New ways to control *Shigella* infection in the zebrafish model

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Submitted for the degree of Doctor of Philosophy
September 2017
Abstract

ABSTRACT

Shigella flexneri is an invasive bacterial pathogen and inflammatory paradigm that has led to key discoveries in innate immunity. The transparent zebrafish (Danio rerio) larva is highly amenable to in vivo microscopy and has emerged as a valuable model for the study of host defence against bacterial infection. In this thesis, I use the zebrafish to explore the innate immune response to Shigella infection, and illuminate new ways to control bacterial infection in vivo.

Septins are an evolutionarily conserved family of cytoskeletal proteins with diverse biological functions. Very recent work has shown a key role for components of the cytoskeleton in inflammation control, yet a role for septins was unknown. In Chapter 3, I develop a zebrafish hindbrain model of Shigella infection and reveal a new role for septins in the restriction of inflammation crucial for host defence.

The global rise in antibiotic resistance amongst human pathogens has prompted an urgent search for novel antimicrobials. Bdellovibrio bacteriovorus are predatory bacteria that invade and kill Gram-negative species. Bacterial predation has been studied extensively in vitro but little is known of its efficacy in vivo. In Chapter 4, I use the Shigella-zebrafish infection model developed in Chapter 3 to show how immune cells and Bdellovibrio can work together to cure infection from multidrug resistant Shigella.

Septins were discovered for their essential roles in cell division. S. flexneri is controlled by neutrophils in vivo, and emergency granulopoiesis is a haematopoietic program of neutrophil production used to counteract immune cell exhaustion during infection. In Chapter 5, I use
Abstract

*Shigella* infection of zebrafish to reveal a cell autonomous role for septins in haematopoietic stem cell-driven emergency granulopoiesis and in enhancing host immunity.

Overall, the findings in this thesis have important implications for the treatment of inflammatory, infectious and haematological disease. As a result of these data, manipulation of the septin cytoskeleton represents a novel strategy by which to control inflammation. Furthermore, results obtained using *Bdellovibrio* are promising for the future consideration of living therapies to treat bacterial infection. Finally, the host cell division machinery can be used to modulate the haematopoietic response to infection and boost host defence. Collectively, results obtained using our *Shigella*-zebrafish infection model are highly translatable to higher vertebrates, including humans.
DECLARATION OF ORIGINALITY

I certify that the data presented in this thesis are my own, or else appropriately referenced in figure legends. Data in Chapter 3 were obtained in collaboration with Maria Mazon-Moya, Vincenzo Torraca, Laurent Boucontet and Emma Colucci-Guyon. Data in Chapter 4 were obtained in collaboration with Christopher Moore, Maria Mazon-Moya, Sina Krokowski, Carey Lambert and Elizabeth Sockett. Data in Chapter 5 were obtained in collaboration with Vincenzo Torraca and Jennifer Shelley.

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ACKNOWLEDGEMENTS

First and foremost, to my supervisor Dr Serge Mostowy: thank you for listening to and considering my opinions on matters science and otherwise. Thank you for taking the time to teach me how to write more succinctly, present more cleanly and publish at all. Thank you for saying yes to most every opportunity that you came across because it made the experience all the richer. Finally, thank you for not showing me that video of a horse. Maybe you can thank me for making it socially acceptable to cry in your office. It’s been real.

To my co-supervisor Dr Cristina Lo Celso: thank you for your unwavering enthusiasm and helpful insight throughout my masters and PhD, and also for letting me babysit Ansel. Thank you to Prof Liz Sockett, who wrote my reference for Imperial and was reincarnated in my second year as my intellectually superior lab partner. It is unique to come across someone so busy, who can still prioritise a zebrafish-themed dinner party. Thank you also to Chris and Carey, who helped make the Bdellovibrio paper possible.

I thank all those gracing the Mostowy lab. Special thanks to: Maria, who introduced me to fried Béchamel sauce and just about every detail of a zebrafish – thank you for your patience, and for contributing very significantly to the work in this thesis; Nic, the youth who may one day ruthlessly discard my CV; Nagisa, who kindly organised the zebrafish and I; Damian, who adopted the role of annoying older brother and Romanian linguistics tutor, and Vincenzo, whose presence made science more magic – thank you for arriving early to inject morpholinos and for staying late to discuss platybelodons and failed diets.
To my unaffiliated Imperial friends: firstly, literally anyone that opened the door to me (not metaphorically): Alex, Peter, Camilla, Megan, Daphne … Special thanks to Carrie, better known as the receiving end – thank you for listening, caring, and for sharing my love of a canapé; David, who formulated poor excuses to Uber home; Regina, whose snacks were occasionally my only sustenance; Julian, who advocated cocktails on school nights and hangovers on weekdays; Robbie, who resolved my existential crises with crosswords, and finally to Bridget, who is excellent and an entirely unwitting role model.

I would like to acknowledge the Wellcome Trust and the Lister Institute of Preventative Medicine for funding the Mostowy Lab during my PhD. I am grateful to be paid to study and my final academic thanks go to the Medical Research Council (MRC) and the Defense Advanced Research Projects Agency (DARPA).

To my London family: Amy, Chloe, Lizzie and Hannah, thank you for being lovely and supportive friends, especially Beth, who suffered to read a scientific paper. Thank you to Jack, who maintained my social life by booking family-run Italians and hotels in Magaluf, and Lil, who shared and halved my problems, my cider and occasionally, my utility bills. To Sina, my more rational counterpart who I feel very lucky to have shared much of my PhD with – thank you for masterminding every ridiculous plan we ever made, you made a wonderful nativity donkey. To Sarah, thank you for spending the last 5 years coming up with grand unifying theories, which could serve as my moral compass for up to 7 days, and for enthusiastically cohabiting space-time with me. To Sophie, thank you for coming to London, making me pasta dishes and looking after me.

