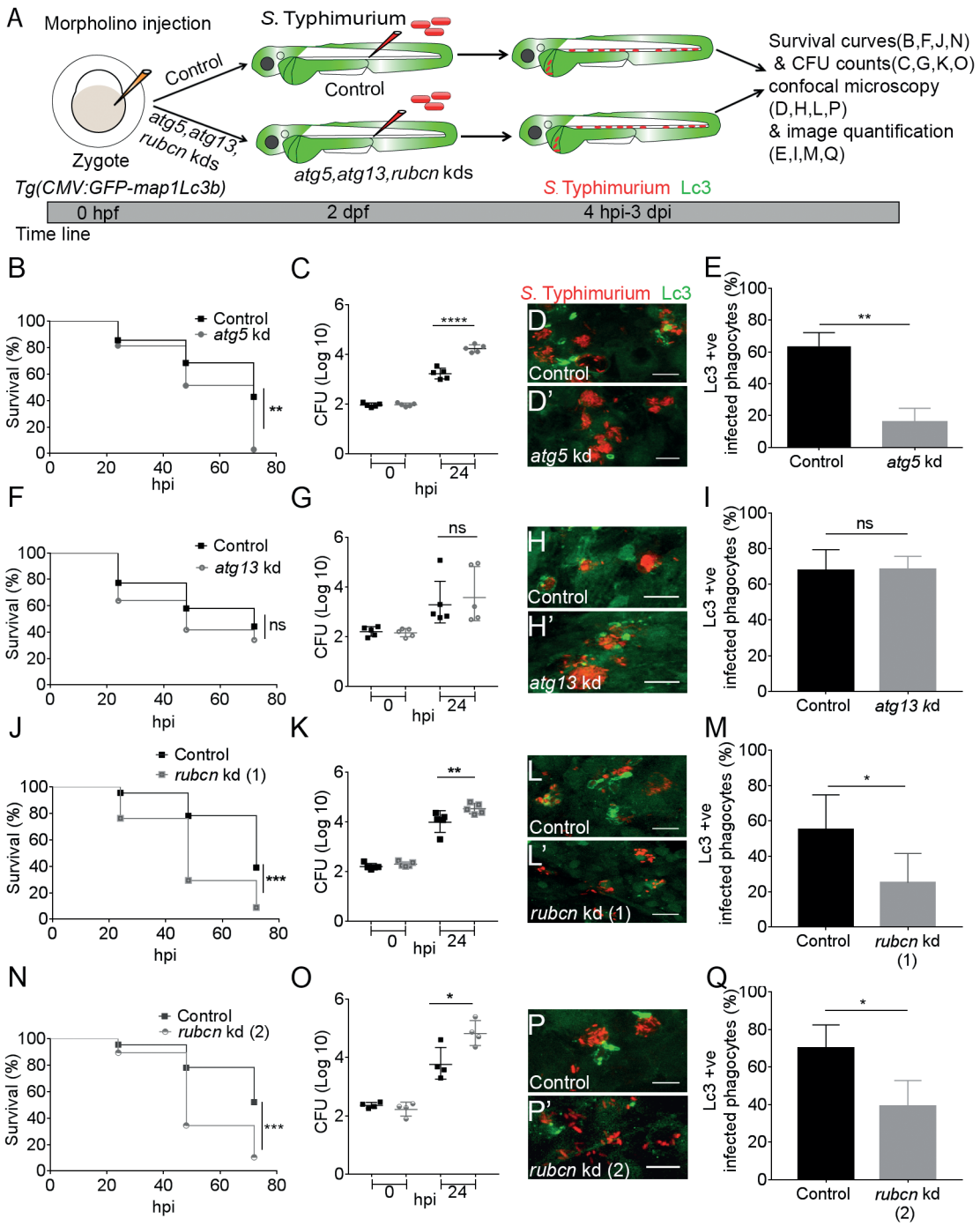

Figure 5: Roles of autophagy-related proteins in the host response to *Salmonella* infection. *(figure legend on previous page).*

The Lc3 response to phagocytosed *Salmonella* is strictly NADPH oxidase-dependent

To provide further evidence that *Salmonella* is targeted by LAP during systemic infection, we investigated the role of the ROS-producing enzyme complex NADPH oxidase, which has been shown to be stabilized on the phagosomal membrane by Rubicon (Yang et al., 2012). To this end, we targeted the p22phox subunit of NADPH oxidase, encoded by *cyba*, using a published morpholino knockdown approach (Niethammeer et al., 2009; Tauzin et al., 2014). In *S*. Typhimurium infection experiments, control embryos showed significantly higher survival rates and were able to curtail bacterial infection better than *cyba*-deficient embryos **(Figure 6B,C)**. In addition, GFP-Lc3 recruitment to *Salmonella* compartments was almost completely abolished by *cyba* knockdown **(Figure 6D-E)**.

To demonstrate that ingested *Salmonella* is targeted by intraphagocyte ROS, we used an *S.* Typhimurium biosensor strain that constitutively expresses *mCherry* and contains a GFP reporter under the control of the katGp promotor, which is activated when bacterial cells are exposed to ROS from the host (Burton et al., 2014). Activation of this ROS biosensor was shown to occur inside phagocytes **(Figure 6G)**, including *mpeg1:mCherry-F* labeled macrophages **(Figure 6K)**, and this activation was strictly dependent on both Rubicon **(Figure 6H,J,L&N)** and Cyba **(Figure 6I,J,M&N)**. Collectively, our results show that Lc3 association with the *Salmonella*-containing compartments as well as ROS production in the infected cells occurs by a mechanism dependent on Rubicon and NADPH oxidase. The essential role of these proteins indicates that LAP is the process that targets *Salmonella* inside phagocytes, predominantly macrophages, in our model. Furthermore, we conclude that LAP is a host-beneficial response that restricts the intracellular replication of *S.* Typhimurium.

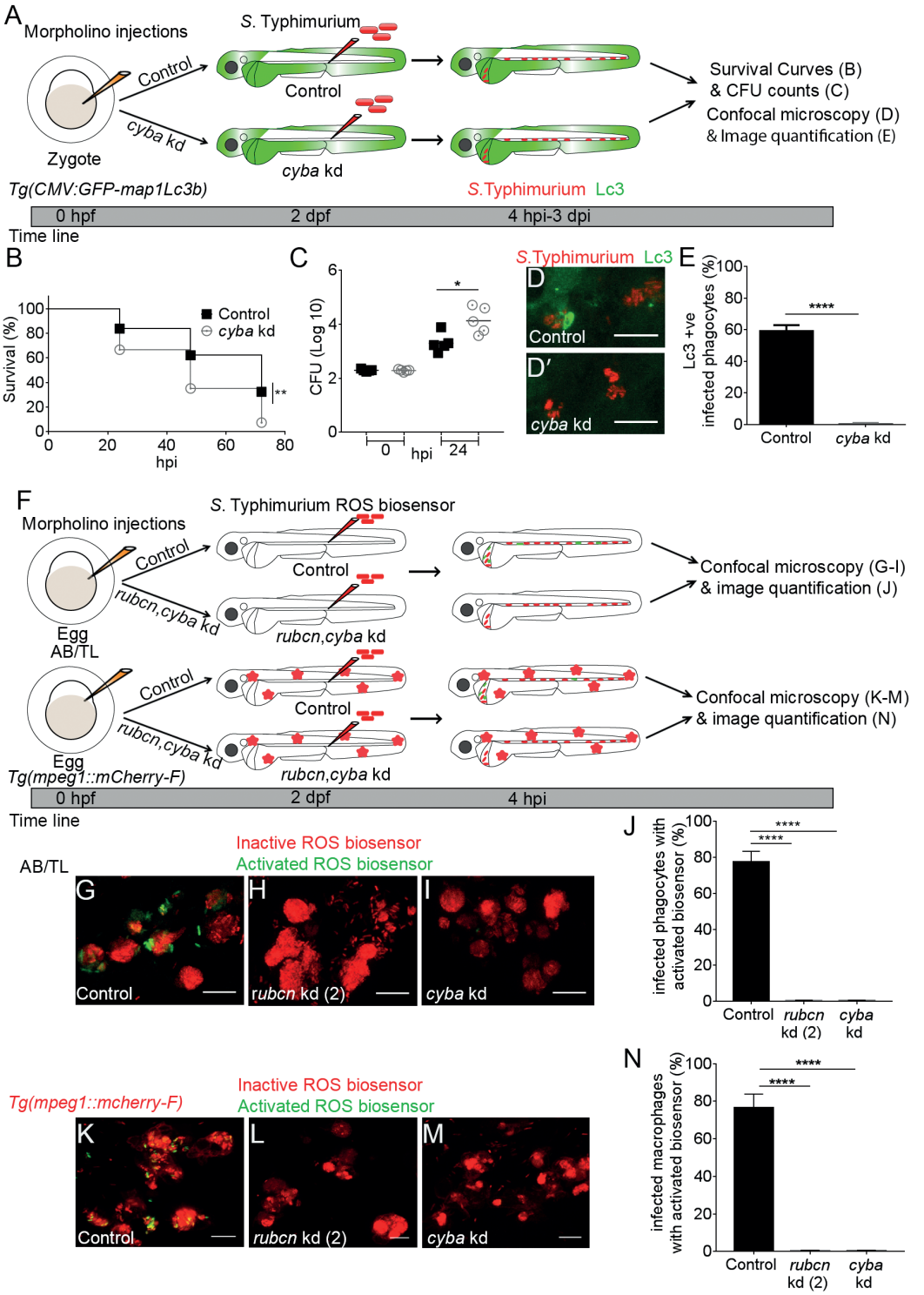

Figure 6: Requirement of NADPH oxidase for Lc3-mediated host defense against *Salmonella*. *(figure legend on next page).*

Figure 6: Requirement of NADPH oxidase for Lc3-mediated host defense against *Salmonella.* *(figure on previous page).* **A:** Work flow and time line of knockdown (kd) experiments in B-E. **B:** Survival curves of *S*. Typhimurium-infected control and *cyba* knockdown embryos. One representative of three replicates is shown. **C:** Representative CFU counts of *S*. Typhimurium-infected control and *cyba* knockdown embryos. Five embryos/larvae per time point were used and the log transformed CFU data are shown with the geometric mean per time point. **D:** Representative confocal images of control (D) and *cyba* (D') knockdown embryos at 4 hpi. **E:** Quantification of GFP-Lc3-*Salmonella* associations in control and *cyba* knockdown embryos at 4 hpi. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive over the total were averaged from five embryos per group. Error bars represent the SD. **F:** Work flow and time line of experiments in G-N. **G-I,K-M:** Representative confocal images of non-transgenic (AB/TL) embryos **(G-I)** or Tg(*mpeg1::mCherry-F*)^{umsF001} embryos **(K-M)** at 4 hpi following injection of a *Salmonella* ROS biosensor strain into control **(G,K)**, and *rubcn* kd(2) **(H,L)** or *cyba* morpholino knockdown groups **(I,M)**. The ROS biosensor strain expresses a constitutive *mCherry* marker and the GFP signal indicates ROS biosensor activation. Macrophages in the *Tg(mpeg1::mCherry-F*)^{umsF001} line express a farnesylated *mCherry* protein localizing to membranes and therefore the signal is separated from bacterial *mCherry* signal. **J,N:** Quantification of ROS biosensor activation at 4 hpi in control, *rubcn* kd(2) and *cyba* morpholino knockdown groups of non-transgenic (AB/TL) embryos **(J)** or *Tg*(*mpeg1::mCherryF*)^{umsF001} embryos **(N)** at 4 hpi. Numbers of total phagocytes **(J)** or macrophages **(N)** showing ROS biosensor activation (GFP and *mCherry* bacterial signals) or without ROS biosensor activation (*mCherry* bacterial signal only) were counted from confocal images and the percentages of ROS biosensor-positive over the total were averaged from five embryos per group. Error bars represent the SD. Scale bars: D,D', G-I, K-M = 10 µm. ****P< 0.0001, **P< 0.01, *P< 0.1.

Collectively, our results show that Lc3 association with the *Salmonella*-containing compartments as well as ROS production in these compartments occurs by a mechanism dependent on Rubicon and NADPH oxidase. The essential role of these proteins indicates that LAP is the process that targets *Salmonella* inside phagocytes, predominantly macrophages, in our systemic infection model. Furthermore, we conclude that LAP is a host beneficial response that restricts the intracellular replication of *S.* Typhimurium.

Discussion

Autophagy and related processes have emerged as important innate immune defense mechanisms against intracellular bacterial infections, and several pathogens, including *Salmonella*, are known to have evolved mechanisms to counteract the host autophagy response (Birmingham et al., 2006; Cemma et al., 2011; Huang & Brumell, 2014; Jia et al., 2009; Riquelme et al., 2016; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Yu et al., 2014; Zheng et al., 2009). Macrophages are the key cell type responsible for dissemination of *Salmonella* during systemic typhoid disease. Yet, it is not known whether the autophagy machinery of macrophages plays a host protective role or might be exploited by pathogenic *Salmonellae*. In this study we used zebrafish embryos as a model host for *S.* Typhimurium to address this question on whole organism level. Using combined *in vivo* imaging and genetic approaches we demonstrate that macrophages target *S*. Typhimurium by an autophagy-related process known as LAP, where Lc3 is directly recruited to phagocytosed bacteria in a manner dependent on the activation of ROS production in the *Salmonella*-containing compartment. This conclusion is supported by data showing that GFP-Lc3-*Salmonella* association and restriction of bacterial growth required the functions of Atg5, Rubicon, and NADPH oxidase, while independent of Atg13, a component of the ULK1 complex whose function is limited to canonical autophagy.

In addition to macrophages, neutrophils are also known to play an important role in host defense against *Salmonella* infection (Burton et al., 2014; Chen et al., 2014; Tyrkalska et al., 2016). However, in our study macrophages stood out as the predominant cell type targeting *Salmonella* and showed higher phagocytic activity against blood borne as well as subcutaneous bacteria. A genetically induced increase in neutrophil numbers at the expense of macrophages was detrimental to host defense against *Salmonella* infection. Furthermore, the majority of Lc3-*Salmonella* associations were observed in macrophages. It has previously been shown that *S.* Typhimurium causes programmed cell death of macrophages in an autophagy-dependent manner (Hernandez et al., 2003). Whether this autophagy-induced macrophage cell death restricts *Salmonella* replication or promotes pathogenesis is not known. We did not study the effects of autophagy or LAP inhibition on the life span of infected macrophages,

and it is well possible that Lc3 activation in our model could be linked with macrophage cell death when these cells reach the stage that they are no longer able to control *Salmonella* growth. However, it is clear from our data that Lc3 activation is an early response that is dependent on phagosomal ROS production, restricting bacterial replication at least for a limited period of time.

S. Typhimurium may defend itself against the observed defense responses of phagocytes by inhibiting Lc3 activation and NADPH oxidase or by expressing genes involved in detoxification of ROS, such as *katG.* Consistent with this hypothesis, Lc3 recruitment was not observed in all infected macrophages, and a subset of bacteria in the infected macrophages expressed a *katGp*-driven ROS biosensor gene. The heterogeneous activation of this biosensor is consistent with studies in mice, which furthermore suggested that sub-lethal ROS production in resident macrophages allows *Salmonella* to spread and persist in the infected host (Burton et al., 2014). In agreement with this work, we find that deficiency in autophagy genes and NADPH oxidase impairs zebrafish host defense, but autophagy and NADPH oxidase competent hosts are unable to fully control *S.* Typhimurium replication as 75% of infected individuals eventually succumb to infection.

That LAP could be involved in the response of phagocytes against *Salmonella* infection follows from an early study, in which targeting of LC3 to phagosomes was observed in neutrophils isolated from wild type but not from *nox2-/-* mice that lack NADPH oxidase activity (Huang et al., 2009). Since then, LAP has been defined to be dependent on NADPH oxidase and Rubicon, and independent of the ULK1-Atg13-FIP200 complex (Martinez et al., 2015; Yang et al., 2012). Lc3-*Salmonella* association in our model fulfilled all these criteria, thereby providing solid evidence for LAP as the underlying mechanism. Furthermore, we showed that deficiency in each of the essential components of LAP impaired the defense response of the zebrafish host against *S*. Typhimurium infection. A critical role for LAP in innate immunity was previously demonstrated in *Aspergillus fumigatus* and *Listeria monocytogenes* infection experiments using Rubicon-deficient or Rubicon-overexpressing mice (Martinez et al., 2015). Together, the studies in two different preclinical animal models, zebrafish and mice,

highlight LAP as a critical and evolutionary conserved host defense mechanism against both fungal and bacterial infections.

The major role for LAP identified in our study does not exclude that canonical forms of autophagy are also involved in the response of the zebrafish host to *S.* Typhimurium. In fact, we observed a variety of GFP-Lc3 patterns associated with *Salmonella* bacteria in infected macrophages. Furthermore, besides bacteria residing in phagosomes, TEM analysis revealed *Salmonella* in multivesicular compartments and in other vesicles containing cytoplasmic material, suggesting the possible involvement of autophagy. Although this cytoplasmic content could be derived from intraluminal vesicles, it might also be the result of fusion between autophagosomes and phagocytic compartments, which generates amphisomes (Fader & Colombo, 2009). It is conceivable that the decoration of phagosomes with Lc3 could promote such fusion processes. We also noted that, at the time point of our analysis (4 hpi), some bacteria were already present freely in the cytosol of the infected phagocytes, and therefore xenophagy, a common process in *Salmonella*-infected epithelial cells, could become important at later stages of infection following the escape of *Salmonella* from LAPosomes (Birmingham et al., 2006; Shahnazari et al., 2011; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). Comparison between the NADPH oxidase and Rubicon knockdown effects in our study also provides an indication for different autophagy-related processes to be at play. NADPH oxidase and Rubicon knockdown were equally effective in abolishing ROS biosensor activation. However, despite that both factors are required for LAP, depleting NADPH oxidase almost completely abolished GFP-Lc3-*Salmonella* associations, whereas GFP-Lc3-*Salmonella* associations were reduced but not absent under Rubicon knockdown conditions. Since Rubicon is known to function as an inhibitor of canonical autophagy and an inducer of LAP, it is possible that depletion of Rubicon increases the escape of *Salmonella* from phagosomes and the subsequent targeting of cytosolic bacteria by xenophagy.

In conclusion, our study provides evidence that LAP serves as a host protective mechanism of macrophages limiting systemic infection with *Salmonella*. Together with the fact that LAP has also been shown to protect against *Aspergillus* and *Listeria* infections (Martinez et al., 2015), this war-

rants further investigation into the development of therapeutic strategies promoting LAP as a host-directed approach to infectious disease treatment.

Materials and methods

Zebrafish lines and maintenance

Zebrafish adults and embryos were handled in compliance with local animal welfare regulations and maintained according to standard protocols (zfin.org). Breeding of zebrafish adults was approved by the local animal welfare committee (DEC) of the University of the Leiden, under license number 10612 and in compliance with international guidelines specified by the EU Animal Protective Directive 2010/63/EU. All studies in this work was performed on embryos/larvae before the free feeding stage, no adult fish were sacrificed, and experiments did not fall under animal experimentation law according to the EU Animal Protection Directive 2010/63/EU.

Fish lines used for the present work were wild-type (wt) strain AB/TL, transgenic lines *Tg(CMV:GFP-map1lc3b)* (He et al., 2009), and *Tg(mpeg1::mcherry-F)*^{umsF001} (Bernut et al., 2014). Embryos from adult fish were obtained by natural spawning and were kept at 28.5^{o}C in egg water (60 µg/ml sea salt, Sera Marin, Heinsberg, Germany). PTU (1-phenyl-2thiourea; Sigma Aldrich, St Louis, MO, USA) was added to egg water at 0.003% to prevent melanization of embryos. For infection delivery and live imaging experiments embryos were anaesthetized in egg water with 0.02% of buffered Tricane (3-aminobenzoic acid ethyl ester; Sigma Aldrich).

Bacterial cultures and infection experiments

Salmonella Typhimurium strains used for this study included wild type (wt) strain SL1344 (Hoiseth & Stocker, 1981) constitutively expressing *mCherry*, and a ROS biosensor strain (SL1344 *sifBp::mCherry/*p*katGp-gfpOVA)* (Burton et al., 2014) constitutively expressing *mCherry* and expressing an unstable GFP variant (GFP-OVA) under an OxyR-activated promoter upon exposure to ROS from the host. The bacterial strains were plated from -80

stocks over LB agar plates with appropriate antibiotics and were left overnight to grow at 37°C. Before the start of infection experiments colonies from LB agar plates were suspended into phosphate buffered saline (PBS) supplemented with 2% polyvinylpyrrolidone-40 (Sigma Aldrich) to obtain the low dose (200-400 CFU, for survival curves and CFU counts experiments) or high dose (2000-4000 CFU, for imaging experiments). Bacterial inoculum was injected systemically into the caudal vein of the anaesthetized embryos at 2 dpf or the local infections were established with infection delivery under the skin layers of a somite in the tail region. To check the inoculum size, the same dose was spotted onto agar plates, and bacterial counts determined following overnight incubation. After infection, embryos were kept individually in egg water in 48-well plates to score survival during larval development up to 5 dpf and to collect individuals for CFU counts at 24 h intervals.

Determination of *in vivo* bacterial (CFU) counts

Five embryos/larvae per time point were sacrificed and homogenized in PBS using the Bullet Blender Tissue Homogenizer (Next Advance Inc.). Homogenates were then serially diluted, and three technical replicates for each embryo/larva were plated on LB solid media with the appropriate antibiotics for *S*. Typhimurium. To determine the CFUs, the resulting colonies were counted manually after 24 h incubation at 37°C.

Morpholino knockdowns, RT-PCR and pharmacological verification

Morpholino oligonucleotides (Gene Tools) were diluted in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6) to obtain the required concentrations. 1 nl volume of morpholino was injected into the yolk of 0 hours post fertilization (hpf) zebrafish embryos with microneedles and a Femtojet injector (Eppendorf) paired with a stereo-microscope (Leica). Morpholino sequences and concentrations used are shown in **supplementary Table 1**. As control 1 nl of the standard control morpholino by Gene Tools was injected in same concentration as the other morpholinos.

Rubicon knockdown was achieved with *rubcn* start morpholino (MO1-rubcn) and *rubcn* splice morpholino (MO2-rubcn). Knockdown of Rubicon

with MO2-rubcn was verified by RT-PCR with a pair of primers flanking the e6i6 boundary, Forward: TCTTATCAGCGCAGCTCAAAC and Reverse: GTGAAAATGGACCACAGCTCTT at several developmental stages up to 120 hpf **(supplementary Figure S3)**.

Morpholino knockdown of *atg13* was verified with functional analysis by incubation with Bafilomycin A1 (B1793-10UG, Sigma Aldrich). Control and *atg13* knockdown embryos (n=24) were bath treated with 100 μM bafilomycin in egg water for 12 hours starting at 48 hpf. In parallel, control and *atg13* knockdown embryos were bathed only in egg water (without bafilomycin) as negative control groups of the treatment. Embryos from all groups were quickly washed with egg water and were immediately fixed in 4 % PFA for 24 hours and imaged afterwards using confocal microscopy **(supplementary Figure S2)**.

Whole mount immunohistochemistry

Identification of macrophages and neutrophils was facilitated by immune-labelling with leukocyte specific anti-L-plastin antibody paired with secondary antibody staining with Alexa Fluor 405, combined with neutrophil specific Tyramide Signal Amplification (TSA) paired with Cyanine-5 (PerkinElmer Inc., Waltham, MA, USA). Embryos were fixed at 4 hpi in 4% paraformaldehyde (PFA) in PBS-TX (1X PBS supplemented with 0.8 % of Triton X-100; Sigma Aldrich) overnight and proceeded for L-plastin antibody staining and TSA as previously described (Cui et al., 2011).

Imaging and image analysis

Stereo Fluorescence images were acquired with an MZ16FA microscope (Leica) equipped with a DFC420C digital color camera. Confocal laser scanning images and time lapse movies were acquired using a 63x water immersion objective (NA 1.2) with a Leica TCS SPE system (micrographs in **Figures, 2, 5, 6, S2**, **supplementary Movies M1 & M2)** or a 63x oil immersion objective (NA xx) Zeiss Observer 6.5.32 (micrographs in **Figures 3&4**).

For live imaging and recording of time lapses, embryos were mounted in 1% low-melting-point agarose (SphaeroQ, Burgos, Spain) and in the case of fixed sample imaging, embryos were fixed in 4% PFA and washed with

PBS before image acquisitions. GFP-Lc3-*Salmonella* associations were determined by imaging the region of the tail just behind the yolk extension. Images were analyzed through Z-stacks in Leica LAS AF Lite software and bacterial clusters were observed and manually counted in the overlay channel. Max projections in the overlay channels were used for representative images. For images acquired under the Zeiss Observer, the Z-stack projection function of Fiji software at maximum intensity was used to make composite images of all channels acquired.

For quantification of GFP-Lc3 recruitment within infected phagocytes, for each embryo, the total number of observable phagocytes were manually counted through the Z-stacks of the acquired confocal image. Phagocytes were identified in the yolk sac circulation valley by bacterial clusters in the *mCherry* channel and cellular boundaries of phagocytes were determined in the light transmission channel. Among these total observable infected phagocytes, the numbers of cells with GFP-Lc3 signal in association with *Salmonella* bacteria were counted and the percentage of Lc3-positive phagocytes over the total observable phagocytes was determined for each embryo. The same approach was used to quantify the percentage of phagocytes showing activation of the *Salmonella* ROS biosensor strain.

For bafilomycin treatment experiments, embryos from all four groups were washed with 1XPBS-Tx and imaged in the caudal fin region using a 63x water immersion objective (NA 1.2) with a Leica TCS SPE system. Five embryos per group were imaged in three separate replicates and GFP-Lc3 signal was quantified as fluorescent pixels with a described pixel count program (Cui et al., 2011; Stoop et al., 2011) for five embryos per group in three replicates.

For transmission electron microscopy embryos were fixed in 2% glutaraldehyde (Polysciences) and 2 % paraformaldehyde in sodium cacodylate buffer pH 7.2 (Agar scientific) at 4 hpi for 3 hours at room temperature followed by fixation at 4^{o}C. Post-fixation was performed in 1 % osmium tetraoxide (SPI supplies) in sodium cacodylate buffer for 1 h at room temperature. After dehydration through a graded series of ethanol, specimens were kept in epoxy resin (Agar scientific, AGR 1043) for 16 h before embedding. Ultrathin sections were collected on Formvar coated 200 mesh

or one hole copper grids (Agar scientific, AGS 162) stained with 2 % uranyl acetate (BDH) in 50 % ethanol and 0,2 % lead citrate (BDH) for 10 min each. Electron microscopy images were acquired with a JEOL JEM-1010 (Tokyo-Japan) transmission electron microscope equipped with an Olympus Megaview camera (Tokyo, Japan). Transmission electron micrographs were viewed and cropped in Fiji software.

Statistics

All data sets were analyzed with Prism 7 software. Survival curves were analyzed with Log rank (Mantel-Cox) test. For CFU counts, one way ANOVA was performed on Log transformed data and was corrected for multiple comparisons using Sidak's multiple comparisons test when required. Percentage GFP-Lc3-positive biosensor-positive phagocyte quantifications and pixel count means were analyzed for significance with unpaired parametric t-test between two groups, and for multiple groups the one way ANOVA test was performed and corrected for multiple comparisons.

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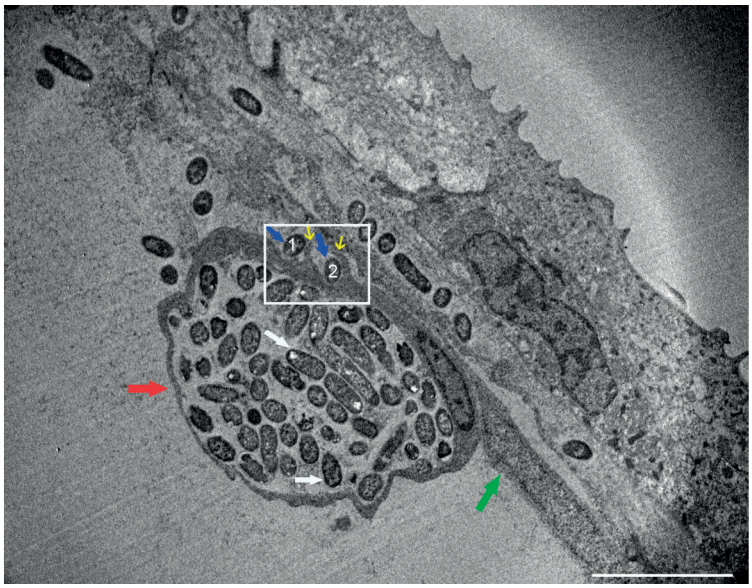
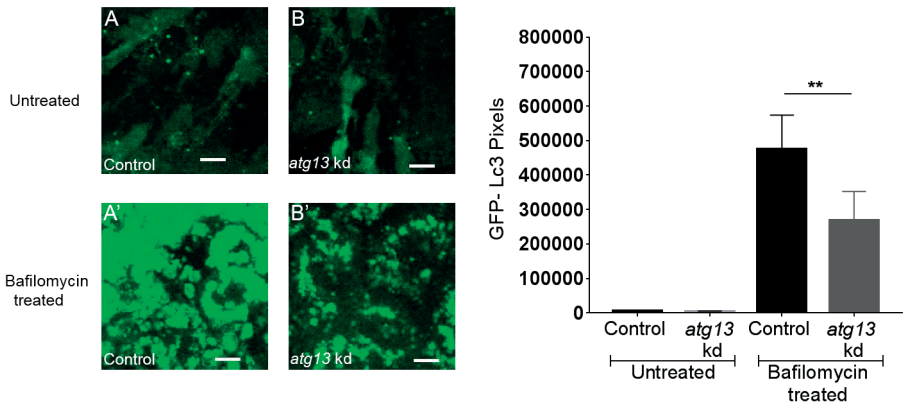
Supplementary material

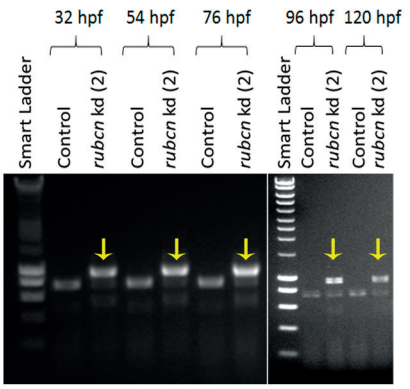


Supplementary Figure S1: Macrophage with phagocytosed *Salmonella* cells. Macrophage (red arrow) containing a large number of phagocytosed Salmonella cells (white arrows indicate two examples) and forming membrane ruffles (yellow arrows) as involved in phagocytosing extracellular Salmonella cells (1 & 2) while attached to the sub-dermal layer (green arrow). The white rectangle outlines the area magnified and shown in Figure2 C'. Scale bar: 5 µm.



Supplementary Figure S2: Functional verification of *atg13* knockdown. *(figure legend on next page).*

Supplementary Figure S2: Functional verification of *atg13* knockdown. *(figure on previous page).* **A,A':** Representative micrographs of the tail fin region of control GFP-Lc3 embryos (60 hpf) without (A) or after (A') bafilomycin treatment. **B,B'**: Representative micrographs of the tail fin region of *atg13* knockdown GFP-Lc3 embryos (60 hpf) without (B) or after (B') bafilomycin A1 treatment. **C**: Quantification of GFP-Lc3 signal with pixel count analysis for control and *atg13* knockdown with and without bafilomycin treatment (n= 5 embryos per group). Scale bars: 5 µm. P**< 0.01.



Supplementary figure S3: RT-PCR verification of *rubcn* splice morpholino efficacy. Electrophoresis gel scan of reverse transcription polymerase chain reaction (RT-PCR) products for control and *rubcn* knockdown groups used for determining splice morpholino **(MO-2 *rubcn*, supplementary Table 1)** efficacy at different developmental stages up to 120 hpf. Yellow arrows point to the shift of PCR product size confirming MO2- *rubcn* efficacy.

Supplementary movies M1 & M2: Time lapse recordings of GFP-Lc3 association dynamics to live and heat-killed S. Typhimurium.
Time lapses of the tail region of GFP-Lc3 embryos intravenously injected with live (S1) or heat-killed (S2) *S.* Typhimurium. Recordings were taken around 4 hpi and are shown 1 frame per second. Bright signals of GFP-Lc3 associations can be observed with live (M1) but not with heat-killed (M2) bacterial cells in the form of puncta and rings inside infected, motile phagocytic cells patrolling the region of focus. The URL to access the time lapses movies is: https://www.dropbox.com/sh/riiq2qrzw5eeo27/AAAXGB4E8gWUReI9JYzikl9la?dl=0

Supplementary Table 1: Morpholino sequences used in this study.

Gene	Name	Location	Sequence	Concentration used	Reference
irf8	MO1-*irf8*	Splice	AATGTTTCGCTTACTTTGAAAATGG	1 mM	Li et al., 2011
atg5	MO1-atg5	start	CATCCTTGTCATCTGCCATTATCAT	0.5 mM	Hu et al., 2011
atg13	MO1-atg13	start	GGCTCAGATCACTATCCATTGTCGC	1 mM	Musso et al., 2014
rubcn	MO1-*rubcn*	start	ATCTTGATCCTCAGGTAATGCAGGT	1 mM	This study
rubcn	MO2-*rubcn*	Splice (Exon 6 > intron 6)	CGCTGTGAAATCTGCTGACCTGAGC	0.25 mM	This study
cyba	MO1-cyba	Splice	ATCATAGCATGTAAGGATACATCCC	1 mM	Niethammer et al., 2009

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Chapter 4

Increased virulence of *Salmonella* mutants in Rubicon-deficient zebrafish

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(Manuscript in preparation)

Abstract

Intracellular pathogens such as *Salmonella* depend on their molecular virulence factors to evade host defense responses like autophagy. Using a zebrafish systemic infection model, we have previously shown that macrophages target *Salmonella* Typhimurium by an autophagy-related pathway known as Lc3-associated phagocytosis (LAP), which is dependent on the host protein Rubicon. Here, we explore the influence of *Salmonella* virulence factors on pathogenicity in the zebrafish model and induction of LAP as a defense response. We investigated five mutant strains, all of which triggered GFP-Lc3 recruitment as puncta or rings around single bacteria or bacterial clusters in a Rubicon-dependent manner. We found that *S.* Typhimurium strains carrying mutations in PhoP or PurA, responsible for adaptation to the intracellular environment and efficient metabolism of purines respectively, displayed attenuated virulence in the zebrafish model. However, both strains showed increased virulence when LAP was inhibited by knockdown of Rubicon. Mutations in type III secretion systems 1 and 2 factors, SipB and SsrB, which are important for invasion and replication in non-phagocytic cells, did not affect the ability to establish successful infection in the zebrafish model. This observation is in line with our previous characterisation of this infection model revealing that macrophages actively phagocytose the majority of *S*. Typhimurium. In contrast to SipB mutants, SsrB mutants were unable to become more virulent in Rubicon-deficient hosts, suggesting that type III system 2 effectors are important for intracellular replication of *Salmonella* in the absence of LAP. Finally, we found that mutation of FlhD, required for production of flagella, rendered *S.* Typhimurium hypervirulent both in wild type zebrafish embryos and in Rubicon-deficient hosts. FlhD mutation also led to lower levels of GFP-Lc3 recruitment compared with the wild type strain, indicating that recognition of flagellin by the host innate immune system promotes the LAP response. Together, our results provide new evidence that the Rubicon-dependent LAP process is an important defense mechanism of macrophages against *S*. Typhimurium.

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the most common causes of foodborne gastroenteritis in humans, claiming more than 150,000 lives each year (Majowicz et al., 2010). Although *S.* Typhimurium infections generally resolve without treatment, immunocompromised patients can develop serious complications, such as bacteremia and other forms of systemic infection. *S.* Typhimurium also causes a systemic infection in mice that resembles typhoid fever, as caused in humans by another *S.* enterica serovar, *S.* Typhi. *S.* Typhimurium and *S.* Typhi are facultative intracellular pathogens that are able to replicate in both phagocytic and non-phagocytic cells. To this end, these pathogens employ a broad range of virulence strategies that mediate host cell invasion, growth in the intracellular environment, and subversion of the host cell's microbicidal mechanisms. These virulence strategies depend for a major part on effector proteins translocated by two type III secretion systems (T3SSs), T3SS1 and T3SS2, encoded by *Salmonella* pathogenicity island (SPI), SPI1 and SPI2 respectively (Ibarra & Steele-Mortimer, 2009). Other factors, such as flagellar motility and the ability to make structural and metabolic adaptations to its environment, also play important roles in *Salmonella* virulence.

S. Typhimurium accomplishes active invasion into non-phagocytic cells via the T3SS1 secretion system, which translocates a limited number of tightly regulated effector proteins (Myeni et al., 2013) that collectively induce dramatic changes to the host cell cytoskeleton leading to membrane ruffling and ultimately resulting in bacterial internalization (McGhie et al., 2009). The delivery of these SPI1 effectors is dependent on translocator proteins like SipB and SipC, which are also encoded in the SPI1 region (Myeni et al., 2013). In contrast, professional phagocytes such as macrophages recognize *S*. Typhimurium-related pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), and utilize their phagocytic abilities for direct uptake of the bacterial cells. This recognition and phagocytosis initiates a number of host signalling responses in phagocytes including a bactericidal oxidative burst, cytokine production, and the activation of autophagy (Dupre-Crochet et al., 2013; Huang et al., 2009; Levine et al., 2011).

Once internalized, *S.* Typhimurium survives by avoiding anti-bacterial host responses and replicates intracellularly inside a modified phagosome, the *Salmonella*-containing vacuole (SCV). The function of the T3SS2 secretion system, translocating SPI2 effector proteins such as SifA, SseF and SseG, is important for the maturation and maintenance of this intracellular niche (Hensel et al., 1998; Kuhle & Hensel, 2002). Expression of the T3SS2 is activated by the two-component system SsrA-SsrB (Walthers et al., 2007). Other factors that play a role in *S.* Typhimurium pathogenesis include flagellar-based motility, which increases invasiveness of *Salmonella* (Schmitt et al., 2001). On the other hand, recognition of flagellin by host PPRs induces the innate immune response (Franchi et al., 2006; Miao et al., 2006). *S.* Typhimurium highly depends on a number of metabolic products to survive intracellularly and cause invasiveness. One such factor is PurA (succinyl AMP synthase), which functions in purine metabolism by converting IMP to AMP (Benson & Gots, 1976; McFarland & Stocker, 1987). PurA mutants grow poorly in vivo and are non-virulent in mice (O'Callaghan et al., 1998). Additionally, *S.* Typhimurium is highly dependent on the PhoP/PhoQ two-component system to survive inside professional phagocytes (Miller et al., 1989; Bijlsma & Groisman, 2005). The PhoP/PhoQ system detects acidification of the phagosome and presence of cationic antimicrobial peptides, leading to essential adaptations of the protein and lipid contents of the outer membrane (Dalebroux & Miller, 2014).

Recently, autophagy has emerged as an important host defense mechanism against *S.* Typhimurium and other intracellular pathogens. Autophagy is a cellular degradative pathway that can target bacterial invaders in a similar manner as it destructs defective organelles or protein aggregates (Huang & Brumell, 2014; Levine et al., 2011). A number of studies in epithelial cells have shown that *Salmonella* bacteria escaping from the SCV into the cytosol are ubiquitinated, recognized by ubiquitin receptors, enclosed in a double membrane autophagosome, and degraded following the fusion with lysosomes (Birmingham et al., 2006 ; Cemma et al., 2011; Huang et al., 2009; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Wild et al., 2011; Zheng et al., 2009). This selective, ubiquitin-dependent, autophagy mechanism, can also detect damage to the SCV membrane, likely functioning as a repair mechanism to delay cytosolic escape. Alternatively, components of the autophagy

machinery can be recruited directly to phagosomes, in a process that has been termed Lc3-associated phagocytosis (LAP) (Huang & Brumell, 2014; Sanjuan et al., 2007). LAP is initiated by PRRs, including Toll-like receptors (TLRs), and characterized by recruitment of the autophagy component LC3 to the phagosomal membrane and subsequent phagosome fusion with lysosomes, leading to rapid acidification and enhanced bacterial killing (Sanjuan et al., 2007).

A key player in LAP is RUN and cysteine rich domain containing Beclin1 interacting protein (Rubicon). Rubicon is found to regulate autophagy by modulating the activity of the PI3 kinase VPS34 (Zhong et al., 2009). Under normal conditions, Rubicon suppresses autophagy by interacting with the Beclin1-VPS34 complex (Matsunaga et al., 2009). However, upon TLR stimulation, Rubicon interacts with the NADPH oxidase complex on phagosomes. It then stabilizes the $p22^{phox}$ and $gp91^{phox}$ components of the NADPH-oxidase, inducing the production of bactericidal reactive oxygen species (ROS) (Yang et al., 2012; Martinez et al., 2015). Thus, Rubicon functions as a molecular switch between canonical autophagy and LAP.

We have recently shown that both Rubicon and NADPH oxidase are required for Lc3 recruitment to *S*. Typhimurium and for control of infection in a systemically challenged zebrafish host **(Chapter 3)**. This led us to conclude that LAP is the major pathway involved in the anti-*Salmonella* autophagy response under conditions where macrophages dominate the defense response. In the present study we investigated which of the *S*. Typhimurium virulence factors, PhoP, PurA, SipB, SsrB, and FlhD, are required for pathogenicity in this systemic infection model, and how these factors affect the LAP response. Our study revealed that PhoP and PurA facilitate *S.* Typhimurium replication in the zebrafish host, while FlhD-regulated expression of the flagellar apparatus benefits host defense. We found LAP to occur as a general response to *S.* Typhimurium internalization, since Rubicon-dependent recruitment of Lc3 was observed in infections with both wild type and mutant strains. With the exception of Δ*ssrB* mutants, all tested strains displayed increased virulence under conditions of Rubicon knockdown, indicating that LAP functions to restrict *S.* Typhimurium growth and suggesting that SPI2 effectors promote *S.* Typhimurium virulence in the absence of LAP.