My final, very important, acknowledgement is to my parents, Elena and John Willis. Thank you for all of your love and support, without which this thesis would of course not exist.
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<th>Description</th>
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<tbody>
<tr>
<td>AGM</td>
<td>Aorta-gonad-mesonephros</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-recruitment domain</td>
</tr>
<tr>
<td>Cb</td>
<td>Carbenicillin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CHT</td>
<td>Caudal haematopoietic tissue</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CRISPR/Cas</td>
<td>Clustered regularly interspaced short palindromic repeats and CRISPR-associated genes</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilisation</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post infection/injection</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>G-MCSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocytic-macrophage progenitors</td>
</tr>
<tr>
<td>HBV</td>
<td>Hindbrain ventricle</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>HPC</td>
<td>Haematopoietic progenitor cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>HSPC</td>
<td>Haematopoietic stem and progenitor cell</td>
</tr>
<tr>
<td>il-1b</td>
<td>interleukin 1 beta</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin 1 receptor</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>lta4h</td>
<td>leukotriene A4 hydrolase</td>
</tr>
<tr>
<td>lyz</td>
<td>lysozyme C</td>
</tr>
<tr>
<td>Mo</td>
<td>Morpholino antisense oligonucleotide</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mpeg1</td>
<td>macrophage-expressed gene 1</td>
</tr>
<tr>
<td>mpx</td>
<td>myeloid-specific peroxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerisation domain</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS, 0.1% (w/v) Tween20®</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTU</td>
<td>1-phenyl-2-thiourea</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>R</td>
<td>Resistant</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse-transcription quantitative PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>spp.</td>
<td>Species</td>
</tr>
<tr>
<td>Sm</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>tnf-α</td>
<td><em>tumour necrosis factor alpha</em></td>
</tr>
<tr>
<td>TSB</td>
<td>Trypticase soy broth</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume in final volume</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight in g per 100 ml</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>YPSC</td>
<td>Yeast-extract-peptone-calcium</td>
</tr>
<tr>
<td>YT</td>
<td>Yeast-tryptone</td>
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</tbody>
</table>
CHAPTER 1. INTRODUCTION

N.B. Owing to the broad range of topics covered in this thesis, this chapter will provide only the background common to Results Chapters 3-5, i.e. *Shigella* pathogenesis and the zebrafish as a model of infection. In-depth introductions of topics specific to a given results chapter will be provided therein.

1.1. *Shigella flexneri* is a causative agent of bacillary dysentery

*S. flexneri* is a Gram-negative, enteroinvasive pathogen closely related to *Escherichia coli*. Following ingestion, *Shigella* invade the colonic mucosa and cause shigellosis (i.e. inflammatory destruction of the gut epithelium) in primates, including humans (Niyogi, 2005). *Shigella* is estimated to cause 163 million disease cases each year, resulting in 1 million deaths (Lima et al., 2015). Furthermore, *Shigella* is included amongst the ESKAPE (Enterobacter spp.) group of pathogens that are considered to be an urgent threat to human health owing to their widespread resistance to many frontline antibiotics (Khaghani et al., 2014, Sivapalasingam et al., 2006, Boucher et al., 2009, Rice, 2010). Given that traditional antibiotics have failed to provide a long-lasting solution to human infection, many research groups are now exploring alternative antibacterial therapies (Taubes, 2008, Reardon, 2015, Spellberg et al., 2013, Peters et al., 2008). In particular, the predatory bacterial species *Bdellovibrio* which hunts Gram-negative prey has been widely studied in vitro, and has the potential to become a novel ‘living therapy’ for bacterial infection in humans (this will be discussed further in Chapter 4) (Sockett and Lambert, 2004, Kadouri et al., 2013, Shatzkes et al., 2017a).
1.2. *Shigella* virulence determinants

*Shigella* pathogenesis is mediated by a large virulence plasmid that encodes a multiprotein needle-like complex called the Type III Secretion System (T3SS) (Deng et al., 2017, Schroeder et al., 2007). Delivery of bacterial effectors to host cells via the T3SS enables pathogen invasion, vacuolar escape, and intracytosolic survival (Phalipon and Sansonetti, 2007, Mattock and Blocker, 2017). Cell-to-cell spread is mediated by IcsA, an autotransporter expressed at the bacterial surface, which promotes actin tail formation and escape from cellular immunity (Bernardini et al., 1989, Stevens et al., 2006) (Fig.1.1).

**Figure 1.1. The intracellular lifestyle of S. flexneri**

*Shigella* secretes bacterial effectors into the host cell via the T3SS. Cytoskeletal rearrangements are triggered at the plasma membrane and promote bacterial uptake into a

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Chapter 1. Introduction
phagocytic vacuole. T3SS effectors mediate vacuole rupture and *Shigella* escape into the cytoplasm where they are able to replicate. Cytosolic bacteria polymerise host actin and form an ‘actin tail’, which enables intracellular motility and escape from host defences. Actin-based motility also promotes the formation of bacterial protrusions, enabling cell-to-cell spread. Rupture of the double-membrane vacuole releases *Shigella* into the cytosol of a neighbouring cell to repeat the infection cycle. Figure adapted from (Krokowski and Mostowy, 2016).