Results

Virulence of *S.* Typhimurium in the zebrafish model relies on PhoP and PurA factors

S. Typhimurium requires a number of effector proteins for infecting a wide range of cell types, including macrophages (Buckner et al., 2011; LaRock et all., 2015). Mutant strains of *S.* Typhimurium that cannot survive inside macrophages are avirulent (Fields et al., 1986), but the interaction of the macrophage defense machinery with *S.* Typhimurium is not well understood (Gog et al., 2012). PhoP, which is part of the PhoP/Q two component regulatory system, has been shown to be an essential virulence factor as bacteria containing an inactive PhoP are defective in intracellular survival in murine macrophages (Miller et al., 1989). We previously showed that *S.* Typhimurium infection in zebrafish embryos/larvae predominantly resides inside macrophages **(Chapter 3)**, providing an in vivo context to determine the importance of PhoP for establishing systemic infection. To this end, we challenged 2 days post fertilization (2 dpf) embryos either with a PhoP-deficient *S.* Typhimurium mutant strain or its wild type counterpart (SL1344) **(Figure 1A)**. We evaluated the relative virulence of both strains on the basis of survival of infected embryos following intravenous injection of 200-400 colony forming units (CFU) and monitored bacterial burdens in infected hosts. We observed that Δ*phoP*-infected larvae showed higher survival rates as compared to individuals infected with the wild type strain **(Figure 1B)**. Similarly, the retrieved bacterial counts for hosts infected with the Δ*phoP* strain were significantly lower at 24 hours post infection (24 hpi) as compared to wild type bacteria **(Figure 1C)**. These results confirm that PhoP is required for *S.* Typhimurium infection establishment and virulence in the zebrafish host.

Another important factor for *S.* Typhimurium virulence in *in vitro* (Grant et al., 2012) and in *in vivo* models (Benson & Gots, 1976; O'Callaghan et al., 1988) is PurA, which is required by *S*. Typhimurium for metabolic adaptation to the host environment (McFarland & Stocker, 1987). We found that a PurA-deficient *S.* Typhimurium (Δ*purA*) strain was avirulent to zebrafish larvae and failed to cause mortalities in comparison to the wild type strain **(Figure 1D)**. Similarly, the Δ*purA* strain could not establish a successful infection as depicted by significantly lower numbers of CFU counts at 24

hpi in Δ*purA* infected larvae when compared to larvae infected with the wild type strain **(Figure 1 E)**.

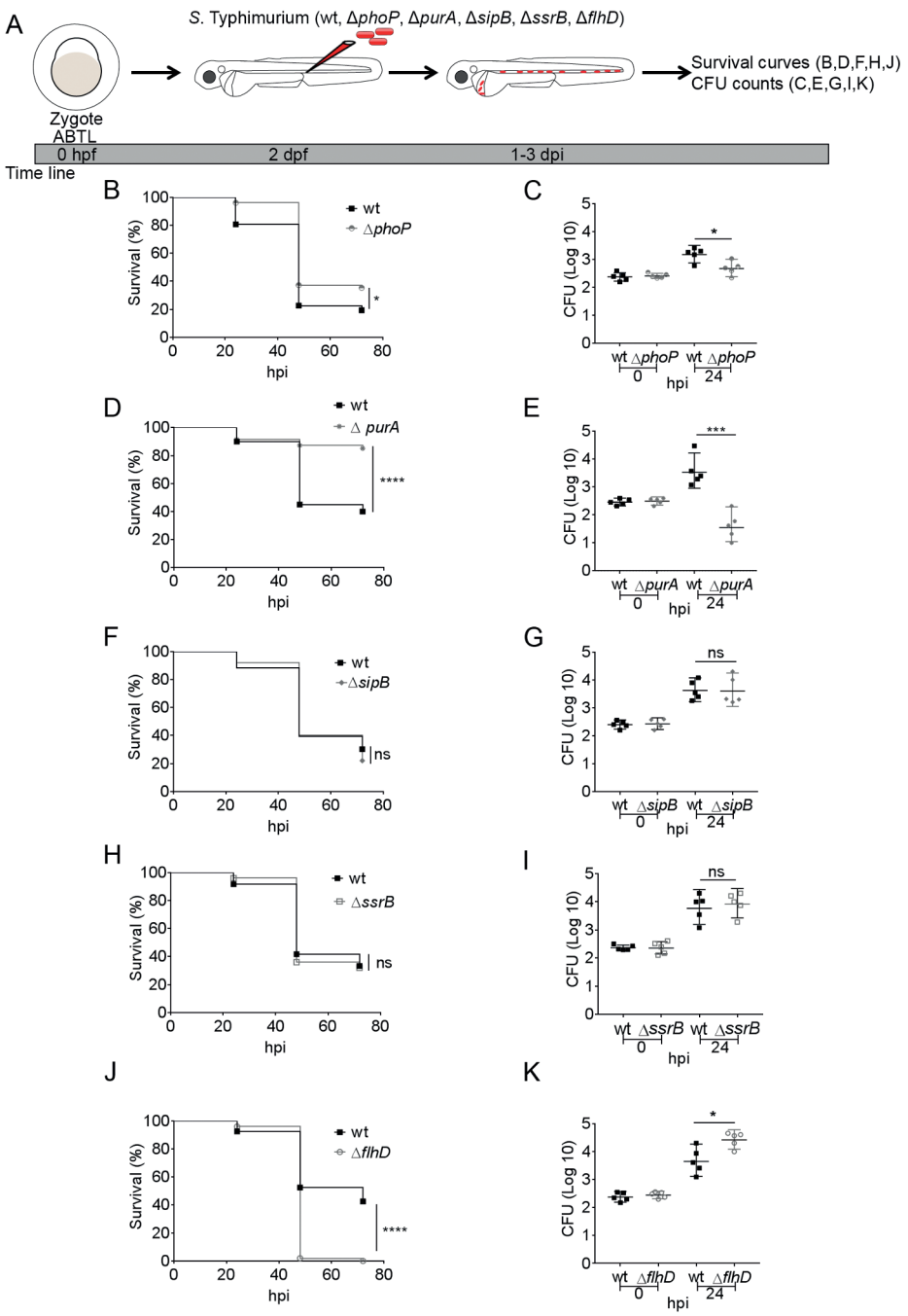

Figure 1: Comparison of virulence and infection progression of *S.* Typhimurium mutants. *(legend on next page).*

Figure 1: Comparison of virulence and infection progression of *S*. Typhimurium mutants. *(figure on previous page).* **A:** Work flow for experiments followed in B-K, displayed along the time line of zebrafish development. **B,D,F,H,J:** Survival curves for systemically challenged zebrafish with *S*. Typhimurium mutant strains, Δ*phoP* (B), Δ*purA* (D), Δ*sipB* (F), Δ*ssrB* (H) and Δ*flhD* (J) with their respective control groups injected with wild type strain SL1344. One representative of three replicates is shown (n= 50 embryos per group). **C,E,G,I,K:** Representative CFU counts of the infections with *S*. Typhimurium mutant strains, Δ*phoP* (C), Δ*purA* (E), Δ*sipB* (G), Δ*ssrB* (I) and Δ*flhD* (K) with their respective control groups injected with wild type strain SL1344 at 24 hpi. One representative of three replicates is shown, where five embryos per time point were used and the log transformed CFU data are shown with the geometric mean per time point. Error bars represent SD. ****P< 0.0001, ***P<0.001, *P<0.05, ns= non-significant.

SPI1 and SPI2 factors, SipB and SsrB, are dispensible for establishing systemic infection in zebrafish embryos

S. Typhimurium depends on active invasion and formation of the SCV to successfully infect and survive inside non-phagocytic host cells (LaRock et al., 2015). However, in our model, active invasion by *S*. Typhimurium is not required due to swift phagocytosis of the bacteria by macrophages and neutrophils following injection into the bloodstream of the zebrafish host. We therefore investigated the hypothesis that SPI1 effectors, required for active invasion, would be dispensable for virulence in this model. To this end, we infected zebrafish embryos with a SipB-deficient *S*. Typhimurium strain, which fails to produce the translocation apparatus for delivery of SPI1 effectors, resulting in severe inability to cause infections in non-phagocytic cells (Kaniga et al., 1995; Myeni et al., 2013). The Δ*sipB* strain and its isogenic wild type strain caused similar mortalities to zebrafish larvae **(Figure 1F)**. Moreover, the *sipB* mutant *S.* Typhimurium was equally virulent as the wild type strain, as CFU counts at 24 hpi for Δ*sipB* and wild type did not differ from each other **(Figure 1G)**.

In order to determine the requirement for SPI2 effectors in our model, we used an SsrB-deficient strain. SsrB is a part of the two component regulatory system SsrA/B and is required for expression of most of the SPI2 proteins, required for maintenance of the SCV (Walthers et al., 2007). We found no significant difference in survival rates **(Figure 1H)** and in CFU

counts **(Figure 1I)** between the groups injected with Δ*ssrB* or wild type *S.* Typhimurium strains. Collectively, our results suggest that both SipB and SsrB are less relevant factors for pathogenicity of *S*. Typhimurium in the zebrafish-*Salmonella* infection model, presumably due to no or little requirement of active bacterial invasion into macrophages.

Mutation of the flagellin transcriptional regulator FlhD increases virulence of *S*. Typhimurium in the systemically infected zebrafish host

FlhD is a part of the *flhDC* master operon responsible for initiating the flagellum production. Mutations in FlhD factor render *S*. Typhimurium immotile due to loss of flagellin (a main component of the bacterial flagellum) synthesis. Flagellin positively and negatively affects *Salmonella* virulence as *Salmonella* requires flagellum-based motility for optimal invasion of the host cells (Ibarra et al., 2010), but on the other hand flagellin is a ligand for TLR pathway detection by the host, activating the innate immune response (Hayashi et al., 2001). In fact, flagellin has already been shown to initiate an immune cascade via TLR5 recognition in zebrafish (Stockhammer et al., 2009). In order to address the role of the flagellum as either a host or bacterial beneficial factor, we infected zebrafish larvae with the Δ*flhD* strain or its wild type counterpart and determined the virulence in terms of CFU counts and survival curves, as above. This revealed that infection with the Δ*flhD* strain leads to reduced host survival **(Figure 1J)** and increased bacterial proliferation **(Figure 1K)** when compared to infection with the wild type strain. These results suggest that flagellin detection by the innate immune system restricts *S.* Typhimurium growth in the zebrafish infection model.

***S.* Typhimurium virulence factor mutants elicit GFP-Lc3 recruitment in similar patterns as the wild type strain**

We recently identified a host protective role of an autophagy-related process known as Lc3-associated phagocytosis (LAP) during infection of zebrafish embryos with *S.* Typhimurium **(Chapter 3)**. In this work, we determined whether LAP also targets *S.* Typhimurium with non-functional PhoP, PurA, SipB, SsrB, and FlhD factors. To visualize the autophagy response we injected a high dose (2000-4000 CFU) of each bacterial mutant strain or the wild type strain into the *Tg(CMV:GFP-map1lc3b)* transgenic zebrafish

line, which expresses a GFP-fusion of the autophagy marker Lc3 **(Figure 2A)**. Evaluation of the autophagy response at 4 hpi showed that GFP-Lc3 associated with *S*. Typhimurium independent of the different virulence factors tested **(Figure 2B-G)**. The GFP-Lc3 localization patterns observed in response to infections with the mutant *S.* Typhimurium strains were similar to those observed with the wild type strain **(Chapter 3 and Figure $2B_{1-4}$)**, as in all cases the autophagy signal appeared either in the form of GFP-Lc3 puncta associated with single bacterial cells or bacterial clusters, or as GFP-Lc3 rings around the bacterial cells or clusters **(Figure 2B-G**). However, differences were observed in the level of GFP-Lc3 recruitment between the strains **(Figure 2H)**. Infection with Δ*phoP* and Δ*SipB* strains elicited slightly higher levels of GFP-Lc3 recruitment, whereas GFP-Lc3 recruitment was reduced in infections with the Δ*FlhD* and Δ*purA* strains **(Figure 2H)**. These differences in GFP-Lc3 recruitment did not correlate with the virulence of the mutants, since Δ*phoP* and Δ*purA* were attenuated and Δ*flhD* was more virulent in the zebrafish model **(Figure 1)**.

Figure 2: Host LAP response to *S.* Typhimurium mutants. *(figure on next page).* **A:** Work flow and time line of experiments followed in B-H. **B-G:** Representative confocal micrographs of GFP-Lc3 positive infected phagocytes with *mCherry* labelled *S.* Typhimurium mutant strains wild type (B), Δ*phoP* (C), Δ*purA* (D), Δ*sipB* (E), Δ*ssrB* (F) and Δ*flhD* (G). Yellow arrows indicate GFP-Lc3 associations with bacterial cells while numbers 1-4 represent four different examples of Lc3 association patterns for each *S.* Typhimurium mutant strain. At the top of each column of images is a symbolic representation of the pattern of GFP-Lc3 and bacterial cell association as reported earlier **(Chapter 3)**. **H:** Quantification of GFP-Lc3-*Salmonella* association for *S.* Typhimurium mutant strains Δ*phoP*, Δ*purA*, Δ*sipB,* Δ*ssrB* and Δ*flhD* along with wild type SL1344 at 4 hpi. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive (Lc3+ve) over the total were averaged from five embryos per group. Error bars represent the SD. One of the two replicates is shown. Scale bars B-G= 5 μm, ****P< 0.0001, **P< 0.01, *P<0.05, ns= non-significant.

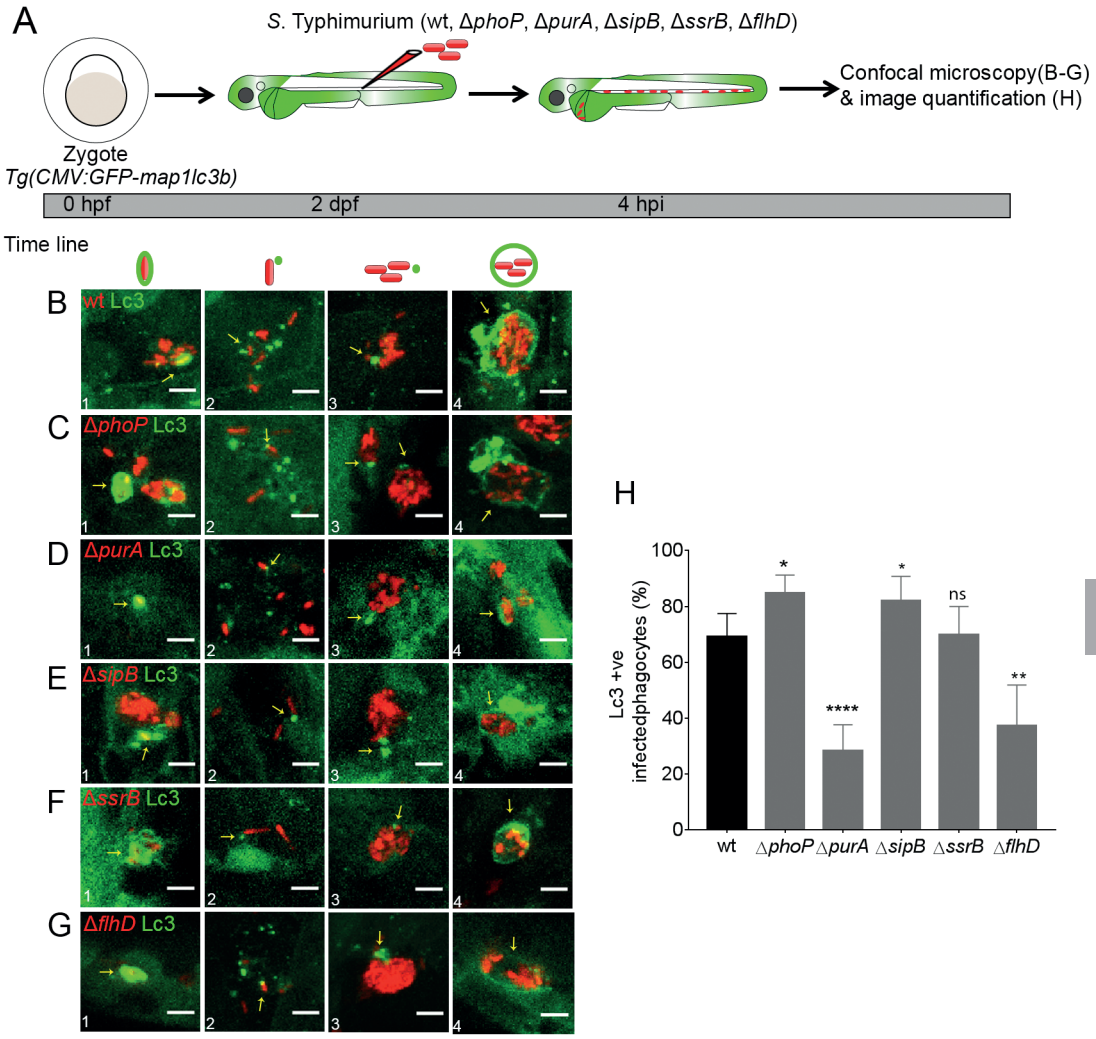

Figure 2: Host LAP response to *S.* Typhimurium mutants. *(figure legend on previous page).*

S. Typhimurium virulence factor mutants are targeted by the Rubicon-dependent LAP pathway

In order to determine if the GFP-Lc3 response to the different *S.* Typhimurium mutant strains can be classified as LAP, we tested for dependency on the host protein Rubicon, which is essential for LAP but not for

canonical autophagy (Martinez et al., 2015). Depletion of Rubicon using a previously described splice blocking morpholino oligonucleotide **(Chapter 3)** resulted in significantly reduced levels of GFP-Lc3 recruitment in response to the Δ*phoP,* Δ*purA,* Δ*sipB* Δ*ssrB,* and Δ*flhD* strains **(Figure 3)**, in agreement with previous evidence for the role of LAP during infection with wild type *S.* Typhimurium **(Chapter 3)**. The results of this experiment confirmed that the *S.* Typhimurium mutants elicited GFP-Lc3 recruitment to different extents. In case of the Δ*purA,* ΔssrB and Δ*flhD* mutants, which elicited lower levels of GFP-Lc3 recruitment **(Figure 2H)**, GFP-Lc3 association was almost completely abolished under conditions of Rubicon knockdown **(Figure 3D,E, H,I and J,K)**. In contrast, during infections with the Δ*phoP* and Δ*sipB* strains, which elicited higher levels of GFP-Lc3-recruitment **(Figure 2H)**, Rubicon-deficient hosts displayed residual levels of GFP-Lc3-*Salmonella* association **(Figure 3 B,C and F,G)**, suggesting that canonical autophagy might target these strains when LAP is inhibited. Together, these results extend our previous findings and strengthen our conclusion that LAP is the main autophagy-related host defense response of macrophages targeting *S.* Typhimurium during systemic infection in zebrafish larvae.

Figure 3: Host LAP response to *S.* Typhimurium mutants in Rubicon-depleted embryos. *(figure on next page)***. A:** Work flow and time line of experiments in B-K. **B,D,F,H,J:** Representative confocal micrographs of GFP-Lc3 positive infected phagocytes with *mCherry*-labelled *S.* Typhimurium mutant strains Δ*phoP* (B), Δ*purA* (D), Δ*sipB* (F), Δ*ssrB* (H), and Δ*flhD* (J) in Rubicon-depleted hosts (B',D',F',H',J') along with respective controls (B,D,F,H,J). **C,E,G,I,K:** Quantification of GFP-Lc3-*Salmonella* association for *S.* Typhimurium mutant strains Δ*phoP* (C), Δ*purA* (E), Δ*sipB* (G), Δ*ssrB* (I), and Δ*flhD* (K) in Rubicon-depleted larvae and their respective controls. Numbers of infected phagocytes positive or negative for GFP-Lc3-*Salmonella* associations were counted from confocal images and the percentages of Lc3-positive over the total were averaged from five embryos per group. Error bars represent the SD. One of the two replicates is shown. Scale bars (B,D,F,H,J)= 10 μm, ****P< 0.0001, ***P< 0.001, **P< 0.01, *P<0.05.