1.3. An overview of the innate immune system

Innate immunity is the first line of defence against invading pathogens (Riera Romo et al., 2016). In contrast to adaptive immunity, the innate immune system recognises molecules that are conserved amongst pathogens and typically functions without memory of past infection. Innate immunity encompasses physical barriers and secreted chemicals that prevent infectious agents entering the body, as well as the pathogen-sensing cell types that release cytokines and the effector leukocytes that are recruited to eliminate the invader (Lacy, 2015). Amongst the effector cells are professional phagocytes such as macrophages and neutrophils (Silva and Correia-Neves, 2012). These highly motile cells engulf bacteria and are central to bacterial clearance. Interactions between *S. flexneri* and macrophages and neutrophils are crucial to bacterial pathogenesis and are described below (Fig 1.2) (Phalipon and Sansonetti, 2007).
Figure 1.2. A model of *S. flexneri* infection at the intestinal epithelium

*Shigella* are taken up by M-cells in the intestine via T3SS-mediated endocytosis. *Shigella* that cross the intestinal lining encounter large numbers of macrophages that phagocytose the bacteria. In macrophages, *S. flexneri* undergo further intracellular replication and promote pyroptotic cell death. Inflammatory cytokines released by macrophages recruit large numbers of neutrophils to the site of infection, which further disrupt host tissue for the benefit of invading *Shigella*. Importantly, neutrophils are able to kill *Shigella*, and finally resolve both infection and inflammation in an immune-competent host. Figure adapted from (Ashida et al., 2015).
1.4. *S. flexneri* interactions cause macrophage cell death

During the initial stages of infection, *S. flexneri* cross the intestinal lining via microfold cells (or M-cells) present in the epithelium, and enter a connective tissue where they encounter large populations of macrophages (Wassef et al., 1989, Sansonetti et al., 1996). *Shigella* taken up into macrophages by phagocytosis are able to escape to the cytosol, replicate intracellularly and promote macrophage killing via bacterial effectors (Zychlinsky et al., 1992, Zychlinsky et al., 1996). Characterisation of this process provided the first example of a bacterium inducing programmed host cell death. Though originally described as apoptosis, subsequent studies have labelled macrophage death ‘pyroptosis’ i.e. a highly inflammatory form of cell death, with a role in orchestrating the antibacterial response (described further in Section 1.9) (Hilbi et al., 1998, Suzuki et al., 2007, Hermansson et al., 2016).

1.5. Neutrophils are key for host defence against *S. flexneri*

Neutrophils are the most abundant circulating leukocyte in humans, and key mediators of acute inflammation (Kolaczkowska and Kubes, 2013, Jones et al., 2016). Macrophage death releases proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Zychlinsky et al., 1994, Sansonetti et al., 2000), which serve to recruit massive numbers of neutrophils to the infection site from nearby vasculature (Sansonetti et al., 1999, Singer and Sansonetti, 2004). Whilst neutrophil migration can benefit the pathogen by causing further tissue damage, thus enabling further bacterial invasion, these effectors are crucial to the resolution of infection (Phalipon and Sansonetti, 2007). Neutrophils use diverse mechanisms to kill *Shigella*, including phagocytosis and degradation of the pathogen, as well as the production of reactive oxygen species (ROS) (Mandic-Mulec et al., 1997). Indeed, the release of neutrophil extracellular traps (NETs), i.e. decondensed nuclear chromatin and granule-derived bactericidal proteins (e.g. neutrophil elastase), by dying neutrophils was first characterised using *Shigella*, where they were shown to immobilise bacteria and degrade...
bacterial effectors (Brinkmann et al., 2004). To counteract bacterial killing, S. flexneri have also evolved ways to subvert neutrophil-mediated immunity. For example, binding of granular antimicrobials to Shigella can improve adherence to epithelial cells, rendering bacteria hyperinvasive (Eilers et al., 2010). S. flexneri can also induce necrosis in neutrophils in a T3SS-dependent manner during the early stages of infection, though the bacterial effectors that mediate killing are not yet known (Francois et al., 2000). Given that neutrophils are essential to the resolution of both infection and inflammation, it is crucial that these cells be replaced for the host to maintain immunocompetency. Replenishment of leukocyte populations requires the proliferation and differentiation of haematopoietic stem and progenitor cells (HSPCs), and thus these precursors can be considered an integral part of the host immune system (Prendergast and Essers, 2014). Infection-induced neutrophil production is termed ‘emergency granulopoiesis’ and has not yet been tested in the context of Shigella infection (this will be discussed further in Chapter 5) (Manz and Boettcher, 2014).

1.6. S. flexneri is used to study cell-autonomous immunity

The intracellular infection process of S. flexneri is well-characterised and as such, this pathogen has become an inflammatory paradigm and a popular tool for cell biology (Sansonetti, 2006). Studies using Shigella have contributed to major advances in immunology and shed light on host defence mechanisms including nucleotide-binding oligomerisation domain (NOD) -like receptors (NLRs), autophagy, and the inflammasome (Girardin et al., 2003, Brinkmann et al., 2004, Ogawa et al., 2005, Hermansson et al., 2016).

1.7. Shigella are recognised by host NLRs

Innate immunity to bacterial infection is largely mediated by inflammatory signalling cascades induced upon host sensing of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Mogensen, 2009). In the case of intracellular
bacteria such as *Shigella*, host cell invasion stimulates a family of cytosolic sensors called NLRs (Kim et al., 2016). NLRs are highly conserved proteins, and homologues of eight key NLRs can be found in most vertebrate species, including the zebrafish. In particular, zebrafish NOD1 and NOD2 share 50.9% and 47.9% sequence identity with their human orthologues. NLRs are comprised of: (i) a pathogen-sensing C-terminal leucine-rich repeat (LRR) domain, (ii) a core NOD domain that mediates oligomerisation upon activation, and (iii) an N-terminal protein-protein interaction domain that varies between receptors, e.g. a caspase-recruitment domain (CARD) or a pyrin domain (PYD) (Ting et al., 2008a). Detection of *Shigella*-derived peptidoglycan fragments by the prototypical NOD1 and NOD2 receptors drives self-oligomerisation and activates mitogen-activated protein kinase (MAPK) and nuclear factor -κB (NF-κB) signalling pathways (Girardin et al., 2001, Kufer et al., 2008, Kufer et al., 2006). Receptor activation can be potentiated by additional NLRs, such as NLRP10 in epithelial cells, or inhibited by host proteins, such as ATG16L (Lautz et al., 2012, Sorbara et al., 2013). Furthermore, NLRs have been shown to directly regulate other aspects of cellular immunity, including: ROS production, inflammasome activity, and bacterial autophagy (Tattoli et al., 2008, Ting et al., 2008b, Travassos et al., 2010).

### 1.8. *Shigella* effectors for evasion of bacterial autophagy

During autophagy, cytoplasmic constituents are sequestered within a double-membrane bound compartment called an ‘autophagosome’, and degraded by lysosome-derived hydrolytic enzymes (Yang and Klionsky, 2010, Bento et al., 2016). As well as recycling host components, autophagy can also be used to target bacteria for destruction (Levine et al., 2011, Huang and Brumell, 2014). In the case of *S. flexneri* infection, autophagy machinery can be recruited: to the plasma membrane upon detection of bacterial peptidoglycan by NOD receptors during pathogen entry (Travassos et al., 2010), to *Shigella*-containing phagocytic vacuoles in a T3SS-dependent manner (Campbell-Valois et al., 2015), to danger-associated molecular patterns (DAMPs) and ubiquitinated remnants of the ruptured
phagocytic vacuole following pathogen escape (Dupont et al., 2009), and to cytosolic
*Shigella* via recognition of the actin-polymerising effector IcsA (Ogawa et al., 2005). To
counteract bacterial autophagy and promote intracellular survival, *S. flexneri* employs a
number of effectors. Early during infection i.e. whilst in the phagocytic vacuole, *Shigella*
effector IcsB binds the host protein Toca-1 to prevent recruitment of key autophagy proteins
including, light-chain 3 (LC3), to the autophagosome (Baxt and Goldberg, 2014). Cytosolic
bacteria also express IcsB, but in this case the effector binds and masks IcsA to inhibit
autophagy; this finding was the first example of a bacterial effector to prevent host
recognition to promote intracellular survival (Ogawa et al., 2005). Finally, VirA is secreted
during invasion of epithelial cells and inactivates host GTPase Rab1; failure of the host to
activate Rab1 disrupts membrane trafficking and suppresses autophagosome formation
(Dong et al., 2012). Interestingly, VirA is one of only two known bacterial effectors able to
directly suppress the host autophagy machinery. Collectively, the studies described here
well-illustrate how *Shigella* can be used to give a broad view of both host defence
mechanisms, and the bacterial mechanisms that have evolved to counter them (Krokowski
and Mostowy, 2016).

1.9. Fine-tuning of the host inflammasome by *S. flexneri*

Inflammasomes are multiprotein complexes that assemble upon infection, initiating
inflammatory signalling cascades and in certain cases, causing pyroptotic cell death (Broz
and Dixit, 2016, Guo et al., 2015, Martinon et al., 2002). Upon the detection of PAMPs or
other indicators of pathogen invasion (e.g. increases in ROS or changes in ion gradient),
NLRs bind and activate the cysteine protease pro-Caspase-1, via adaptor proteins. Active
Caspase-1 then mediates the maturation of proinflammatory cytokines, including the
cleavage of pro-IL-1β to IL-1β, and enables their secretion (Martinon et al., 2002). Manipulation of the inflammasome by *Shigella* is key to establishing infection, and was first
observed in macrophages. Here, following phagosomal escape, bacterial effector IpaB binds
directly to Caspase-1, triggering formation of the NLRC4 inflammasome and macrophage pyroptosis (Hilbi et al., 1998, Suzuki et al., 2007). Macrophage cell death induces tissue damage and releases bacteria for further host cell invasion. In contrast, Shigella dampens inflammasome activity in epithelial cells. In this case, remodelling of the bacterial lipopolysaccharide (LPS) enables Shigella to replicate more silently and for longer within the cytoplasm prior to eventual cell death (Paciello et al., 2013). These findings serve to highlight the complex relationship between inflammation control and host defence against bacterial infection.

1.10. The cytoskeleton as a regulator of cell-autonomous immunity

Recently, components of the cytoskeleton, i.e. the host’s structural proteins, have been linked to processes of cellular immunity (Mostowy and Shenoy, 2015). In particular, a family of cytoskeletal proteins known as septins have been shown to respond to bacterial pathogens, including Shigella, and mediate bacterial autophagy (Mostowy and Cossart, 2012, Mostowy et al., 2010, Sirianni et al., 2016, Torraca and Mostowy, 2016). Actin interacts extensively with septins and has been heavily implicated in inflammatory control (Mostowy and Shenoy, 2015, Schmidt and Nichols, 2004b); however, a role for septin in inflammation has not yet been tested (this will be discussed further in Chapter 3).