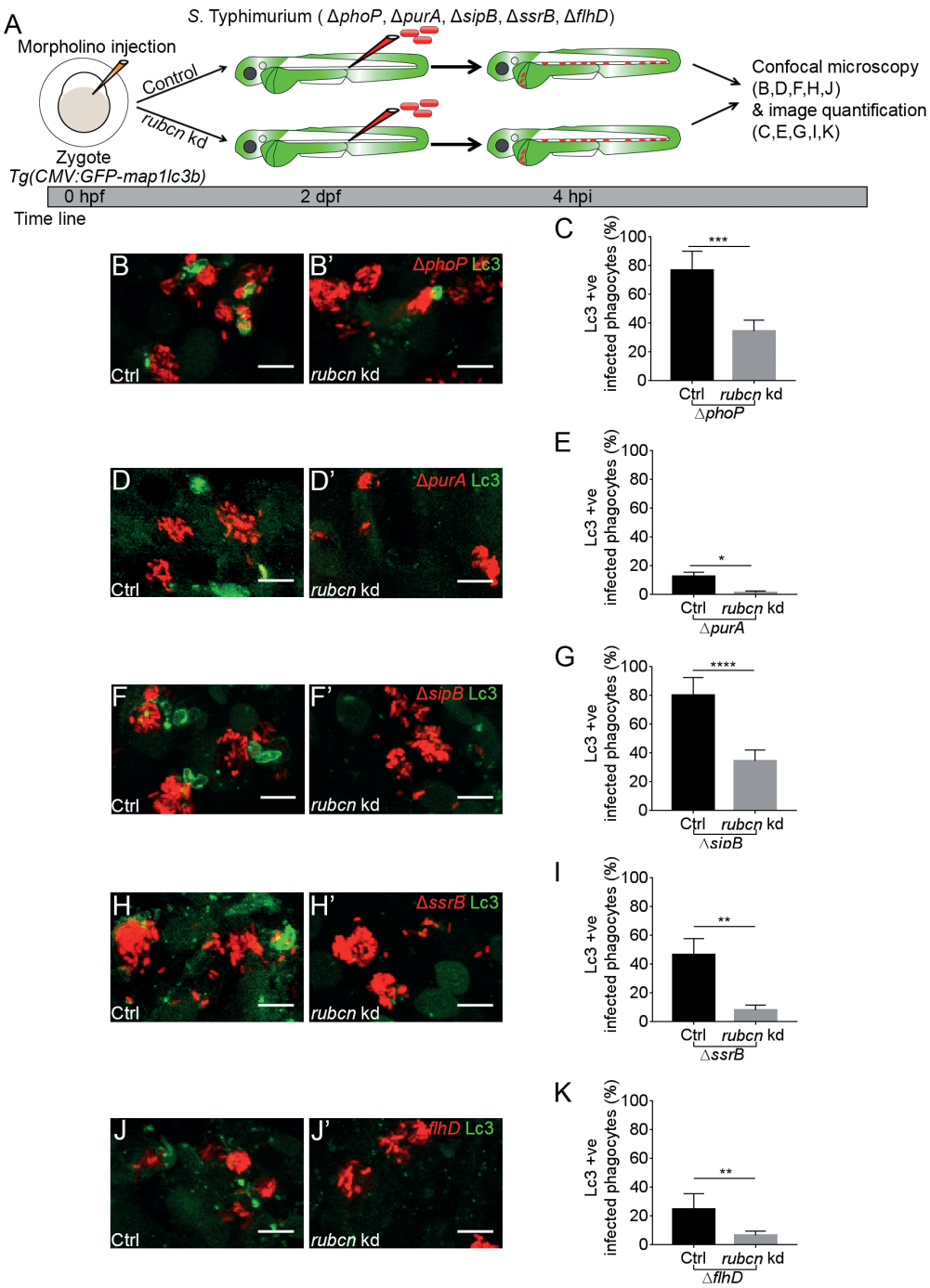

Figure 3: Host LAP response to *S.* Typhimurium mutants in Rubicon-depleted host. *(legend on previous page).*

S. Typhimurium mutants show increased virulence in a LAP-deficient host with the exception of Δ*ssrB*

Our previous results showed that LAP-deficient zebrafish embryos are more susceptible to wild type *S*. Typhimurium infection **(Chapter 3)**. To expand on this finding, we investigated the importance of LAP in defense against the *S*. Typhimurium mutant strains. In order to inhibit LAP we knocked down host Rubicon with the splice blocking morpholino approach and assessed the effect on host survival and bacterial burden **(Figure 4A)**. We found that infections with Δ*phoP*, Δ*purA*, Δ*sipB*, and Δ*flhD* **(Figure 4B,D,F,J)** resulted in significantly increased mortality of Rubicon-deficient hosts as compared to control embryos. In agreement, the CFU counts for these strains were significantly increased in Rubicon-deficient embryos **(Figure 4C,E,G,K,)**. In contrast, the virulence of the Δ*ssrB* strain and its infection establishment were independent of Rubicon levels of the host, as there were no significant differences between the survival curves of Rubicon-deficient and control larvae **(Figure 4H)** and the CFU counts **(Figure 4I)**. Therefore, we conclude that the Rubicon-dependent LAP response is required to restrict growth of *S.* Typhimurium strains carrying mutations in critical virulence factors and that only a strain deficient in expression of SPI2 factors (Δ*ssrB*) is unable to display increased virulence in a LAP-deficient host.

Figure 4: Virulence and infection progression of *S.* Typhimurium mutants in Rubicon-depleted hosts. *(figure on next page)***. A:** Work flow and time line of experiments followed in B-K. **B,D,F,H,J:** Survival curves for systemically injected *S*. Typhimurium mutant strains, Δ*phoP* (B), Δ*purA* (D), Δ*sipB* (F), Δ*ssrB* (H), and Δ*flhD* (J) in Rubicon-depleted larvae with their respective control groups. One representative of two replicates is shown (n= 50 embryos per group). **C,E,G,I,K:** Representative CFU counts of the infections with *S*. Typhimurium mutant strains, Δ*phoP* (C), Δ*purA* (E), Δ*sipB* (G), Δ*ssrB* (I), and Δ*flhD* (K) in Rubicon-depleted zebrafish with their respective control groups at 24 hpi. One representative of two replicates is shown, where five embryos per time point were used and the log transformed CFU data are shown with the geometric mean per time point. Error bars represent SD. ****P< 0.0001, **P< 0.01, *P<0.05, ns= non-significant.

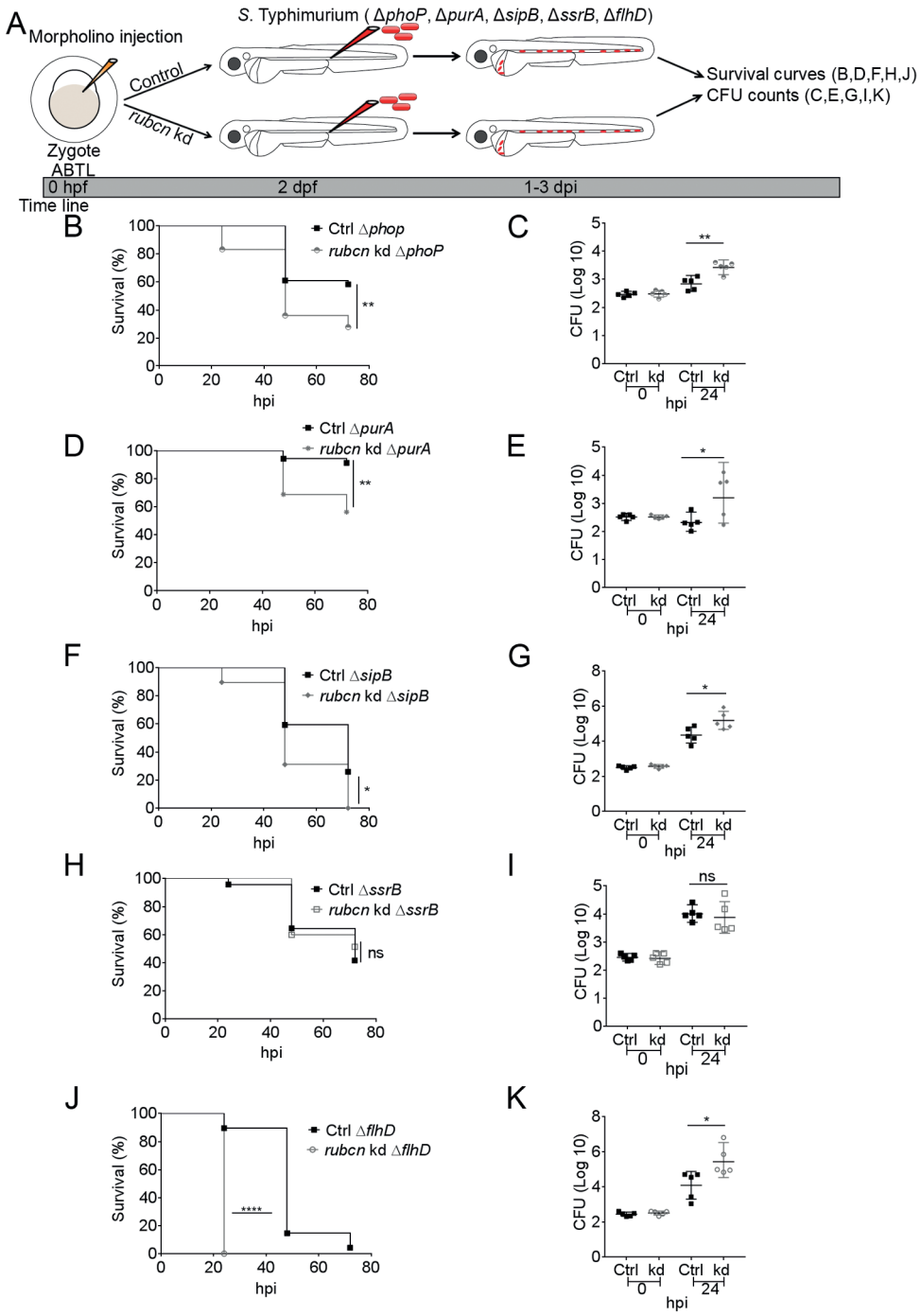

Figure 4: Virulence and infection progression of *S.* Typhimurium mutants in Rubicon-depleted hosts. *(legend on previous page).*

Discussion

The functions of *S.* Typhimurium virulence factors in invasion of host cells and intracellular replication are well described as a result of many years of studies in various cell culture and mouse models (Ibarra & Steele-Mortimer, 2009; van der Heijden & Finlay, 2012). However, how these factors induce or inhibit the host autophagy machinery is only beginning to be understood (Mesquita et al., 2012; Owen et al., 2014; Tattoli et al., 2012a; Tattoli et al., 2012b). Recently, we exploited GFP-Lc3 transgenic zebrafish to study the dynamics of activation of the autophagy machinery *in vivo*. Following intravenous infection in zebrafish embryos, *S.* Typhimurium is mainly contained by macrophages and as such this model mimics the advanced stages of systemic infections in humans. We found that internalization of live *S.* Typhimurium by macrophages in the zebrafish host is associated with rapid induction of an autophagy-related response known as LAP, whereby Lc3 associates with phagosomes in a Rubicon and NADPH oxidase dependent manner and independent of the ULK1 pre-initiation complex **(Chapter 3)**. Here we provide new evidence for the host-protective function of LAP, showing that *S.* Typhimurium strains carrying mutations in virulence factors PhoP, PurA, SipB, SsrB, and FlhD are all able to trigger LAP and that all mutants except the SsrB-deficient strain become more virulent in a LAP-deficient host.

It has previously been shown that lipopolysaccharide mutants are less virulent than wild type *S.* Typhimurium in the zebrafish model (van der Sar et al., 2003). However, the functions of other critical virulence factors had not yet been well characterized. In this study we show the importance of two virulence factors, PhoP and PurA, for *S.* Typhimurium infection in the zebrafish host. The PhoP/Q regulon, which controls outer membrane composition, has been reported to inhibit phagolysosomal fusion and also to enable adaptation to other intramacrophage stresses (Garvis et al., 2001; Thompson et al., 2011). In addition, it has been suggested to decrease immune responses, by reducing TLR activation (Dalebroux & Miller, 2014). Infection with a PhoP-deficient strain led to attenuated infection in the zebrafish model, which is in agreement with studies in murine macrophages and *in vivo* infection in mice (Miller et al., 1989; Miller & Mekalanos, 1990; Thompson et al., 2011), and with the fact that PhoP-deficient *S.* Typhi has been found safe for human vaccination (Hohmann

et al., 1996). We observed an even stronger attenuation in the case of the PurA-deficient strain, which was largely avirulent in the zebrafish host. This observation is consistent with avirulence of PurA-deficient *S.* Typhimurium *in vivo* in mice (O'Callaghan et al., 1988). These results support the validity of the zebrafish model for studying *S.* Typhimurium infection.

When LAP was inhibited by knockdown of Rubicon, both the PhoP-deficient and the PurA-deficient strains induced more severe infections in the zebrafish host. That virulence of attenuated strains can be (partially) restored in the absence of LAP extends our previous finding that LAP is a host-protective response against *S*. Typhimurium **(Chapter 3)**. While both attenuated strains triggered recruitment of GFP-Lc3 in a Rubicon-dependent manner, they had opposite effects on the level of GFP-Lc3 recruitment, which was increased in the infection with PhoP-deficient bacteria and reduced in response to PurA-deficient bacteria. Since we examined only a single time point, this difference might be due to different kinetics in the clearance of bacteria. We have previously found that no GFP-Lc3 response to heat-killed bacteria is present at this time point **(Chapter 3)**. Therefore, the lower levels of GFP-Lc3 in response to Δ*purA S.* Typhimurium could be due to rapid clearance of this largely avirulent strain and failure to induce signals for LAP. The higher levels of GFP-Lc3 targeting the Δ*phoP* mutant could point towards a role for PhoP in LAP evasion, which adds to its other reported functions in escaping host defense mechanisms (Dalebroux & Miller, 2014; Garvis et al., 2001; Miller & Mekalanos, 1990; Thompson et al., 2011). Therefore, the PhoP deficiency might enhance intraphagosomal killing of *S.* Typhimurium in zebrafish macrophages possibly due to higher LAP activity.

SipB-deficient bacteria, defective in the translocation of SPI1 effectors, showed similar virulence as wild type *S.* Typhimurium in the zebrafish model. SPI1 effectors are essential for gaining access to non-phagocytic target cells such as those in the gut epithelium (Watson & Holden 2010), but this invasion step is bypassed in the intravenous infection route used for infecting zebrafish embryos. Therefore, the non-attenuated phenotype of Δ*sipB* infection in zebrafish is consistent with studies in mice showing that virulence of SPI1 mutants is unaffected when the bacteria are delivered intraperitoneally, whilst administration by the oral route affects the ability to establish systemic infection (Galan & Curtiss, 1989).

In macrophages, SPI1 effectors can trigger caspase-1 dependent pyroptosis and SPI1 mutants, including Δ*sipB*, lack the ability to induce this response (Fink & Cookson et al., 2007). We cannot exclude that SipB deficiency might affect macrophage cell death in the zebrafish host. When *Salmonella* bacteria are released from macrophages, they can continue to replicate extracellularly by forming microcolonies on the endothelial cells of the blood vessels (van der Sar et al., 2003). Therefore, it is possible that SipB mutation could have altered the ratio between intra- and extracellular bacteria, without alteration in overall bacterial burden or effect on host survival. Our observation of a small but significant increase in GFP-Lc3 recruitment by Δ*sipB* bacteria compared with the wild strain suggests increased targeting of the mutant strain by LAP, but apparently insufficient to impact on the overall infection development. However, as in the case of Δ*phoP* and Δ*purA* mutants, depletion of Rubicon revealed the host-protective function of LAP during infection with the Δ*sipB* strain.

Impaired SPI2 function, by mutation of SsrB, did not significantly affect GFP-Lc3 recruitment and had no detectable effect on virulence in the zebrafish host. The lack of a virulence phenotype was unexpected, since Δ*ssrB Salmonella* bacteria are attenuated in several *in vitro* and *in vivo* models, including human epithelial cells, murine and porcine macrophages, orally and intravenously infected mice, and intravenously infected pigs (Cirillo et al., 1998; Boyen et al., 2008; Grant et al., 2012). However, a study in mice reported that the main cause of the attenuated phenotype of SPI2 mutants is their inability to leave infected cells, whereas growth of SPI2 mutants inside phagocytes exceeds that of wild type *Salmonella* (Grant et al., 2012). Therefore, the lack of an attenuated phenotype of Δ*ssrB Salmonella* in zebrafish embryos might be explained by the fact that phagocytes are the main replication site in this model. Interestingly, a function for SsrB in the zebrafish model was revealed when we inhibited LAP. We found that Δ*ssrB* bacteria, unlike all other mutants tested, were unable to display increased virulence under conditions of Rubicon knockdown. That only SsrB-competent strains become more virulent in a Rubicon-deficient host might be an indication that SPI2 effector proteins controlled by the SsrA/B system promote *S.* Typhimurium virulence in the absence of LAP.

We found that loss of the FlhD regulator, which deprives *S.* Typhimurium from the formation of flagella, leads to increased virulence in both wild type and Rubicon-deficient zebrafish hosts. These results are in agreement with studies in mice showing that non-flagellated mutants of *Salmonella*, including Δ*flhD*, are equally or more virulent than the wild type strains, despite that these mutants lack the ability to invade epithelial cells (Lockman & Curtiss, 1990; Schmitt et al., 2001). The hypervirulence of Δ*flhD* bacteria can be explained by escape from recognition by TLR5, which is required for activation of the innate immune response to flagellin in mice as well as zebrafish (Fournier et al., 2009; Stockhammer et al., 2009). An additional explanation is the escape from inflammasome activation and interleukin 1 beta secretion (Franchi et al., 2006; Lai et al., 2013; Winter et al., 2009). In agreement with the fact that LAP induction has been shown to depend on TLR signaling (Sanjuan et al., 2007), we observed significantly lower GFP-Lc3 association with Δ*flhD* than with wild type bacteria. Considering the evidence for a host-protective function of LAP provided by our study, we propose that the increased virulence of Δ*flhD S.* Typhimurium resides at least partly in lower activation of LAP through TLR5-mediated signalling.

In conclusion, we have demonstrated attenuated virulence of Δ*phoP* and Δ*purA S.* Typhimurium mutants in the zebrafish systemic infection model in contrast to hypervirulence of the non-flagellated Δ*flhD* mutant. Furthermore, our data support that the Rubicon-dependent LAP pathway plays an important role in host defense, since all tested mutant strains were able to cause more severe infections when LAP was inhibited, except for one strain deficient in the expression of SPI2 effectors. While LAP is the predominant autophagy-related response during the macrophage-dominated infection in zebrafish, selective autophagy mediated by ubiquitin receptors (xenophagy) has been found to restrict growth of *S.* Typhimurium in epithelial cells (Huang & Brumell, 2014). Therefore, there is accumulating evidence for the function of autophagy proteins in host defense against *S*. Typhimurium, encouraging further exploration of autophagy modulating drugs for host-directed therapy of antibiotic resistant *Salmonella* infections.

Materials and methods

Zebrafish lines and maintenance

Zebrafish were handled in compliance with local animal welfare regulations and maintained according to standard protocols (zfin.org). Breeding of zebrafish was approved by the local animal welfare committee (DEC) of the University of the Leiden, under license number 10612. All experiments were performed on embryos/larvae before the free feeding stage and did not fall under animal experimentation law in line with the EU Animal Protection Directive 2010/63/EU.

Fish lines used for the present work were the wild type (wt) strain AB/TL, and the transgenic line *Tg(CMV:GFP-map1lc3b)* (He et al., 2009). Embryos from adult fish were attained by natural spawning and were kept at 28.5°C in egg water (60 µg/ml sea salt, Sera Marin, Heinsberg, Germany). PTU (1-phenyl-2thiourea; Sigma Aldrich) was added to egg water at 0.003% to prevent melanization of embryos. For infection delivery and live imaging experiments embryos were anaesthetized in egg water with 0.02% of buffered Tricane (3-aminobanzoic acid ethyl ester; Sigma Aldrich).

Bacterial cultures and infection experiments

Salmonella Typhimurium strains used in this study are listed in **supplementary Table 1** along with their respective mutations and their description. The bacterial strains were plated fresh from -80°C stocks over LB agar plates with respective selection markers and were incubated overnight to grow at 37°C. Before the start of infection experiments colonies from LB agar plates were suspended in phosphate buffered saline (PBS) supplemented with 2% polyvinylpyrrolidone-40 (Sigma Aldrich) to obtain the low dose (200-400 CFU, for survival curves and CFU counts experiments) or the high dose (2000-4000 CFU, for imaging experiments). Bacterial inoculum was injected systemically into the caudal vein of the anaesthetized embryos at 2 dpf. To check the inoculum size, the same dose was spotted onto agar plates, and bacterial counts determined following overnight incubation. After infection, embryos were kept individually in egg water in 48-well plates to score survival during larval development up to 5 dpf and to collect individuals for CFU counts at 24 h intervals.

Determination of *in vivo* bacterial (CFU) counts

Five larvae per time point were sacrificed and homogenized in PBS using the Bullet Blender Tissue Homogenizer (Next Advance Inc.). Homogenates were then serially diluted, and three technical replicates for each embryo/larva were plated on LB solid media with the appropriate antibiotics for *S*. Typhimurium. To determine the CFUs, the resulting colonies were counted manually after 24 h incubation at 37°C.

Morpholino knockdown

Morpholino oligonucleotides (Gene Tools) used for Rubicon (*rubcn*) knockdown and control were diluted in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6) to obtain the required concentrations. *rubcn* knockdown was achieved by injecting 1 nl volume of 0. 25 mM of previously described splice morpholino MO2-rubcn **(Chapter 3)**, similarly 1 nl of standard control morpholino was injected into the yolk of 0 hpf zebrafish embryos with microneedles and a Femtojet injector (Eppendorf) paired with a stereo-microscope.

Imaging and Image analysis

Confocal laser scanning images were acquired using at 63x water immersion objective (NA 1.2) with a Leica TCS SPE system, Samples were fixed at 4 hpi in 4% PFA and washed with PBS before image acquisitions. For Lc3-*Salmonella* associations, images acquired were analyzed through Z-stacks in Leica LAS AF Lite software and bacterial clusters were observed and manually counted in the overlay channel. Max projections in the overlay channels were used for representative images.