1.11. Animal models to study Shigella in vivo

Shigella is a human pathogen, and our inability to faithfully replicate the symptoms of shigellosis in a non-primate animal model has limited our understanding of the disease (Kim et al., 2013). Whilst rabbit, guinea pig and mouse models of colitis have all been used to study S. flexneri infection in vivo previously, to establish disease requires complex experimental approaches that limit the translatability of findings. Examples of techniques used to enhance Shigella virulence include the infection of neo-nates, antibiotic-mediated
clearance of the host microbiome prior to infection, and the transplant of human intestine into mice (Fernandez et al., 2003, Rabbani et al., 1995, Shim et al., 2007, Perdomo et al., 1994, Singer and Sansonetti, 2004, Zhang et al., 2001). The recent development of a murine model of intraperitoneal infection is promising; here, S. flexneri can invade and colonise cells of the large intestine, as well as induce a strong proinflammatory response (Yang et al., 2014). This new mouse model is an attractive candidate for the future investigation of bacillary dysentery in vivo, and can be expected to replace existing Shigella infection models, such as the guinea pig cornea and murine lung, for the evaluation of vaccine efficacy (Hartman et al., 1991, Mallett et al., 1993). Interestingly, the zebrafish has emerged as a novel animal model to study Shigella infection (Mostowy et al., 2013).

1.12. The zebrafish model organism to study vertebrate biology

The zebrafish (Danio rerio) is a powerful non-mammalian model for the study of vertebrate biology in vivo (Grunwald and Eisen, 2002, Lin et al., 2016). Zebrafish are highly fecund and have a short generation time, ensuring the availability of a large number of externally fertilised eggs. The recent release of a fully-annotated reference genome highlighted the zebrafish as a model of translational research and revealed zebrafish orthologues for 70% of human genes, including 82% of human disease genes (Howe et al., 2013). Collectively, these attributes support simple genetic manipulation of the zebrafish using morpholino antisense oligonucleotides (for transient knockdown of a target gene), mRNAs (for transient overexpression of a given protein), and transcription activator-like effector nucleases (TALENS) and clustered regularly interspaced short palindromic repeats and CRISPR-associated gene 9 (CRISPR/Cas9) (for targeted genome editing) (Bill et al., 2009, Yuan and Sun, 2009, Huang et al., 2016, Li et al., 2016a). Such methods have been applied for the rapid screening of gene function and have contributed to the generation of an array of fluorescent transgenic lines (Shah et al., 2015). Despite major advances in the field of in vivo imaging, it remains difficult to visualise biological processes non-invasively at high resolution.
in a mammalian model (Zelmer and Ward, 2013). In contrast, the zebrafish larva is optically accessible and highly amenable to non-invasive \textit{in vivo} imaging (Godinho, 2011). Moreover, studies conducted in zebrafish present significantly less ethical concern than comparable studies in higher vertebrates.

### 1.13. The zebrafish larva as a model to study infection \textit{in vivo}

The transparent zebrafish larva is a highly suitable and innovative model to image infection and innate immunity \textit{in vivo} (Kanther and Rawls, 2010, Renshaw and Trede, 2012, Meijer et al., 2014). Live imaging of fluorescently-tagged bacteria offers immediate insight into the spatiotemporal events underlying infection, and transgenic larvae with fluorescent leukocytes can be used to dissect the host immune response in real time. The zebrafish larva is uniquely suited to study innate immunity because an adaptive immune system only becomes functional from ~30 days post fertilisation (dpf) (Renshaw and Trede, 2012). The innate immune cells of zebrafish and humans are highly homologous, both phenotypically and functionally, and zebrafish studies have provided important insight into neutrophil and macrophage function (Harvie and Huttenlocher, 2015, Torraca et al., 2014). As in humans, zebrafish macrophages can be identified by expression of \textit{macrophage-expressed gene 1} (\textit{mpeg1}) (Spilsbury et al., 1995, Ellett et al., 2011). Similarly, neutrophils can be distinguished by expression of \textit{lysozyme C} (\textit{lyz}) and \textit{myeloid-specific peroxidase} (\textit{mpx}), as well as their segmented nuclei and antimicrobial granules (Hall et al., 2007, Renshaw et al., 2006, Lieschke et al., 2001). Importantly, zebrafish macrophages and neutrophils are able to phagocytose and kill bacteria, and neutrophils can also generate respiratory bursts and release NETs in order to destroy a pathogen (Herbomel et al., 1999, Meijer et al., 2008, Yang et al., 2012, Palic et al., 2007).
1.14. Insights into host-pathogen interactions using the zebrafish model

The zebrafish larva has previously been used to model infection by a number of important viral, fungal and bacterial pathogens, including *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, *Mycobacterium marinum* and *S. flexneri* (van der Sar et al., 2003, Levraud et al., 2009, Myllymaki et al., 2016, Mazon Moya et al., 2014). Studies using the *Salmonella*-zebrafish infection model have revealed novel bacterial effectors for the suppression of autophagy (Li et al., 2016b), discovered new inflammasome components important for bacterial clearance (Tyrkalska et al., 2016), and identified infection-inducible host proteins important for mitochondrial ROS production and bactericidal activity (Hall et al., 2013). Furthermore, infection of zebrafish with *L. monocytogenes*, has shown important roles for macrophages in inflammasome-mediated host defence (Vincent et al., 2016) and immune factors crucial for protecting the host against pore forming bacterial toxins (Mesquita et al., 2017). Finally, high resolution imaging of zebrafish larvae has been useful in visualising and characterising the autophagic response to *M. marinum* (Hosseini et al., 2014, Meijer and van der Vaart, 2014), and has shown lysosomal function, the neutrophil inflammasome and reprogramming of macrophages, to be key determinants in disease control and granuloma formation, with implications for the treatment of *Mycobacterium tuberculosis* (Berg et al., 2016, Kenyon et al., 2017, Cronan et al., 2016).