For quantification of the autophagic response within infected phagocytes, for each embryo, a total number of observable phagocytes were manually counted through the Z-stacks of the acquired confocal image. Phagocytes were identified in the yolk sac circulation valley by bacterial clusters in the *mCherry* channel and cellular boundaries of phagocytes were determined in the light transmission channel. Among these total observable infected phagocytes, the numbers of cells with GFP-Lc3 signal in association with *Salmonella* bacteria were counted and the percentage of Lc3-positive

phagocytes over the total observable phagocytes was determined for each embryo.

Statistical Analyses

All data sets were analyzed with Prism 7 software. Survival curves were analyzed with Log rank (Mantel-Cox) test. For CFU counts, one way ANOVA was performed on Log transformed data and was corrected for multiple comparisons using Sidak's multiple comparisons test when required. Percentage Lc3-positive phagocyte quantifications was analyzed for significance with unpaired parametric t-test between two groups, and for multiple groups the one way ANOVA test was performed and corrected for multiple comparisons.

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Supplementary material

Supplementary Table S1: List of *Salmonella* strains used for study.

Serial no.	Name of strain[1]	Mutation
1	SL1344	None (wild type)
2	Δ*phoP*	Mutation in PhoP/Q two-component sensor of the host intracellular environment
3	Δ*ssrB*	Mutation in SsrB regulator of SPI2-proteins
4	Δ*flhD*	Mutation in flagellar transcription regulator
5	Δ*sipB*	Mutation in SPI1 translocator protein SipB
6	Δ*purA*	Mutation in adenylosuccinate synthetase (purine metabolism)

[1] All strains were provided by Dirk Bumann (University of Basel). All mutant strains are in SL1344 background.

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Chapter 5

Autophagy modulator Dram1 promotes Lc3-associated phagocytosis of *Salmonella*

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Abstract

Dram1 is a stress and infection inducible autophagy modulator gene that functions downstream of transcription factors p53 and NFκB. Using a zebrafish embryo infection model, we have previously shown that Dram1 provides protection against the intracellular pathogen *Mycobacterium marinum* by promoting anti-bacterial autophagy via a p62-dependent mechanism. However, the possible interplay between Dram1 and other anti-bacterial autophagic mechanisms remains unknown. Recently, LC3-associated phagocytosis (LAP) has emerged as an important host defense mechanism that requires components of the autophagy machinery and targets bacteria directly in phagosomes. Our previous work established LAP as the main autophagy-related mechanism by which macrophages restrict growth of *Salmonella* enterica serovar Typhimurium (*S*. Typhimurium) in a systemically infected zebrafish host. We therefore employed this infection model to investigate the role of Dram1 in LAP. Morpholino knockdown or CRISPR/Cas9-mediated mutation of Dram1 led to reduced host survival and increased bacterial burden during *S*. Typhimurium infections. In contrast, overexpression of *dram1* by mRNA injection curtailed *Salmonella* replication and reduced mortality of the infected host. Imaging the early response to infection in GFP-Lc3 transgenic zebrafish revealed a strong correlation between LAP induction and levels of *dram1* of the host, with over two-fold reduction of GFP-Lc3-*Salmonella* association in *dram1* knockdown or mutant embryos and an approximately 30% increase by *dram1* overexpression. Since LAP is known to require the activity of the phagosomal NADPH oxidase, we used a *Salmonella* biosensor strain to detect bacterial exposure to reactive oxygen species (ROS) and found that the ROS response was largely abolished in the absence of *dram1*. Together, these results demonstrate the host protective role of Dram1 during *S*. Typhimurium infection and a functional link between Dram1 and the induction of LAP.

Introduction

Autophagy has long been known as a fundamental housekeeping process wherein dysfunctional cellular components are captured inside double membrane vesicles that fuse with lysosomes to degrade and recycle the contents (Levine & Klionsky, 2004; Mizushima, 2007). More recently, autophagy has also become recognized as an integral part of the immune system, functioning not only as a direct anti-microbial mechanism but also contributing to regulation of the immune response (Deretic & Levine, 2009; Levine & Deretic, 2007; Levine et al., 2011). Autophagy can function as a non-specific bulk process or as a selective mechanism mediated by receptors that recognize molecular degradation signals like ubiquitin. The selective autophagy of invading microbes, referred to as xenophagy, is an innate immune effector mechanism targeting invading microbes either when present inside membrane-bound compartments or when they escape into the host cytosol (Huang & Brumell, 2014). A hallmark of all forms of autophagy is the conjugation of microtubule-associated protein 1 light chain 3 (MAP1LC3, hereafter LC3) with phosphatidylethanolamine on the autophagosomal double membrane structures (Mizushima et al., 2004). However, the autophagy machinery can also recruit LC3 to single membrane compartments, specifically phagosomes, in a process known as LC3-associated phagocytosis (LAP) (Mehta et al., 2014; Sanjuan et al., 2007). Both xenophagy and LAP have been shown to be important for resistance against infections, but pathogens have also evolved mechanisms to subvert these host autophagic defenses (Huang & Brumell, 2014; Huang et al., 2009; Hubber et al., 2017; Martinez et al., 2015).

There are various mechanisms by which autophagy can be induced in response to internal and external stress factors, such as nutrient restriction, DNA damage, and microbial invaders. One factor implicated in the activation of autophagy is DNA damage regulated autophagy modulator gene 1 (*DRAM1*), which encodes a member of an evolutionary conserved family of six transmembrane proteins (Mah et al., 2012). *DRAM1* was first reported as direct target gene of the tumor suppressor protein p53 and shown to play a role in p53-mediated autophagy and apoptosis (Crighton et al., 2006). Expression of *DRAM1* is dysregulated in several types of cancers (Galavotti et al., 2013; Ryan, 2011). Overexpression of *DRAM1* has been shown to increase basal levels of autophagosome numbers, indi-

cating that DRAM1 can act early in the autophagy process, contributing to autophagosome formation (Mah et al., 2012). However, the DRAM1 protein predominantly localizes to lysosomes and there is evidence also for functions at later steps in the process, showing that DRAM1 enhances autophagic flux and promotes ATPase activity and lysosomal acidification (Zhang et al., 2013). Furthermore, DRAM1 is thought to mediate crosstalk between autophagy and apoptosis by interacting with the proapoptotic protein BAX (Guan et al., 2015). Other molecular interactions of DRAM1 that could explain its mode of action at early as well as late steps of the autophagy pathway remain to be elucidated.

In addition to DNA damage, *DRAM1* is also induced by infection (Laforge et al., 2013; van der Vaart et al., 2014). In HIV-infected T-cells, DRAM1 has been shown to function downstream of p53, triggering lysosomal membrane permeabilization and cell death (Laforge et al., 2013). In contrast, we have shown that the induction of *DRAM1* in macrophages infected with *Mycobacterium tuberculosis* is independent of p53 and mediated instead by transcription factor NFκB, which functions downstream of pathogen recognition by Toll-like receptors (TLR) (van der Vaart et al., 2014). Similarly, induction of the zebrafish homolog of *DRAM1* (*dram1*) by *Mycobacterium marinum* infection relies on the TLR adaptor molecule MyD88 that mediates downstream activation of NFκB (van der Vaart et al., 2014). *M. marinum*-infected zebrafish embryos develop a tuberculosis-like disease and we have shown that Dram1 plays a host protective role in this model (van der Vaart et al., 2014; Meijer & van der Vaart, 2014). Specifically, we found that knockdown of *dram1* strongly reduces the co-localization of Lc3 with *M. marinum,* whereas overexpression increases Lc3-*M. marinum* co-localization in a manner dependent on the selective autophagy receptor p62 (Meijer & van der Vaart, 2014).

In the present study we sought to investigate the role of Dram1 during infection with another intracellular pathogen, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). A zebrafish infection model for *S.* Typhimurium has previously been established and *dram1* expression is inducible in this model (Stockhammer et al., 2010; van der Sar et al., 2003). In our recent work, we have shown that LAP is the main autophagy-related process targeting *S.* Typhimurium following phagocytosis of the pathogen by macrophages in the zebrafish host **(Chapter 3)**. Furthermore, we found

that inhibition of LAP, by knockdown of factors specific for this process (Rubicon and NADPH oxidase), impaired host resistance **(Chapter 3)**. Here we demonstrate that Dram1 promotes this autophagy based anti-*Salmonella* defense mechanism, thereby providing the first evidence that links Dram1 function to LAP.

Results

Knockdown and overexpression of *dram1* indicates its function in host defense against *Salmonella* infection

In order to investigate the role of Dram1 during *Salmonella* infections we used the zebrafish systemic infection model previously described in **Chapter 3** and modulated levels of *dram1* by splice blocking morpholino knockdown or mRNA injection at the 1-2-cell stage. We challenged the host at 2 days post fertilization (2 dpf) with *S*. Typhimurium by intravenous injection and recorded progression of the resulting infection by survival curves and CFU determination **(Figure 1A)**. We found that *dram1* knockdown resulted in hypersusceptibility to *Salmonella* infection, where at 48 hours post infection (hpi) nearly 74% of hosts succumbed to *S*. Typhimurium infection in significant contrast to 54 % of mortality in control hosts **(Figure 1B)**. Moreover, only 4 % of *dram1*-deprived hosts were alive at 72 hpi compared to 29 % of the control group **(Figure 1B)**. Additionally, under knockdown conditions of *dram1*, infected hosts contained significantly higher *S*. Typhimurium bacterial counts at 24 hpi **(Figure 1C)**. Conversely, the overexpression of *dram1* by mRNA injection resulted in higher survival rates **(Figure 1B)** and lower numbers of *S.* Typhimurium bacteria at 24 hpi **(Figure 1C)**. These results indicate that Dram1 restricts *Salmonella* infection.

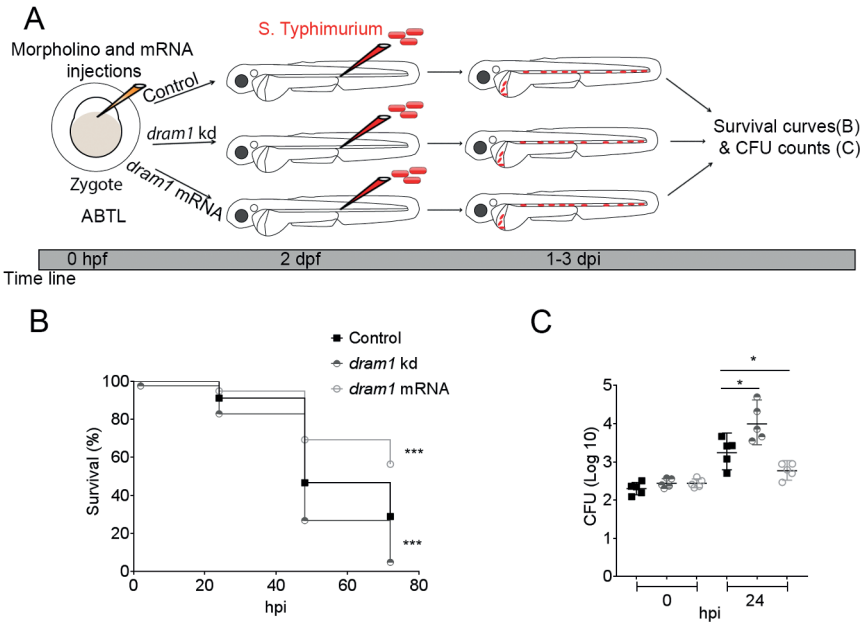

Figure 1: Dram1 is required for effective host defense during *Salmonella* infections. A: Workflow of experiments followed in B&C along with the time line of developing embryos. **B**: Survival curves of embryos with *S.* Typhimurium infection expressing different levels of *dram1*. Survival curves of control embryos were compared to *dram1* knockdown and overexpressing groups. One representative of three replicates is shown (n=50). **C:** CFU counts for infected larvae groups in B. For each of the three groups five embryos/larvae per time point were used and the log transformed CFU data are shown with the geometric mea mean per time point. One representative of three replicates is shown. Error bars represent SD. ***P< 0.001, *P< 0.05.

Dram1 is required for the LAP response of the host that targets *Salmonella* phagocytosed by macrophages

We have previously shown that the autophagy response of macrophages towards *Salmonella* in our model occurs mainly as Lc3-associated phagocytosis (LAP) **(Chapter 3)**. We therefore asked whether Dram1 has a possible role in LAP targeting of *Salmonella*. To this end we injected *dram1* morpholino into embryos of the *Tg(CMV:GFP-maplc3b1)* line (hereafter referred to as GFP-Lc3) and infected these with *mCherry*-expressing *S.*

Typhimurium **(Figure 2A)**. We observed that GFP-Lc3 associations with *Salmonella* cells are significantly Dram1 dependent, since morpholino knockdown resulted in more than two-fold reduction of GFP-Lc3-positive infected phagocytes **(Figure 2B,C,E)**. Thus, the increased susceptibility of *dram1* knockdown embryos **(Figure 1)** is associated with diminished levels of LAP. Next, we investigated if the positive effect of mRNA-mediated *dram1* overexpression on restricting infection **(Figure 1)** is accompanied by an increased LAP response. Indeed, infection of control and *dram1* overexpressing GFP-Lc3 embryos showed a significant increase of GFP-Lc3 associations with *S.* Typhimurium bacterial cells in the overexpression group **(Figure 2 D and E)**. These results indicate that Dram1 promotes the LAP response during *Salmonella* infections, resulting in a more effective host defense.

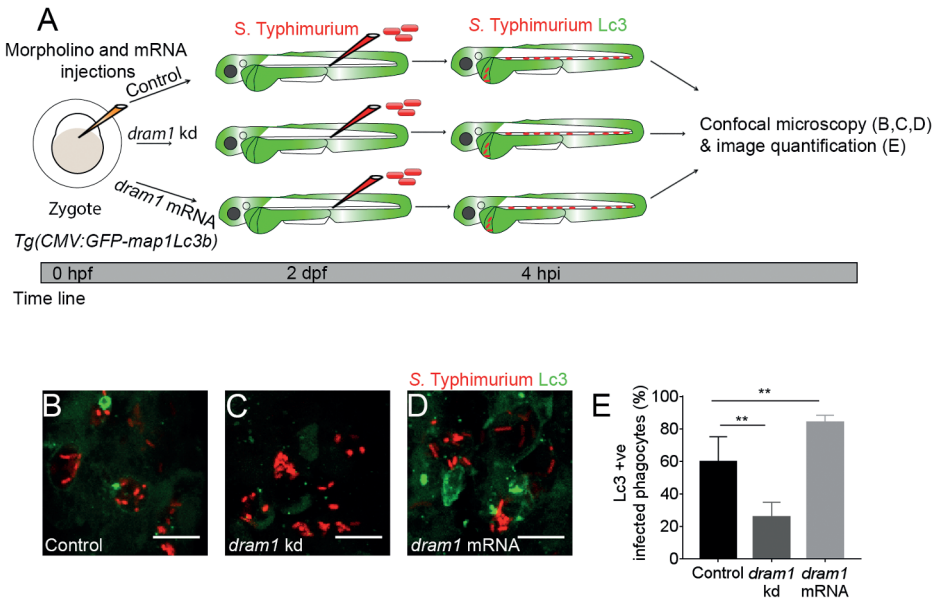

Figure 2: Dram1 modulates GFP-Lc3 associations with *S*. Typhimurium. **A:** Workflow and time line of experiments in B-E. **B-D:**. Representative confocal micrographs of *Tg(CMV:GFP-Lc3map1b)* embryos from control (B), *dram1* kd (C), and *dram1* mRNA (D) groups infected with *mCherry*-expressing *S.* Typhimurium at 4 hpi. **E:** Quantification of GFP-Lc3-*Salmonella* associations at 4 hpi. Five embryos per group were imaged over the yolk sac circulation valley and quantified as percentages of infected phagocytes positive for GFP-Lc3-*Salmonella* associations (Lc3+ve)

over the total number of infected phagocytes. Error bars represent SD. One representative of three replicates is shown. Scale bars (B-D) = 10µm. **P<0.01.

Dram1 mutation recapitulates the infection phenotype of *dram1* morpholino-induced knockdown

In order to verify the results obtained using morpholino-mediated knockdown, we studied *S*. Typhimurium infection in a recently established *dram1* mutant line (Zhang et al., unpublished). The *dram1* mutation was introduced with CRISPR/Cas9-guided RNA and homozygous *dram1*-/- mutants carrying a deletion of 19 nucleotides were established in GFP-Lc3 and *mpeg1*:mCherry transgenic backgrounds **(Figure 3A)**. The deletion disrupts the *dram1* sequence in the region encoding the first of the six transmembrane regions of the Dram1 protein **(Figure 3B,C)**. The survival of uninfected *dram1* -/- and *dram1* +/+ embryos until 5 dpf was similar **(Figure 3D)**, indicating no effect of *dram1* mutation on viability of larval zebrafish. In addition, the *dram1* -/- larvae showed no detectable morphological differences with their wild type siblings and had similar numbers of macrophages **(Figure 3E)** and neutrophils **(Figure 3F)** as *dram1+/+* larvae. Having established that *dram1* mutation does not affect larval development, we infected *dram1* -/- and *dram1* +/+ embryos with *S*. Typhimurium **(Figure 4A)** and observed that *dram1*-/- hosts showed increased mortality during *S*. Typhimurium infection as compared to *dram1*+/+ controls **(Figure 4B)**. Bacterial growth determination confirmed the *Salmonella*-restricting role of Dram1 as significantly higher bacterial counts were retrieved from *dram1-/-* embryos at 24 hpi as compared to *dram1+/+* embryos **(Figure 4C)**. The *dram1* mutants were subsequently tested for the ability to induce the LAP response during *S.* Typhimurium infection. We observed significantly less GFP-Lc3 associations with *Salmonella* cells in *dram1-/-* embryos compared to *dram1+/+* embryos **(Figure 4D-F)**, further confirming our initial observations using morpholino knockdown. The consistent results obtained in Dram1-deficient hosts achieved by knockdown or stable mutation support the function of Dram1 in LAP-mediated host defense during *Salmonella* infections.

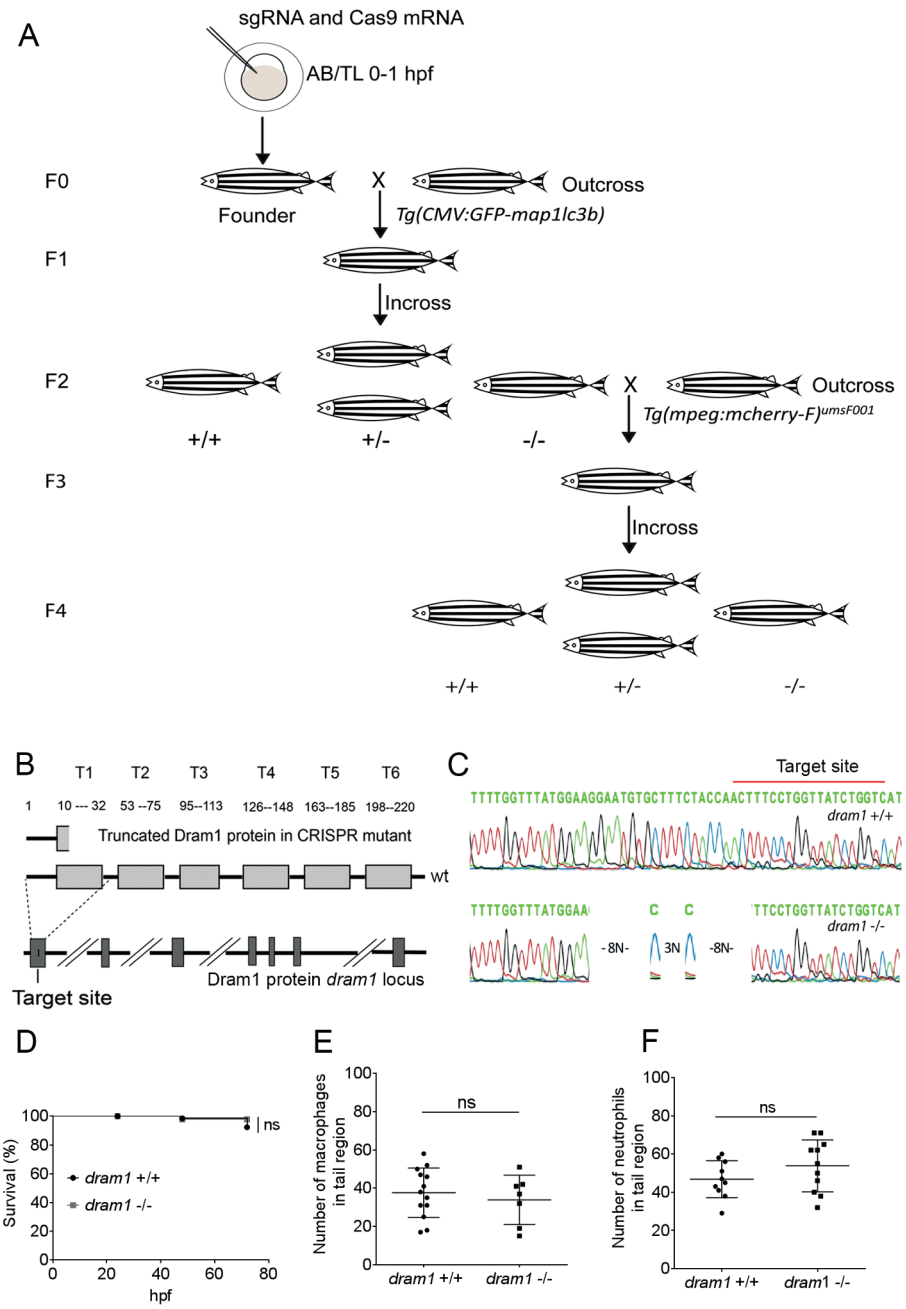

Figure 3: *dram1* mutant generation and characterization.
(figure legend on next page).