1.15. The Shigella-zebrafish infection model

In recent years, the zebrafish has been developed to investigate the cell biology of *S. flexneri* infection *in vivo* (Mostowy et al., 2013). Although not a natural fish pathogen, *Shigella* is able to recapitulate many of the key aspects of human pathogenesis in zebrafish larvae, including: host cell invasion, phagosomal escape, intracytosolic replication and rearrangement of the host cytoskeleton. Critically, *S. flexneri* can induce zebrafish death in a dose-dependent manner when injected systemically, and pathogenicity is dependent upon a
functional T3SS. Microscopy analysis of host-pathogen interactions showed a crucial role for neutrophils in bacterial clearance and eliminating macrophages that, as in humans, had succumbed to infection. Moreover, the Shigella-zebrafish model was used to study bacterial autophagy in vivo. In this study, both suppression of autophagy (using a p62 morpholino antisense oligonucleotide) and upregulation of autophagy (using rapamycin), increased bacterial burden and reduced host survival. These results highlight the requirement for very precise manipulation of bacterial autophagy for therapeutic purposes.

1.16 Summary and aims of this study

S. flexneri is a human pathogen and an inflammatory paradigm used to reveal novel aspects of host cell biology and bacterial pathogenesis (Sansonetti, 2006). The zebrafish larva is an important model to visualise processes of development and infection (Detrich et al., 2010, Kanther and Rawls, 2010, Renshaw and Trede, 2012, Meijer et al., 2014). In this thesis, I use Shigella infection of zebrafish as a system to discover novel approaches to control bacterial infection in vivo. My specific aims are detailed below.

1. Develop a Shigella-zebrafish infection model to study in vivo the role of septins in inflammation and infection control (Chapter 3).

2. Investigate the therapeutic potential of the predatory bacteria, Bdellovibrio against antimicrobial resistant infection, using our Shigella-zebrafish infection model (Chapter 4).

3. Use the Shigella-zebrafish infection model to study the role of emergency granulopoiesis in host defence, and septins in haematopoietic stem cell biology (Chapter 5).


Data to support Aim 3 is a manuscript to be submitted in October 2017. **Willis, A.R.**, Torraca, V., Shelley, J., Mazon-Moya, M., Lo Celso, C., Mostowy, S. Septin-mediated emergency granulopoiesis rescues zebrafish larvae from secondary infection.
CHAPTER 2. MATERIALS AND METHODS

2.1. Zebrafish maintenance

2.1.1. Ethics statement

Animal experiments were in accordance with the Animals (Scientific Procedures) Act 1986. Experiments conducted at Imperial College London and the University of Nottingham were approved by the Home Office under Project Licenses: PPL 70/7446 and PPL 30/3378, respectively.

2.1.2. Zebrafish husbandry

Wild type (WT) AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR, USA). Details of transgenic zebrafish lines are provided in Table 2.1. Embryos were collected from naturally spawning zebrafish, bleached with 0.003% sodium hypochlorite for 5 min and reared in Petri dishes containing Embryo Medium (15 mM NaCl, 1 mM MgSO₄, 500 μM KCl, 150 μM KH₂PO₄, 50 μM Na₂HPO₄, 0.3 μg/ml methylene blue). Embryos, including infected larvae, were maintained at 28.5°C and developmentally staged according to Kimmel et al (1995). Larval development is depicted in Fig. 2.1. For live-imaging and injections, larvae were anaesthetised in Embryo Medium containing 200 μg/ml Tricaine (ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich, St Louis, MO, USA). For injections or administration of drugs prior to 3 dpf, larvae were dechorionated manually. Step-wise protocols are available for many of the zebrafish techniques described (Willis et al., 2016a).
Table 2.1. Details of transgenic zebrafish lines

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(lyz:dsRed)&lt;sup&gt;nz50&lt;/sup&gt;</td>
<td>Neutrophils</td>
<td>(Hall et al., 2007)</td>
</tr>
<tr>
<td>Tg(mpx:GFP)&lt;sup&gt;i114&lt;/sup&gt;</td>
<td>Neutrophils</td>
<td>(Gray et al., 2011)</td>
</tr>
<tr>
<td>Tg(il-1b:GFP-F)&lt;sup&gt;zf550&lt;/sup&gt;</td>
<td>il-1b-expressing</td>
<td>(Nguyen-Chi et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>il-1b-expressing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
</tr>
<tr>
<td>Tg(mpeg1:Gal4-FF)&lt;sup&gt;g225&lt;/sup&gt;/Tg(UAS-E1b:nfsB.mCherry)&lt;sup&gt;c264&lt;/sup&gt;</td>
<td>Macrophages</td>
<td>(Ellett et al., 2011)</td>
</tr>
<tr>
<td>Tg(mpeg1:YFP)&lt;sup&gt;w200&lt;/sup&gt;</td>
<td>Macrophages</td>
<td>(Roca and Ramakrishnan, 2013)</td>
</tr>
<tr>
<td>Tg(runx:mCherry)</td>
<td>HSPCs</td>
<td>(Tamplin et al., 2015)</td>
</tr>
<tr>
<td>Tg(runx:eGFP)</td>
<td>HSCs</td>
<td>(Tamplin et al., 2015)</td>
</tr>
</tbody>
</table>
Figure 2.1. Life cycle of the zebrafish

Zebrafish undergo rapid development from a single-celled zygote positioned on top of a yolk sac, to a free-swimming larva within 2 days. Zebrafish reach sexual maturity at ~3 months post fertilisation. Genetic manipulations (morpholino, mRNA injections) are injected up to ~1 hours post fertilisation. Bacterial injections in this thesis are performed from 2 dpf. Figure adapted from (D’Costa and Shepherd, 2009).
Chapter 2. Materials and Methods

2.2. Bacterial work

2.2.1. Bacterial preparation

Details of bacterial strains are provided in Table 2.2. Protocols requiring sterility were performed using aseptic technique; reagents were filter sterilised with a 0.2 µm filter or autoclaved at 121° for 15 min. Where appropriate, bacteria were cultured with 25 µg/ml kanamycin or 50 µg/ml carbenicillin.

Strains of Shigella were streaked and maintained on plates of Tryptic Soy Broth (TSB; 17 g / L casein peptone, 5 g / L NaCl, 3 g / L soya peptone, 2.5 g / L K2HPO4, 2.5 g / L glucose; 12 g / L; Sigma-Aldrich) and Bacteriological Agar (15 g / L; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.01 % (w/v) Congo Red Dye (Sigma-Aldrich). Strains of S. flexneri serotype 5a M90T were cultured at 37°C, shaking (209 rpm). For infection assays, Shigella strains were grown in 5 ml TSB overnight from a single bacterial colony, diluted 32-fold in 8 ml fresh TSB and grown until OD600nm = 0.6. Bacteria harvested via centrifugation (5 min, 4°C, 4500 x g) were washed and resuspended in phosphate-buffered saline (PBS) for a final concentration of 2.0 x 10^3 colony forming units (CFUs) / nl. Preparations were maintained on ice pending injection.

Strains of B. bacteriovorus were cultured at 30°C, shaking (200 rpm). For infection assays, Bdellovibrio ‘starter cultures’ grown overnight by predation on E. coli S17-1 pZMR100 were observed microscopically to confirm purity (i.e. an absence of E. coli) as well as motility of Bdellovibrio and passed through a 0.45 µm filter to remove residual prey. Bdellovibrio were prepared in 50 ml volumes comprising: 10 ml Bdellovibrio starter culture, 10 ml culture of stationary phase E. coli S17-1 pZMR100 grown overnight in Yeast-Tryptone medium (YT; Sigma Aldrich) from a single colony on a YT agar plate, 30 ml CaHEPES buffer (25 mM HEPES, 2 mM CaCl2, pH 7.6). Cultures were grown for 3 h and bacteria harvested by
centrifugation (20 min, 23°C, 1600 x g), washed and resuspended in PBS for a final concentration of $1.0 \times 10^5$ plaque forming units (PFUs) / nl. Preparations were maintained at room temperature (RT) pending injection. Strains of *Bdellovibrio* comprising plasmid-encoded fluorescent proteins were used for experiments in Fig. 4.2H, 4.3, 4.4D-4.4F, 4.5A; chromosomally-tagged fluorescent strains were used in all other experiments.

**Table 2.2. Details of bacterial strains**

<table>
<thead>
<tr>
<th>Species</th>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em></td>
<td>WT M90T</td>
<td>BUG 2505, Serotype 5a, Sm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Mostowy et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>GFP</td>
<td>M90T, expresses GFP from chromosome, Sm&lt;sup&gt;R&lt;/sup&gt;, Cb&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Mostowy et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>mCherry</td>
<td>M90T, pFPV25.1-mCherry construct, Sm&lt;sup&gt;R&lt;/sup&gt;, Cb&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Salgado-Pabon et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Crimson</td>
<td>M90T, pE2-Crimson construct, Sm&lt;sup&gt;R&lt;/sup&gt;, Cb&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Siriani et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>T3SS mCherry</td>
<td>ΔmxiD strain M90T, Sm&lt;sup&gt;R&lt;/sup&gt;, Cb&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Salgado-Pabon et al., 2013)</td>
</tr>
<tr>
<td><em>B. bacteriovorus</em></td>
<td>HD100:pk18</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Willis et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>Bd0064mTeal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HD100:pk18</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Willis et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>Bd0064mCherry</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bd0064mTeal</td>
<td></td>
<td>(Willis et al., 2016b)</td>
</tr>
<tr>
<td></td>
<td>chromosomal</td>
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</tr>
</tbody>
</table>
2.2.2. Infection of zebrafish larvae

Sites of hindbrain ventricle, caudal vein and tail muscle injection are indicated (Fig. 2.2.). Bacteria was delivered by borosilicate glass capillaries (Harvard Bioscience, Holliston, MA, USA) prepared with a P-87 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Up to 2 nl bacterial suspension was microinjected into larvae using an IM-300 Microinjector (Narshige, London, UK) and a M-152 micromanipulator (Narshige). For ease of injection, embryos were aligned in channels of a 1 % (w/v) agarose (Bioline Reagents, London, UK) injection plate; visual protocols for larval injection are available (Mazon Moya et al., 2014). Where applicable, control larvae were injected with an equivalent volume of PBS to the corresponding bacterial injection. In Chapter 3, 3 dpf larvae were injected in the hindbrain or caudal vein with a low dose (0.5-3.0 x 10^3 CFUs) or high dose (1.0-2.0 x 10^4 CFUs) of S. flexneri. In Chapter 4, for assays testing Bdellovibrio alone, 3 dpf larvae were injected in the hindbrain, tail muscle or caudal vein with 1.0-100.0 x 10^4 PFUs of Bdellovibrio. For coinfection assays, larvae were injected in the tail muscle or hindbrain with either 1.0 x 10^3 or >5.0 x 10^3 CFUs of Shigella respectively, and subsequently with 1.0-2.0 x 10^5 PFUs of Bdellovibrio 30-90 min after initial infection (i.e. an MOI (multiplicity of infection) of 5-40 in the hindbrain or an MOI of ~100 in the tail muscle). To test Bdellovibrio replication, a reduced MOI of 10^4-10^1 (i.e. 2.0-6.0 x 10^5 CFUs of Shigella, 1.0-30.0 x 10^2 PFUs of Bdellovibrio) was used to detect Bdellovibrio progeny without being masked by a large standing number
of ‘excess’ non-invasive *Bdellovibrio*. In **Chapter 5**, for single infections, 2 dpf larvae were injected in the hindbrain or caudal vein with a low dose (0.5-3.0 x 10\(^3\) CFUs) of *S. flexneri*. For dual infection assays, 2 dpf larvae were injected with PBS or ‘primed’ with a low dose of *S. flexneri* and subsequently infected with a high dose (1.0-2.0 x 10\(^4\) CFUs) of *S. flexneri* at 4 dpf i.e. 2 days post infection.