Figure 3: *dram1* mutant generation and characterization. *(figure on previous page).* **A:** Workflow describing the generation of *dram1* mutants and *dram1* mutant transgenic lines: (*dram1*-/- /*Tg(CMV:GFP-Lc3map1b)* and *dram1* -/-/*Tg(mpeg1::mcherry-F)*^{umsF001}. **B:** Schematic representation of Dram1 protein structure and the effect of the CRISPR mutation. The light grey boxes indicate the six transmembrane domains of the wild type (WT) Dram1 protein and the truncated first transmembrane domain of the predicted protein resulting from *dram1* CRISPR mutation. The amino acid (aa) numbers of the transmembrane regions are indicated on top and the structure of the *dram1* genomic locus is shown below. Exons in the *dram1* locus are indicated as dark grey boxes and introns (not drawn to scale) as black lines. The CRISPR target site is located in the first exon, causing a 19 nucleotide deletion in the sequence encoding the first transmembrane domain of the Dram1 protein. **C:** Confirmation of the 19 nucleotide deletion in the *dram1* mutant line by Sanger sequencing of *dram1*+/+ and *dram1*-/- **D:** Survival curves for uninfected *dram1*+/+ and *dram1*-/-. One of the three replicates (n=50) is shown. **E & F**: Numbers of macrophages (E) and neutrophils (F) in the tail region of 3 dpf *dram1*+/+/ and *dram1*-/- larvae in*Tg(mpeg1::mcherry-F)*^{umsF001} background (n=7-13). Neutrophils were identified by TSA staining. One representative of three replicates is shown. ns= non-significant.

Dram1 mediates ROS generation to induce LAP

LAP strictly depends on NADPH oxidase and Rubicon-mediated generation of ROS **(Chapter 3)**. Therefore, to strengthen the conclusion that Dram1 is required for the LAP response to *S.* Typhimurium, we investigated the effect of Dram1 deficiency on reactive oxygen species (ROS) generation. To this end we utilized an *S.* Typhimurium ROS biosensor strain that contains a constitutively expressed *mCherry* reporter and a GFP reporter that is activated when bacterial cells are exposed to ROS (Burton et al., 2014) **(Figure 5A)**. We have previously shown that the activation of this ROS biosensor is strictly dependent on the expression of Rubicon and the NADPH oxidase component Cyba, demonstrating that this biosensor is a reliable indicator of the occurrence of LAP **(Chapter3)**. We observed that *dram1*-/- hosts were unable to activate the ROS biosensor in contrast to *dram1*+/+ individuals **(Figure 5B-D)**. Together, our results demonstrate that Dram1 deficiency impairs both GFP-Lc3 recruitment and ROS generation and provide evidence that Dram1 is required for induction of LAP.

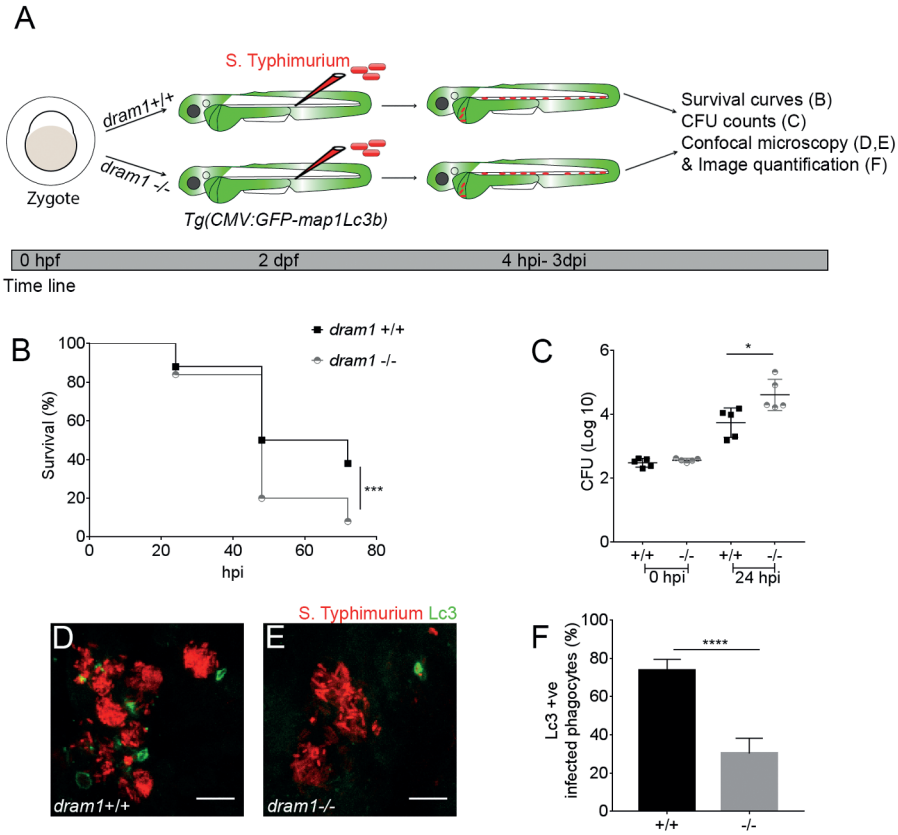

Figure 4: LAP induction is impaired in *dram1* mutants. *(figure on next page).* **A:** Workflow and time line of experiments in B-F. **B:** Survival curves of *dram1*+/+ and *dram1*-/- embryos infected with *S.* Typhimurium. One representative of two replicates is shown (n=50). **C:** CFU counts recovered from *dram1* +/+ and *dram1* - /- infected individuals. Five embryos/ larvae per time point were used and the log transformed CFU data are shown with the geometric mean per time point. One representative of three replicates is shown. Error bars represent SD. **D,E:** Representative confocal micrographs of GFP-Lc3-*Salmonella* associations in *dram1*+/+ (D) and *dram1*-/- (E) embryos in *Tg(CMV:GFP-Lc3map1b* background. **F**: Quantification of GFP-Lc3-*Salmonella* associations in *dram1*+/+ and *dram1* -/- embryos. Five embryos were imaged over the yolk sac circulation valley and infected phagocytes positive for GFP-Lc3-*Salmonella* associations were quantified as percentage over the total number of infected phagocytes. Error bars represent SD. One representative of three replicates is shown. One of the two representative is shown. Scale bars (D,E) = 10 µm, ****P<0.0001. **P<0.01, *P<0.05.

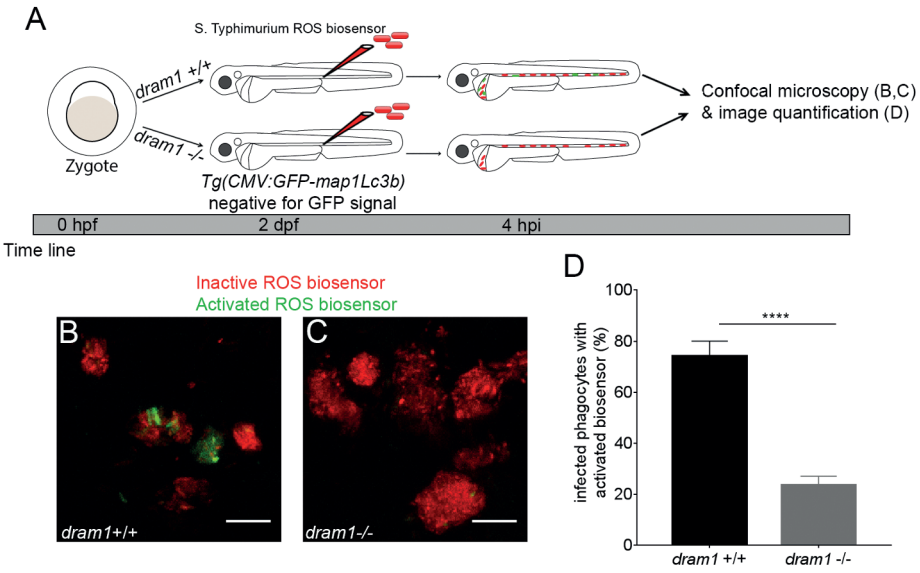

Figure 5: ROS generation is mediated by Dram1 during *Salmonella* infection. A: Workflow and time line of experiments in B-D. **B-C:**. Representative micrograph for *dram1*+/+ (B) and *dram1*-/- (C) where embryos were infected with a *Salmonella* biosensor strain for ROS. Inactive biosensor constitutively expresses *mCherry* and activated biosensor expresses GFP in addition to *mCherry*. Images were taken at 4 hpi. **D:** Quantification of ROS biosensor activation in *dram1*+/+ and *dram1*-/- at 4hpi. Numbers of phagocytes showing ROS biosensor activation (GFP and *mCherry* bacterial signals) or without ROS biosensor activation (*mCherry* bacterial signal only) were counted from confocal images and the percentages of ROS biosensor-positive over the total were averaged from five embryos per group. Error bars represent the SD. Scale bars (B-C) = 10 µm. ****P<0.0001.

Discussion

In this study, we have used a zebrafish infection model to demonstrate that the autophagy modulator Dram1 promotes host resistance to systemic *Salmonella* infection. This work expands on our previous finding that Dram1 mediates autophagic host defense in the zebrafish tuberculosis model. Importantly, our study links Dram1 to LAP, an autophagy-related pathway, recently shown to function as a crucial host defense mechanism in bacterial as well as fungal infections **(Chapter 3)**, (Hubber et al., 2017; Martinez et al., 2015; Sprenkeler et al., 2016).

DRAM1 has been shown to promote autophagy and autophagic flux, but has not previously been implicated in LAP (Crighton et al., 2006; Zhang et al., 2013). In LAP, the autophagy marker LC3 is recruited to phagosomes by a mechanism that requires essential components of the autophagy machinery, but is independent of the ULK1 preinitiation complex (Martinez et al., 2015). We have previously shown that macrophages in systemically infected zebrafish embryos target *S.* Typhimurium almost exclusively by LAP **(Chapter 3)**. Lc3 recruitment to *S.* Typhimurium in zebrafish macrophages is dependent on two proteins that are essential for LAP: Rubicon and p22Phox, a component of the NADPH oxidase (Martinez et al., 2015) and; **Chapter 3**. Depletion of either of these proteins abolishes GFP-Lc3 association with *S.* Typhimurium and prevents activation of a bacterial ROS biosensor gene **(Chapter 3)**. We found that both these responses are also inhibited by knockdown or mutation of *dram1*, indicating that Dram1 is required for the targeting of *S.* Typhimurium by LAP. This Dram1-mediated process is host-protective, since we found that *dram1*-deficient zebrafish embryos are more susceptible to *S.* Typhimurium infection, while *dram1* overexpression promotes host resistance.

The molecular mechanism of action of Dram1 is currently unknown. Our data show that Dram1 is required not only for Lc3 recruitment but also for the ROS response in phagosomes. This suggests that Dram1 functions upstream of Rubicon, which has been shown to stabilize the NADPH oxidase. An attractive hypothesis is that Dram1 interacts directly with components of the Beclin1-VPS34-UVRAG-Rubicon complex to promote the association of Rubicon with phagosomes. This hypothesis is consistent with a recent study on a member of the human DRAM family, DRAM2,

which has been shown to stimulate autophagy by disrupting the association of Rubicon with the Beclin1/UVRAG complex (Kim et al., 2011). The displacement of Rubicon relieves its inhibitory function from this complex and thereby promotes the activity of the class III phosphatidylinositol 3-kinase, VPS34, which is required for autophagosome formation and maturation. If Dram1 functions by a similar mechanism in the response to *Salmonella* infection, disruption of the Rubicon-Beclin1/Uvrag interaction would liberate Rubicon to associate with the *Salmonella*-containing phagosomes. This hypothesis would explain how Dram1 is able to stimulate autophagy and LAP at the same time. In addition, it is possible that Dram1 stimulates the maturation of autophagosomes and LAPosomes by associating with lysosomal proteins, such as LAMP1 and LAMP2 , similar to DRAM2 (Kim et al., 2011).

In addition to promoting LAP, it is likely that the activity of Dram1 can enhance other autophagy-related mechanisms against intracellular *Salmonella*, including the selective autophagy process that is mediated by ubiquitin receptors. Selective autophagy has been shown to function as an important defense mechanism against *Salmonella* in epithelial cells, which this pathogen enters by active invasion (Huang et al., 2009). Selective autophagy specifically targets cytosolic bacteria or detects membrane damage on the bacterial replication niche. In zebrafish, we have shown that this response is elicited by the escape of *M. marinum* from phagosomes and in this model Dram1 depends on the ubiquitin receptor p62 for its autophagy enhancing effect (van der Vaart et al., 2014). The above proposed interaction of Dram1 with Rubicon is consistent with positive effects on selective autophagy as well as LAP. In addition, the microbicidal capacity of Lc3-marked bacterial compartments might be enhanced by Dram1 activity, regardless of whether these compartments are of autophagosomal or phagosomal origin. This could be facilitated by delivery of ubiquitinated antimicrobial peptides through fusion with autophagosomes, which are present in increased numbers when Dram1 is overexpressed (Ponpuak et al., 2010; van der Vaart et al., 2014).

Different members of the DRAM family might have partially overlapping functions in host defense. In addition to a single copy of *dram1*, the zebrafish has two copies of the gene for Dram2, *dram2a* and *dram2b*. Based on our RNA sequencing data of leukocyte populations sorted from

zebrafish larvae, *dram1* is the most abundantly expressed gene in macrophages, while the expression level of *dram2b* is at least 10-fold lower and *dram2a* is not detectably expressed (Rougeot et al., unpublished results). Furthermore, we have only found expression of *dram1* to be inducible by infection (Benard et al., 2014; Stockhammer et al., 2010). Our study did not provide any indication that other members of the zebrafish Dram family might be involved in LAP, since mutation of *dram1* abolished ROS biosensor activation in the *Salmonella*-containing phagosomes almost completely. However, the possible role of Dram2a/b in zebrafish host defense requires further investigation in the light of the recent report that *Mycobacterium tuberculosis* evades autophagy by inducing the expression of a microRNA (miR144*) that targets human *DRAM2* (Kim et al., 2017). This study has revealed that DRAM2 enhances antimicrobial activity in human monocyte derived macrophages, similar to the function that we have shown for Dram1 in the zebrafish host during infections with *M. marinum* and *S*. Typhimurium (van der Vaart et al., 2014); and this study. Therefore, accumulating evidence positions the members of the DRAM family as key players in defense against intracellular pathogens and it will be of great interest to further explore how these autophagy modulators might work in concert and how they could be therapeutically targeted.

Materials and methods

Zebrafish lines and maintenance

Zebrafish were handled in compliance with local animal welfare regulations and international guidelines specified by the EU Animal Protective Directive 2010/63/EU and maintained according to standard protocols (zfin.org). All studies were performed on embryos/larvae before the free feeding stage. Fish lines used for this study are AB/TL (wild type strain), transgenic lines *Tg(CMV:GFP-map1lc3b)* (He & Klionsky, 2010) *Tg(mpeg1::mcherry-F)*^{umsF001} (Bernut et al., 2014) and *dram1-/-* and *dram1*+/+ in *Tg(CMV:GFP-map1lc3b)* and *Tg(mpeg1::mcherry-F)*^{umsF001} backgrounds (Zhang et al., unpublished). Embryos from adult fish were handled and treated pre-infection and post infection as described **(Chapter 3)**.

Bacterial strains and infection experiments

S. Typhimurium SL1344 strains were used for this study, constitutively expressing *mCherry* or the *mCherry* marker in combination with a GFP biosensor for ROS (p*katGp-gfpOVA)* (Burton et al., 2014). Culturing of bacteria, preparation of infection inocula and infection delivery were performed as described before **(Chapter 3)**. Briefly, bacteria were resuspended in PBS supplemented with 2% polyvinylpyrrolidone-40 (PVP) to obtain the low dose (200-400 CFU, for survival curves and CFU counts experiments) or high dose (2000-4000 CFU, for imaging experiments). Bacterial inoculum was injected systemically into the caudal vein of 2 dpf anaesthetized embryos. Survival of infected larvae was recorded at 24 hour intervals.

Determination of *in vivo* bacterial (CFU) counts

The *in vivo* bacterial counts were determined as described in **Chapter 3**. Briefly, homogenized infected embryos were serially diluted and plated out on solid media to enumerate bacterial colonies.

Dram1 knockdown and overexpression experiments

Dram1 expression was altered by injecting 1 nl of a 0.1 mM solution of a previously described antisense morpholino MO1-dram1 (AAGGCTG-GAAAACAAACGTACAGTA) or by injecting at a concentration of 100 pico grams of *dram1* mRNA in 1 nl (van der Vaart et al., 2014).

Dram1 Genome editing

CRISPR/Cas9 genome editing was used to generate a mutation in *dram1* according to established methods (Varshney et al., 2015). Briefly, Cas9 mRNA was co-injected into one-cell stage embryos with a short guide RNA containing the following sequence targeting *dram1*: ACTTTCCTGGT-TATCTGGTC. F0 founders were outcrossed to *Tg(CMV:GFP-map1lc3b)* and F1 fish carrying the same deletion of 19 nucleotides were incrossed to obtain *dram1*+/+ and *dram1* -/- F2 families carrying the GFP-Lc3 marker. The F3 embryos of these families were used for experiments in **Figure 3D** and **Figure 4 B-F**. F3 embryos from the same families but negative for the GFP-

Lc3 marker were used for experiments in **Figure 5 B-C**. GFP-Lc3 negative F2 *dram1* -/- fish were outcrossed to *Tg(mpeg1:mcherry-F)*^{umsF001} and the resulting F3 family was incrossed to obtain *dram1* +/+ and *dram1*-/- lines carrying the *mpeg1:mCherry* marker. F5 embryos of these lines were used for experiments in **Figure 3E,F**. In all families used, the 19N deletion was verified by Sanger sequencing (Baseclear). A detailed characterization of the *dram1* mutant will be described elsewhere (Zhang et al., in preparation).

Quantification of macrophages and neutrophils

Macrophages in *dram1* +/+ and *dram1* -/- embryos were quantified by expression of the mpeg1:mCherry transgene. For quantification of neutrophils Tyramide Signal Amplification (TSA) paired with Cyanine-5 (PerkinElmer Inc., Waltham, MA, USA) was used. Embryos were fixed at 4 hpi in 4% paraformaldehyde (PFA) in PBS-TX (PBS supplemented with 0.8 % (v/v) of Triton X-100; Sigma Aldrich) overnight and processed for TSA staining as previously described (Cui et al., 2011).

Image acquisition and image analysis

Infected embryos were fixed at 4 hpi for image acquisitions over the yolk sac region. A 63x water immersion objective (NA 1.2) with a Leica TCS SPE system was used. For quantification of GFP-Lc3-*Salmonella* associations and ROS biosensor activation, the images acquired were analyzed through Z-stacks in Leica LAS AF Lite software and bacterial clusters were observed and manually counted in the overlay channel as described in Chapter 3. Max projections in the overlay channels were used for representative images.

Statistical analysis

All data sets were analyzed with Prism 7 software. Survival curves were analyzed with Log rank (Mantel-Cox) test. For CFU counts, one-way ANOVA was performed on Log-transformed data. Data of GFP-Lc3-positive infected phagocytes and biosensor-positive phagocytes were analyzed with unpaired parametric t-test between two groups and for multiple groups the one-way ANOVA test was performed and corrected for multiple comparisons.

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Chapter 6

Summary and discussion

Summary and discussion

The control of infectious diseases continues to pose major challenges to the human health sector, even with the availability of modern drug treatments. Ineffective control of antibiotic-resistant bacterial strains emphasizes the need for alternative therapies, which might include targeting of host factors involved in the immune defense mechanisms that are subverted by pathogens. Developing such host-directed therapies will require a better understanding of host-pathogen interactions and the underlying mechanisms involved. Autophagy is a fundamental process that promotes cell survival by degradation and recycling of waste products, and recently, this process has emerged also as an important host defense mechanism against intracellular infections (reviewed in **Chapter 1**). Pharmacological stimulation of autophagy is therefore considered as a possible therapeutic approach for controlling drug-resistant infectious agents. However, it remains to be established if stimulating autophagy will be effective against pathogens that have evolved virulence mechanisms to restrict the host autophagy response, evade it, or even manipulate it to facilitate intracellular survival and replication. The dual role of autophagy as a host-protective or pathogen-promoting mechanism demands further investigation into host pathogen interactions in order to determine the therapeutic potential of autophagy modulating strategies. The zebrafish provides a useful model to study the host autophagy response to human pathogens. The transparent embryos of this model organism are widely used for studies of the innate immune system (as reviewed in **Chapter 2**) and are especially useful for non-invasive imaging of autophagy activation with the aid of a transgenic reporter for autophagosome formation (GFP-Lc3). Furthermore, the activity of host factors that mediate the autophagic defenses can be efficiently studied in zebrafish using gene knockdown or knockout technologies. In this thesis the zebrafish model has been applied to study the role of the autophagy machinery in host defense against *Salmonella* infection.

The zebrafish-*Salmonella* model

Salmonella enterica serovarTyphimurium (*S*. Typhimurium) infection is a common cause of food-borne disease affecting millions of people per year and there is an alarming rise in drug-resistant strains both in devel-

oping and developed parts of the world (Threlfall, 2002). *S*. Typhimurium is a facultative intracellular pathogen that can invade and persist in both non-phagocytic and phagocytic cell types (Finlay & Brumell, 2000; Ibarra & Steele-Mortimer, 2009; LaRock et al., 2015). The virulence mechanisms of *Salmonella* have been widely studied using a number of *in vitro* and *in vivo* models, to which the zebrafish is a recent addition. The zebrafish-*Salmonella* model was first described by van der Sar and coworkers, who demonstrated that *S*. Typhimurium can replicate inside macrophages following intravenous delivery into embryos at 1 day post fertilization (dpf) (van der Sar et al., 2003). With doses of just 50 bacterial cells, this infection also spreads extracellularly and becomes lethal within one day. Earlier work in our laboratory has shown that *S*. Typhimurium infection elicits a strong pro-inflammatory innate immune response under these conditions that is majorly TLR-Myd88 dependent (Stockhammer et al., 2010; Stockhammer et al., 2009; van der Vaart et al., 2013).

In this study we modulated the zebrafish-*Salmonella* infection model to investigate the host autophagic defense response over a longer period of infection **(Chapter 3)**. To this end, we introduced systemic infection at 2 dpf and followed the host response until 5 dpf. We preferred infections at 2 dpf over 1 dpf as this infection model allowed better discrimination between knockdown effects of host factors **(Figure 1A)**, which was not possible with the rapidly lethal infections at 1 dpf. When embryos are infected at 2 dpf with 200 bacteria, this leads to up to a 10^3 exponential rise of *Salmonella* cells, where 60-70 % of hosts succumb to this infection burden and 30-40 % survivors are left at 3 days post infection (dpi) **(Chapter 3)**. The increased immune competence of embryos at 2 dpf in comparison with 1 dpf might be explained by the presence of higher numbers of phagocytes at this stage or by further development of the anti-microbial properties of these cells.

We used the 2 dpf infection model to study a set of *S.* Typhimurium strains with mutations in virulence factors **(Chapter 4)** and the results are summarized in **Figure 1B**. Consistent with results in mouse models (Miller et al., 1989; Miller & Mekalanos, 1990; O'Collaghan et al., 1998; Thompson et al., 2011), we found that *S*. Typhimurium requires the metabolic factor PurA and the two-component sensor PhoP for effective infection establishment in zebrafish embryos. Mutation in the flagellin regulator

FlhD causes hypervirulence in the zebrafish host, which is also in agreement with studies in mouse models (Lockman & Curtiss, 1990; Schmitt et al., 2001). This observation suggests that flagellin recognition by the TLR pathway is important for mounting an effective immune response by the zebrafish host. This assumption is in line with previous results of our laboratory showing that injection of *Salmonella* flagellin induces expression of pro-inflammatory genes in zebrafish embryos, which is reduced under knockdown conditions of the zebrafish homologs of the flagellin receptor TLR5 (Stockhammer et al., 2009). Finally, the virulence of *S.* Typhimurium was not significantly affected by mutation of SPI1 and SPI2 type III secretion system (T3SS) factors **(Chapter 4)**, for which the likely explanation is that *Salmonella* parasitism of macrophages during systemic infection does not require active T3SS-mediated invasion.

The role of macrophages and neutrophils in host defense against *Salmonella*

In zebrafish embryos it is generally observed that macrophages are more efficient in phagocytosing intravenously injected bacteria, whereas subcutaneously injected bacteria are better taken up by neutrophils as their phagocytic behavior requires microbes to be surface-associated (Colucci-Guyon et al., 2011). However, in our comparison of intravenous and subcutaneous injections we observed macrophages to be the main phagocytes containing *Salmonella* bacteria independent of the route of infection **(Chapter 3)**. By knockdown of the transcription factor *irf8* the developmental fate of macrophages can be shifted to the production of neutrophils (Li et al., 2011). Using this strategy, we found that macrophage-depleted embryos with increased neutrophil numbers were unable to curtail *S*. Typhimurium infections and could not survive more than 1 dpi, indicating that macrophages are essential for the zebrafish host to cope with *S*. Typhimurium infection **(Chapter 3)**. In agreement, a previous study using a zebrafish hindbrain infection model demonstrated an important role in host defense against *S.* Typhimurium for immunoresponsive gene 1 (*irg1*), which is specifically expressed in macrophages (Hall et al., 2013). Irg1-deficient macrophages are impaired in the production of the antimicrobial compound itaconic acid and also fail in the effective utilization of fatty acids as an energy source for mitochondrial reactive oxygen species (ROS) production (Hall et al., 2013).

A

Host factor	Function	Infection phenotype in factor-deficient host
wt	-	
irf8	Transcription factor responsible for macrophage fate determination	
atg5	Membrane elongation during autophagy	
atg13	Member of ULK1 complex	
rubcn	Induces LAP and suppresses xenophagy	
cyba	Component of NOX2-complex	
dram1	Promotes autophagy	

B

Bacterial factor	Function	Infection phenotype in wt host	Infection phenotype in *rubcn*-deficient host
wt	-		
Δ*purA*	Purine metabolism		
Δ*phoP*	Defense against anti-microbial peptides		
Δ*flhD*	Flagellin formation		
Δ*sipB*	SPI1 translocator		
Δ*ssrB*	SPI2 regulator		

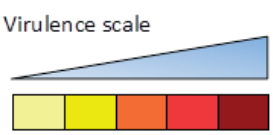

Figure 1: Heat map representations of infection phenotypes caused by deficiencies in host or bacterial factors. A: Summary of the loss-of-function effects of the zebrafish host factors studied in this thesis. The severity of *S.* Typhimurium infection in zebrafish embryos deficient in the indicated host genes is shown in comparison to wild type embryos. **B:** Summary of the infection phenotypes induced by different *S.* Typhimurium strains in wild type hosts and Rubicon-deficient hosts. The severity of infections caused by the indicated mutant strains is shown in comparison with the wild type strain. The virulence scale below shows the color coding for increasing severity of the infection phenotype, based on host survival rates and bacterial burden.

In another zebrafish-*Salmonella* infection model a role for a neutrophil-specific gene (*gbp4)* has been shown (Tyrkalska et al., 2016). This gene encodes guanylate-binding protein 4a, a CARD-domain containing GTPase that has been identified as a mediator of inflammasome activation (Tyrkalska et al., 2016). In contrast to our work, this study used the yolk of the zebrafish embryo as the site of injection with *S.* Typhimurium. In our experience with yolk infection models for other pathogens (*Staphylococcus epidermidis* and *Mycobacterium marinum*), bacteria injected via this route initially replicate extracellularly as the yolk is not accessible to phagocytes, and only at later stages the yolk infection spreads systemically into the host (Veneman et al., 2013). Therefore, the role of *gbp4* in this model may be associated with the inflammatory response of neutrophils attracted to the tissue surrounding the intra-yolk infection. We consider the intravenous and subcutaneous infection routes used in our work more suitable to study the direct interaction of phagocytes with *S.* Typhimurium bacteria. The fact that macrophages are the predominant cell type containing *Salmonella* under these infection conditions makes the zebrafish embryo a highly useful model to study systemic Salmonellosis, since macrophages also constitute the major replication site of *Salmonella* during systemic infections in mammalian hosts (Watson & Holden, 2010).

Association of the autophagy marker Lc3 with intracellular *Salmonella*

In order to study the autophagy response to *S.* Typhimurium we took advantage of a transgenic reporter line constitutively expressing a GFP fusion of Lc3 (He et al., 2009). Under basal conditions this reporter displays a diffused signal of cytosolic GFP-Lc3, but when autophagy is activated, Lc3 is lipidated at the C-terminus and recruited to autophagosomal membranes. We studied the dynamics of GFP-Lc3 association over the first hours of *S.* Typhimurium infection and observed bacteria enclosed in different types of GFP-Lc3 positive vesicles as well as the presence of small GFP-Lc3 vesicles (puncta) in close vicinity of the bacterial cells **(Chapter 3)**. GFP-Lc3 puncta in association with bacterial cells are also frequently observed during *M. marinum* infection; however, the presence of *M. marinum* bacteria inside GFP-Lc3 positive vesicles has only been detected at later stages of infection from 1 dpi and not during the first hours (Hosseini et al., 2014; van der Vaart et al., 2014). In contrast, the level of GFP-

Lc3 recruitment to *S.* Typhimurium peaked at 4 hpi and several different types of Lc3-decorated vesicles containing bacterial cells were observed. These structures appeared as tight or spacious GFP-Lc3 rings enclosing single bacterial cells, but also as spacious GFP-Lc3 rings surrounding larger bacterial clusters. In some cases the *mCherry* fluorescent label of the bacterial cells was diffused, suggesting degradation of the bacteria inside the larger GFP-Lc3 vesicles. GFP-Lc3 association with *S.* Typhimurium at 4 hpi was observed in approximately 60% of infected phagocytes and the response was not triggered by heat-killed bacteria. Although the different types of GFP-Lc3 positive rings or puncta were detected both in macrophages and neutrophils, their presence was at least twice more frequent in macrophages. Five *S.* Typhimurium mutant strains tested in our study **(Figure 1B)** triggered the formation of similar diverse GFP-Lc3-decorated vesicles with different degrees of this response **(Chapter 4)**.

Correlative light and electron microscopy would be required to define the nature of the GFP-Lc3 positive vesicles in which *Salmonella* resides. In our initial non-correlative electron microscopy analysis we observed macrophages containing many bacteria inside a large phagosome, while other cells had smaller phagosomes with one or few bacteria. In addition, we observed bacteria freely in the cytoplasm, in multivesicular compartments, and in compartments that also contained cytoplasmic material suggestive of an autophagic origin **(Chapter 3)**. However, we did not detect *Salmonella* inside the typical double membrane vesicles that are characteristic for the early stages of canonical autophagy.

Lc3-associated phagocytosis as a host-protective mechanism

The observation that GFP-Lc3 recruitment to *Salmonella* follows rapidly after ingestion by phagocytes suggested that a recently discovered autophagy-related pathway, named Lc3-associated phagocytosis (LAP), might be involved. To discriminate between canonical autophagy and LAP we performed knockdown studies of host factors specific for the two processes **(Figure 2A)**. In LAP, Lc3 is recruited directly to the single membrane of the phagosome by a mechanism that relies on components of the autophagy conjugation systems, including ATG5, but does not require the ULK1 preinitiation complex, including ATG13 (Martinez et al., 2011; Martinez et al., 2015). Furthermore, LAP induction is dependent on

phagosomal production of ROS and this requires stabilization of NADPH oxidase on the phagosomal membrane by the RUN and cysteine-rich domain containing Beclin1 interacting protein, Rubicon (Huang et al., 2009; Martinez et al., 2015; Matsunaga et al., 2009), **(Figure 2A)**. We found that knockdown of Atg5, Rubicon, or the $p22^{phox}$ component (Cyba) of NADPH oxidase reduced GFP-Lc3 association with *Salmonella* **(Chapter 3)**. In contrast, Atg13 knockdown had no effect on GFP-Lc3 recruitment. These results are consistent with the hypothesis that *Salmonella* is targeted by LAP. To corroborate this view, we infected embryos with a *Salmonella* biosensor strain that emits green fluorescence when bacteria are exposed to ROS (Burton et al., 2014). In wild type embryos we observed activation of the ROS biosensor in a subpopulation of bacteria contained by phagocytes. This activation was completely abolished not only in the absence of NADPH oxidase function, but also by deficiency of Rubicon. The dual requirement of NADPH oxidase and Rubicon for the ROS response and GFP-Lc3 recruitment provides strong evidence for the involvement of LAP. Furthermore, we conclude that LAP functions as a host-protective response towards *Salmonella*, as hosts deficient in Atg5, Rubicon, or NADPH oxidase, but not Atg13-deficient hosts, are hypersusceptible to infection **(Chapter 3 & Figure 1A)**.

Our results do not exclude that canonical autophagy might also contribute to host defense in our model. In our electron microscopy analysis we observed that a fraction of *Salmonella* bacteria were localized in the cytosol of infected phagocytes. Although we have not investigated ubiquitination or co-localization with selective autophagy receptors, it is likely that these cytosolic bacteria are targeted by xenophagy, as demonstrated by many studies in cultured epithelial cells but also macrophages (Birmingham et al., 2006; Cemma et al., 2011; Hubber et al., 2017; Thurston et al., 2016; Thurston et al., 2009; Thurston et al., 2012; Watson & Holden, 2010; Wild et al., 2011; Zheng et al., 2009). A recent study suggest that xenophagy also targets *Salmonella* in zebrafish, since knockdown of the ubiquitin receptor Optineurin was found to increase susceptibility of zebrafish embryos to infection (Chew et al., 2015). However, in our model, LAP appears to be the initial response of the zebrafish phagocytes, predominantly macrophages but also neutrophils, that take up *Salmonella*. It is likely that mammalian phagocytes also target *Salmonella* by LAP, as suggested by a study showing that GFP-Lc3 recruitment to *Salmonella*-

containing phagosomes in mouse neutrophils was dependent on NADPH oxidase (Huang et al., 2009). Demonstration of Rubicon dependency and independency of the ULK1 complex would be required to formally prove the role of LAP in other systems.

Role of Lc3-associated phagocytosis during infections with virulence mutants

Similar to wild type *S.* Typhimurium, several mutant bacterial strains also elicit GFP-Lc3 recruitment in phagocytes of the zebrafish host in a Rubicon-dependent manner **(Chapter 4)**. The level of GFP-Lc3 recruitment was slightly but significantly enhanced in response to strains carrying mutations in the PhoP regulator of outer membrane composition or in the SipB translocator of the SPI1 T3SS. This suggests that these virulence factors could be involved in evasion of LAP. In contrast, a recent study of *Legionella dumoffi* showed that the recruitment of Lc3 to Legionella-containing vacuoles in cultured macrophages requires the Dot/ICM type IV secretion system (T4SS) of this pathogen (Hubber et al., 2017). This T4SS-dependent process was defined as LAP, based on requirement for Rubicon and NADPH-oxidase and independency from ULK1, similar as we have shown for the response to *S.* Typhimurium in the zebrafish host. Furthermore, the LAP response to *L. dumoffi* was also shown to serve a host-protective role (Hubber et al., 2017). The T4SS of *L. dumoffi* is also required for inhibiting endosome maturation and establishing a replication niche in an endoplasmic reticulum (ER)-like compartment. Therefore, expression of the T4SS involves a trade-off between intracellular pathways promoting (maturation of the LCV) or limiting (LAP) the intracellular survival of this pathogen. In the case of *S.* Typhimurium, we found that LAP is significantly reduced by mutations in genes required for purine metabolism (Δ*purA*) and for the expression of flagella (Δ*flhD*). The Δ*purA* mutant is rapidly cleared by the zebrafish host and therefore might fail to induce signals for LAP. In contrast, the Δ*flhD* mutant is hypervirulent. As LAP has been shown to occur downstream of TLR signaling (Sanjuan et al., 2007), we hypothesize that Tlr5-Myd88-mediated detection of *Salmonella* flagellin contributes to the induction of LAP and other host defense mechanisms that restrict growth of flagellated strains. In future work, it would be of interest to test this hypothesis using previously established

genetic tools and zebrafish mutants for probing TLR signaling (Stockhammer et al., 2009; van der Vaart et al., 2014; Yang et al., 2015).

The hypervirulence of the Δ*flhD* mutant was further increased when LAP was inhibited by Rubicon knockdown. Moreover, Rubicon knockdown also increased virulence of the Δ*sipB* mutant, and mutants with attenuated infections (Δ*purA*, Δ*PhoP*) became virulent in LAP-deficient embryos **(Chapter 4 & Figure 1B)**. Interestingly, virulence of Δ*ssrB*, a strain impaired in function of the SPI2 T3SS, remained unchanged in LAP-deficient embryos **(Chapter 4 & Figure 1B)**. This result, together with the fact that all other SsrB-competent strains tested showed increased virulence under Rubicon knockdown conditions, suggests that SPI2 function is required for intracellular replication and/or spreading from infected cells in the absence of LAP. When LAP fails to restrict *Salmonella* growth, SPI2 function could mediate maturation of the SCV as a replication niche and/or contribute to escape from the SCV resulting in cytosolic replication, subsequently causing macrophage cell death and extracellular spreading. This hypothesis is in line with a study in mice suggesting that SPI2 function mediates the exit from infected cells and bacterial spreading into the host tissues (Grant et al., 2012). It was beyond the scope of the present study to investigate the possible effects of SsrB mutation by electron microscopy, but in case of wild type *S*. Typhimurium we could record an example of an SCV forming tubular extensions into the cytoplasm, which might represent SPI2-dependent formation of the *Salmonella* induced filaments (Sifs) that are characteristic for SCV maturation **(Chapter 3)**. Although SsrB mutation did not detectably alter virulence of *S.* Typhimurium, this possible example of Sif formation is consistent with the expression of SPI2 effectors during infection in the zebrafish host and with the increased virulence of SsrB-competent strains in the absence of LAP **(Figure 1B)**. *Salmonella* possesses a number of other virulence factors that would be relevant to study in connection with LAP. For example, the ADP-ribosyltransferase spvB has been shown to inhibit autophagosome formation through depolymerization of actin filaments (Chu et al., 2016; Li et al., 2016; Wu et al., 2016). Therefore, it is an interesting question whether or not spvB can also promote *S.* Typhimurium pathogenity by the avoidance of LAP.

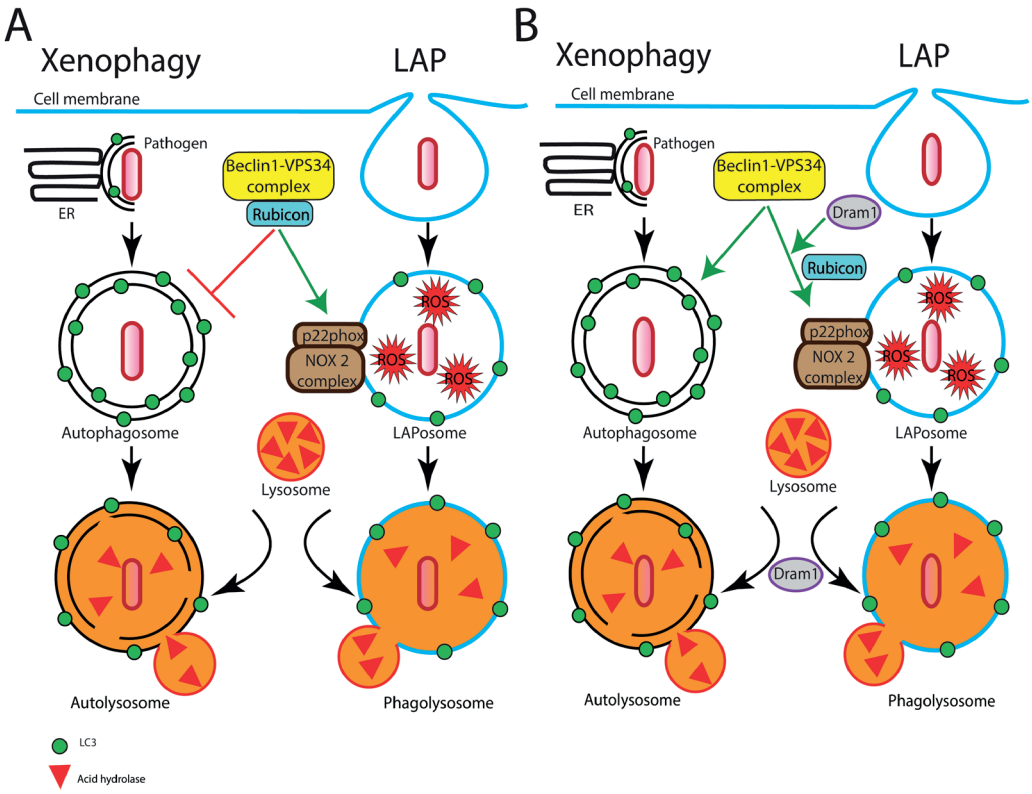

Figure 2: Working model for the role of Dram1 in promoting host defense responses dependent on the autophagy machinery. A: Graphical comparison of xenophagy and LC3-associated phagocytosis (LAP). Rubicon acts as a molecular switch between the two processes, where it negatively regulates xenophagy by binding to the Beclin 1-VPS34 PI3 kinase complex, which also includes the UV resistance associated gene protein UVRAG. On the other hand, Rubicon induces LAP by promoting NADPH oxidase assembly on phagosomes. In turn, NADPH oxidase generates reactive oxygen species (ROS) responsible for Lc3 recruitment resulting in the formation of LAPosomes. Both xenophagy and LAP ultimately result in lysosomal-mediated killing of microbial invaders. **B:** Graphical presentation of the possible roles of Dram1 during xenophagy and LAP. Dram1 might act at different steps during both processes. First, it might promote the release of Rubicon from the Beclin1-VPS34-UVRAG complex on autophagosomal membranes, thereby facilitating the progression of xenophagy and the induction of LAP at the same time. Second, Dram1 might promote both processes by increasing fusion of lysosomes to autophagosomes or LAPosomes. The signaling processes mediated by the Beclin1-VPS34 complex, Rubicon, and Dram1 are localized to membranes of the different organelles in the figure, which is not indicated for simplicity.

Host-protective function of the autophagy modulator Dram1

Previous work in our laboratory had shown that *S.* Typhimurium infection leads to transcriptional induction of *dram1*, which codes for DNA-damage regulated autophagy modulator 1 (Stockhammer et al., 2010). Dram1 belongs to an evolutionary conserved family of six-transmembrane proteins, which includes DRAM1, DRAM2, TMEM150A, TMEM150B/DRAM3, and TMEM150C in human and Sfk1 in yeast (Chung et al., 2015; Crighton et al., 2006; O'Prey et al., 2009). Currently, three of the human DRAM proteins have been identified as positive regulators of autophagy, and the corresponding genes are induced under different stress conditions, being the p53-mediated DNA damage response in case of *DRAM1*, amino acid starvation in case of *DRAM2*, and glucose restriction in case of *DRAM3* (Crighton et al., 2006; Mrschtik et al., 2015; O'Prey et al., 2009; Yoon et al., 2012). In addition, *DRAM1* expression is induced during infections with HIV and *Mycobacterium tuberculosis* (Laforge et al., 2013; van der Vaart et al., 2014). In contrast, *M. tuberculosis* infection has also been shown to result in induction of a microRNA (miR144*) that down-regulates *DRAM2* expression and thereby contributes to evasion of autophagy (Kim et al., 2017). In zebrafish embryos, *dram1* protects against *M. marinum* infection by promoting the Sting- and p62-dependent autophagic mechanism against bacteria following their escape into the cytosol, which is mediated by secreted virulence factors (van der Vaart et al., 2014). Since *S.* Typhimurium infection triggers LAP rather than xenophagy in zebrafish embryos **(Chapter 3)**, it was of interest to investigate if Dram1 is also involved in this autophagy-related process.

To establish a possible functional link between Dram1 and the induction of LAP, we performed loss-of-function analyses by morpholino-mediated knockdown and CRISPR/Cas9 mutation as well as gain-of-function analysis by mRNA injection **(Chapter 5)**. The results led to the conclusion that Dram1 is required for host defense against *S.* Typhimurium infection, since increased bacterial burden and host mortality was observed under loss-of-function conditions, while gain-of-function enabled embryos to restrict *S.* Typhimurium replication and improved survival rates. This host-protective function of Dram1 correlated with a reduction of GFP-Lc3-*Salmonella* association in *dram1*-deficient embryos and an increase under overexpression conditions of *dram1*. Furthermore, activation of a

Salmonella ROS biosensor construct was largely abolished in *dram1* mutants, indicating that the presence of Dram1 is required for activation for NADPH oxidase, which is an essential step in LAP. Therefore, it can be concluded that Dram1 function is required to promote the targeting of *S.* Typhimurium bacteria by LAP.

The question remains how Dram1 can stimulate different anti-bacterial autophagy responses, including xenophagy and LAP. The answer could lie in an interaction with components of the Beclin1-VPS34 complex, in agreement with a recent study on DRAM2 (Kim et al., 2017). Beclin1 forms a complex with the class III phosphatidylinositol 3-kinase, VPS34, and this complex can take different compositions by including regulatory proteins. The Beclin1-VPS34-Atg14L complex functions in early steps of autophagosome formation, whereas the Beclin1-VPS34-UVRAG complex promotes the later steps of the maturation process. Rubicon, which functions as a negative regulator, associates specifically with a subset of Beclin1-VpPS34-UVRAG complexes (Matsunaga et al., 2009). The interaction between Rubicon and UVRAG has not only been shown to block autophagosome maturation, but also endosome maturation, which is due to inhibition of Rab7 activation (Sun et al., 2010). This agrees with the predominant localization of Rubicon on endosomes/lysosomes and with accumulating evidence that autophagosome maturation involves fusion with endocytic vesicles (Sun et al., 2010). Like Rubicon, human DRAM1 and zebrafish Dram1 have also been shown to localize to lysosomes (Crighton et al., 2006; van der Vaart et al., 2014). Therefore, similar to what has been proposed for DRAM2, interactions of Dram1 with the Beclin1-VPS34-UVRAG complex might facilitate the dissociation of Rubicon and thereby promote autophagosome maturation **(Figure 2B)**. This is consistent with other studies that have provided evidence for a role of DRAM1 in stimulating autophagic flux (Guan et al., 2015; Zhang et al., 2013). The Beclin1-VPS34-UVRAG complex but not the Beclin1-VPS34-Atg14L complex is involved In LAP, where Rubicon acts as a positive regulator (Martinez et al., 2015). Therefore, an interaction of Dram1 with the Beclin1-VPS34-UVRAG complex might also be the mechanism underlying the stimulation of LAP **(Figure 2B)**. Possibly, Dram1-mediated dissociation of Rubicon from the Beclin1-VPS34-UVRAG complex is required for Rubicon to stabilize the NADPH oxidase, which precedes LC3 recruitment. However, molecular interactions of Dram1 with other proteins involved in

endosomal maturation and lysosomal function can also be envisaged and further research will be required to elucidate the precise function of this autophagy modulator. An important step in this research will be to study the co-localization of Dram1 with its proposed interaction partners under different stress and infection conditions that rely on Dram1-mediated responses. The zebrafish embryo provides a useful model to study the dynamics of these responses *in vivo*. To this end, a zebrafish line is under construction in our laboratory, in which the endogenous *dram1* is tagged with GFP using CRISPR/Cas9 technology.

Concluding remarks

The work described in this thesis has provided new insights into the role of the autophagy machinery in host defense against *Salmonella* that are particularly relevant for the systemic stages of infection when the pathogen parasitizes macrophages. We demonstrate that an autophagy-related process, named Lc3-associated phagocytosis or LAP, targets *Salmonella* bacteria when they are engulfed by phagocytes, predominantly macrophages, of the zebrafish embryo. Rubicon and NADPH oxidase are essential host factors for this response. Depletion of these factors leads to increased virulence of both wild type and attenuated strains, showing that LAP functions as a host-protective mechanism against *Salmonella* infection. Our studies also revealed a novel functional link between the autophagy modulator Dram1 and induction of LAP. The results in this thesis provide evidence that host resistance to systemic *Salmonella* infection can be promoted by genetically increasing Dram1 activity, which encourages further studies aimed at the identification of autophagy modulating drugs for host-directed therapy of antibiotic-resistant *Salmonella* infections.

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Dutch summary / samenvatting

Samenvatting

Bacteriële infectieziekten vormen nog altijd een wereldwijd gezondheidsprobleem, ook al zijn er antibiotica tegen ontwikkeld. Omdat er steeds vaker antibioticum-resistente bacteriestammen opduiken, is de urgentie om alteratieve behandelingsmethoden te vinden groot. Hierbij kan gedacht worden aan medicijnen die aangrijpen op gastheerfactoren die een rol spelen bij de afweerreacties van het immuunsysteem waaraan pathogene bacteriën weten te ontsnappen. De ontwikkeling van zulke gastheer-gerichte therapieën vereist een beter begrip van de moleculaire mechanismen die betrokken zijn bij gastheer-pathogeen-interacties. In dit proefschrift is de zebravis gebruikt voor onderzoek naar de rol van autofagie als afweermechanisme tegen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), een intracellulaire pathogeen die een voorname oorzaak is van besmettelijke darminfecties.

Autofagie is een fundamenteel proces dat de levensduur van cellen bevordert door beschadigde onderdelen en afvalproducten af te breken en te recyclen. Zoals besproken in **Hoofdstuk 1**, is het recentelijk duidelijk geworden dat autofagie ook een belangrijk afweermechanisme is tegen intracellulaire infecties. Farmacologische stimulatie van autofagie wordt daarom gezien als een mogelijk alternatief voor de behandeling van infecties met antibioticum-resistente bacteriën. Het zal echter nog moeten blijken of deze strategie effectief is tegen pathogenen die virulentiemechanismen bezitten om de autofagierespons van hun gastheer terug te dringen, te ontwijken of juist zodanig te manipuleren dat het hun overleving en replicatie in de gastheercel ten goede komt. Gezien de dubbele rol van autofagie als een gastheer-beschermend of een virulentie-bevorderend mechanisme, is het duidelijk dat verder onderzoek naar gastheer-pathogeen-interacties nodig is om inzicht te krijgen in de therapeutische waarde van stoffen die autofagie stimuleren.

De zebravis is een bruikbaar modelorganisme om de rol van autofagie bij de afweer tegen pathogenen te bestuderen. Onderzoek naar het immuunsysteem van de zebravis, waarvan **Hoofdstuk 2** een overzicht geeft, heeft aangetoond dat er grote overeenkomsten zijn met het immuunsysteem van de mens. De transparante embryo's van de zebravis lenen zich uitstekend voor microscopisch onderzoek, waarbij de cellen

van het aangeboren immuunsysteem (macrofagen en neutrofielen) en autofagieprocessen (gekenmerkt door accumulatie van het eiwit Lc3) met fluorescerende merkers kunnen worden gevolgd. Bovendien zijn er eenvoudige genetische technieken beschikbaar om de gastheerfactoren te bestuderen die een rol spelen bij autofagieprocessen. Voor het onderzoek in dit proefschrift kon gebruik worden gemaakt van een al beschikbaar zebravisembryomodel voor infectie met *S.* Typhimurium.

De rol van autofagie bij afweer tegen *Salmonella*-infecties is tot nu toe voornamelijk bestudeerd in epitheelcellen, die het doelwit zijn van invasie door *Salmonella* bij darminfecties. Er is echter nog weinig bekend over de interactie van *Salmonella* met de autofagiemachinerie van macrofagen. Deze immuuncellen zijn de belangrijkste dragers van de pathogeen wanneer een *Salmonella*-infectie niet beperkt blijft tot het darmkanaal, maar systemisch wordt, zoals bij paratyfus. In **Hoofdstuk 3** is het zebravisembryomodel gebruikt om de interactie van *S.* Typhimurium met immuuncellen tijdens een systemische infectie te bestuderen. Dit onderzoek heeft uitgewezen dat de fagocytose van levende, maar niet van dode, *Salmonella*-bacteriën gepaard gaat met de accumulatie van de fluorescentgelabelde autofagiemerker GFP-Lc3 op de blaasjes waarin de bacteriën door de immuuncellen zijn opgenomen. Hierbij werden verschillende patronen waargenomen, variërend van ringvormige tot puntvormige GFP-Lc3-signalen rond enkele bacteriën of rond clusters van bacteriën. Ook met behulp van transmissie-electronmicroscopie werden verschillende typen van bacteriële compartimenten gedetecteerd. De GFP-Lc3-patronen werden zowel in geïnfecteerde macrofagen als in geïnfecteerde neutrofielen waargenomen. Uit genetische modulatie van de ratio tussen deze twee celtypen bleek echter dat macrofagen het belangrijkste celtype zijn voor de Lc3-afhankelijke afweerreactie.

Hoewel Lc3 een veelgebruikte merker voor autofagie is, kan fagocytose ook gepaard gaan met Lc3-accumulatie. Dat proces wordt Lc3-geassocieerde fagocytose genoemd oftewel LAP ("Lc3-associated phagocytosis") en is afhankelijk van een groot deel van de componenten van de autofagiemachinerie (bijvoorbeeld het Atg5-Atg12-Atg16L-complex), maar niet van alle componenten (bijvoorbeeld het Ulk1-Atg13-Fip200-complex). Bovendien fungeert het eiwit Rubicon als een remmer van autofagie, terwijl het juist essentieel is voor de inductie van LAP. Rubicon stabiliseert

NADPH-oxidase op de membraan van het fagosoom en dat is noodzakelijk voor de productie van reactieve zuurstofmoleculen oftewel ROS ("reactive oxygen species"), waardoor de accumulatie van Lc3 wordt opgewekt. Om te onderzoeken of autofagie danwel LAP het mechanisme is achter de Lc3-respons tegen *Salmonella* werd het onderzoek in **Hoofdstuk 3** vervolgd met knockdown-experimenten, waaruit bleek dat deze respons afhankelijk is van Atg5, Rubicon en NADPH-oxidase, maar onafhankelijk van Atg13. Al deze factoren, behalve Atg13, bleken ook bepalend te zijn voor de resistentie van de zebravisgastheer tegen de infectie. Ten slotte kon aangetoond worden dat zowel Rubicon als NADPH-oxidase essentieel zijn om een *Salmonella*-biosensor voor ROS-productie te activeren. Uit deze resultaten kan geconcludeerd worden dat LAP het belangrijkste autofagie-gerelateerde proces is dat verantwoordelijk is voor de afweer van macrofagen tegen een systemische *Salmonella*-infectie.

Vervolgens werd in **Hoofdstuk 4** onderzocht welke virulentiefactoren van *Salmonella* invloed hebben op de pathogeniciteit in het zebravismodel en op de inductie van LAP als afweerrespons. Hierbij werden vijf *Salmonella*-mutanten bestudeerd die allemaal de accumulatie van GFP-Lc3 rond gefagocyteerde bacteriën konden opwekken via een Rubicon-afhankelijk mechanisme. Stammen met mutaties in de virulentiefactoren PhoP of PurA, respectievelijk betrokken bij adaptatie aan de intracellulaire omgeving en bij het metabolisme van purines, waren minder goed in staat om zebravisembryo's te infecteren dan de wildtype-stam. Echter, de virulentie van deze twee verzwakte stammen nam toe wanneer LAP werd uitgeschakeld door middel van genetische inhibitie van Rubicon. Het is bekend dat mutaties in factoren van de type-III-secretiesystemen (T3SS), waaronder SipB (T3SS-systeem 1) en SsrB (T3SS-systeem 2), belangrijk zijn voor invasie van en replicatie in niet-fagocytische cellen. Mutaties in deze factoren hadden echter geen invloed op de capaciteit van *Salmonella* om infectie te veroorzaken in het zebravismodel, waarin de bacteriën actief door macrofagen gefagocyteerd worden. In tegenstelling tot de SipB-mutant, vertoonde de SsrB-mutant geen toegenomen virulentie in een Rubicon-deficiënte gastheer, wat suggereert dat effectoren van het T3SS-systeem 2 belangrijk zijn voor de intracellulaire replicatie van *Salmonella* in afwezigheid van LAP. Ten slotte bleek mutatie van FlhD, noodzakelijk voor de productie van flagellen, te resulteren in hypervirulentie van *Salmonella* in zowel wildtype-zebravisembryo's als in Rubicon-deficiënte gas-

theren. Mutatie van FlhD resulteerde tevens in een verlaagd niveau van GFP-Lc3-accumulatie in vergelijking met de wildtype-stam, waaruit opgemaakt kan worden dat herkenning van flagellen door het aangeboren immuunsysteem van de zebravisgastheer de LAP-respons stimuleert. Deze resultaten versterken het bewijs dat het Rubicon-afhankelijke LAP-proces een belangrijk afweermechanisme is van macrofagen tegen *S*. Typhimurium.

Het membraaneiwit Dram1 is een door stress en infectie induceerbare modulator van autofagie, waarvan de genexpressie wordt aangestuurd door de transcriptiefactoren p53 en NFκB. Met behulp van een zebravismodel voor tuberculose is eerder aangetoond dat Dram1 bescherming biedt tegen de intracellulaire pathogeen *Mycobacterium marinum*. Bij deze infectie stimuleert Dram1 de p62-afhankelijke autofagie gericht tegen bacteriën die uit fagosomen ontsnapt zijn naar het cytoplasma. Het was echter nog onbekend of Dram1 ook andere autofagie-gerelateerde processen, zoals LAP, kan beïnvloeden. Het onderzoek in **Hoofdstuk 5** was erop gericht om deze vraag te beantwoorden met behulp van het systemische *Salmonella*-infectiemodel, waarin de Lc3-respons door LAP bepaald wordt. Genetische inhibitie (via morpholino-knockdown) of mutatie van het *dram1*-gen (via CRISPR/Cas9-technologie) resulteerde in een toegenomen groei van *Salmonella*-bacteriën en een verhoogde mortaliteit van de geïnfecteerde zebravisembryo's. In overeenstemming hiermee kon de replicatie van Salmonella en de mortaliteit van de geïnfecteerde embryo's juist beperkt worden door *dram1* tot overexpressie te brengen (via mRNA-injectie). Microscopische analyse van GFP-Lc3-accumulatie liet een sterke correlatie zien tussen de mate van LAP-inductie en het expressieniveau van *dram1*, met ongeveer 50% verlaging van GFP-Lc3-*Salmonella*-associaties bij uitschakeling van *dram1* (knockdown of mutatie) en ongeveer 30% verhoging bij overexpressie van *dram1*. Door middel van experimenten met een *Salmonella*-biosensor werd aangetoond dat ook ROS-productie in het fagosoom, wat bepalend is voor de inductie van LAP, grotendeels afwezig is in *dram1*-mutanten. Deze resultaten tonen aan dat Dram1 belangrijk is voor de afweer tegen *S*. Typhimurium en dat er een functioneel verband is tussen Dram1 en de inductie van LAP.

Zoals samengevat en bediscussieerd in **Hoofdstuk 6**, heeft het onderzoek dat in dit proefschrift wordt beschreven nieuwe inzichten opgeleverd

in de rol van de autofagiemachinerie bij afweer tegen *Salmonella*. Deze inzichten zijn in het bijzonder relevant voor de systemische stadia van *Salmonella*-infecties waarbij de pathogeen de macrofagen van de gastheer parasiteert. Het onderzoek heeft aangetoond dat een aan autofagie gerelateerd mechanisme, namelijk Lc3-geassocieerde fagocytose of kortweg LAP, aangrijpt op Salmonella-bacteriën wanneer deze worden opgenomen door de fagocyten, met name de macrofagen, van het zebravisembryo. Rubicon en NADPH-oxidase zijn essentiële gastheerfactoren voor deze respons. Deficiëntie in deze factoren doet de virulentie toenemen van zowel wildtype-*Salmonella* als van verzwakte stammen, waaruit blijkt dat LAP fungeert als een beschermend mechanisme van de gastheer tegen *Salmonella*-infecties. Het onderzoek heeft bovendien een nieuw functioneel verband aangetoond tussen de autofagiemodulator Dram1 en het proces van LAP-inductie. De resultaten hebben uitgewezen dat resistentie van zebravisembryo's tegen systemische *Salmonella*-infecties verbeterd kan worden door de Dram1-activiteit genetisch te verhogen. Dit is een aanmoediging voor vervolgonderzoek gericht op de identificatie van medicijnen om de autofagiemachinerie te stimuleren als therapie tegen antibioticum-resistente *Salmonella*-infecties.

Curriculum vitae

Curriculum vitae

Samrah Masud was born on November 7th 1978 in Layyah, Pakistan, and received her basic education in her home country. She obtained her High school certificate in 1993 from the Federal Government High School, Jehlum Cantt. She attained her Secondary High school certificate in 1996, at the Government College for girls, Muzaffer Garh. She received her Bachelor degree in Biology in 1998 with Botany, Zoology and Chemistry as major subjects. She obtained her Master degree in Zoology in 2000, from the Bahaudin Zakriya University, Multan. During her Master study she wrote a dissertation on the subject of aquaculture entitled "Dynamics of body composition and growth rate of Thaila carp (*Catla catla*)" under the supervision of prof.dr. Abdus Salam. After her master study, she obtained a government teaching position in 2002, as a lecturer in Biology at the Government College for girls, Layyah, and moved on in 2003 to a permanent position as university lecturer at the Bahaudin Zakriya University, Multan, in the area of Zoology. While teaching, she continued her higher education at the same university, where she achieved the M.Phil degree in Zoology (18 years of education) in 2005 with a dissertation "Effect of feed cycling on specific growth rate, condition factor and body composition of grass carp (*Ctenopharyngodon idella*)", under the supervision of prof.dr. Muhammad Ali. She continued teaching the principles of genetics, cell biology and developmental biology to Master and M.Phil students until 2012. In the meantime, she won a competitive foreign PhD scholarship under the faculty development program of the Bahaudin Zakriya University, Multan, financed by the Higher Education Commission of Pakistan (HEC). In December 2012, she joined the group of prof.dr. A.H. Meijer (Institute of Biology, Leiden University), as a PhD candidate with this scholarship. During her initial training period, she mastered infection models of zebrafish with *Mycobacterium marinum* and *Salmonella Typhimurium.* Subsequently, she focused her research efforts on investigating the dynamics of autophagy and related processes during *Salmonella* infections, resulting in the work described in this thesis, which has revealed Lc3-associated phagocytosis as the main autophagy-related defense response of the zebrafish host against *Salmonella*. She will resume her university lecturer position at the Bahaudin Zakriya University, Multan, in November 2017.

List of publications

List of publications

Masud, S., Prajsnar, T. K., Gerda, E.M. Lamers., Benning, M., Torraca, V., & Meijer., A. H. Macrophages target *Salmonella* by Lc3-associated phagocytosis in a systemic infection model. *Submitted*.

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