![Figure 2.2. Infection sites in the zebrafish larva](image)

**Figure 2.2. Infection sites in the zebrafish larva**

Zebrafish larvae were injected in the hindbrain ventricle (HB), tail muscle (TM), or caudal vein (CV), shaded in red. Figure taken from (Willis et al., 2016b).

### 2.2.3. Quantification of inocula and bacterial burden in the zebrafish

To determine *Shigella* inocula and infection burdens, larvae were homogenised mechanically in 200 µl Lysis Buffer (PBS 0.1 % Triton™ X-100; Sigma-Aldrich). Triton™ X-100 enabled recovery of invasive intracellular *Shigella*. Homogenates were serially diluted in PBS and plated on Lennox Lysogeny Broth (LB; 10 g / L tryptone, 5 g / L yeast extract, 5 g / L NaCl, pH 7.5; Thermo Fisher Scientific) - Bacteriological Agar (15 g / L). Plates were incubated at 37°C overnight prior to enumeration of CFUs. To minimise contribution of the microflora to counts, only fluorescent colonies of the appropriate morphology were scored. For dual infection assays performed in **Chapter 5**, *Shigella* burden was assessed by fluorescence stereomicroscopy. Here, the larval hindbrain was defined as a ‘region of interest’ and was subject to ‘threshold’ and ‘measure area percentage’ functions on ImageJ.
Final values for single larvae are given as percentage fluorescence of the hindbrain. For assessment of bacterial burden by either plating of CFUs or image analysis, only viable larvae were considered; non-viable larvae were excluded from analyses.

To determine *Bdellovibrio* inocula and bacterial burden, larvae were homogenised in 200 µl PBS, serially diluted and plated on yeast-extract-peptone-calcium (YPSC) soft agar overlay plates comprising *E. coli* S17-1 for provision of a prey lawn (Stolp and Starr, 1963). Plates were incubated at 30°C for 5-10 days to enable plaque formation; *Bdellovibrio* were enumerated as PFUs. Only viable larvae were assessed for bacterial burden; non-viable larvae were excluded from analyses.

In cases where surplus larvae were unavailable, inocula was determined by direct injection of bacterial suspension into PBS prior to serial dilution and plating of CFUs or PFUs.

### 2.2.4. Zebrafish survival assays

Single larvae were maintained in wells of a 12-well plate and imaged using a light stereomicroscope at time points following infection. Larvae failing to produce a heartbeat or larvae in which bacteria had compromised the hindbrain ventricle were considered non-viable.

### 2.2.5. *In vitro* bacterial predation assays

For automated plate-reader assays, $1.0-3.5 \times 10^5$ CFUs / µl of GFP-*Shigella* were incubated at 37°C with $4.0-5.0 \times 10^7$ PFUs / µl of mCherry-*Bdellovibrio* in a total volume of 200 µl CaHEPES buffer per well of a 96-well multi-well plate (Greiner Bio-One, Kremsmünster,
Austria). For each experiment, samples were run in triplicate wells and \( \text{OD}_{600\text{nm}} \) and fluorescence intensity measured at regular intervals using a microplate reader (TECAN Infinite M200 Pro, TECAN, Männedorf, Switzerland). For manual viable counting assays, 5.0-12.0 x 10^4 CFUs / \( \mu l \) of GFP-\textit{Shigella} were incubated with 6.0 x 10^6 PFUs / \( \mu l \) of mCherry-\textit{Bdellovibrio} in a total volume of 10 ml CaHEPES buffer at 29°C. At time points following incubation, 200 \( \mu l \) of co-culture was sampled for \textit{S. flexneri} enumeration by serial dilution and plating on LB agar as described in Section 2.2.3. Predation from the described co-cultures was visualised by wide-field microscopy as described in Section 2.4.2.

2.3. Zebrafish methodology

2.3.1. Morpholino and mRNA injection of zebrafish embryos

Morpholino antisense oligonucleotides were purchased from GeneTools (www.gene-tools.com; Philomath, OR, USA). Details of morpholinos are provided in Table 2.3. To control for non-specific effects of morpholino injection, a standard control morpholino with no known target in the zebrafish genome was used alongside experimental morpholinos, at a similar concentration (Mostowy et al., 2013). Stock concentrations of morpholino were prepared according to manufacturer’s instruction and diluted to a working concentration of 0.5-1 mM in 0.1-0.2 % Phenol Red (Sigma-Aldrich) in PBS immediately prior to injection. For overexpression of \textit{lta4h}, 200 ng/\( \mu l \) RNA was prepared as previously described and 1.2 nl / embryo injected (Tobin et al., 2012). Solutions were microinjected into 1-8 cell stage embryos using borosilicate glass capillaries, an IM-300 Microinjector and a M-152 Micromanipulator as described in Section 2.2.2. For ease of injection, embryos were aligned in channels of a 1 % (w/v) agarose injection plate; visual protocols for morpholino injection are available (Yuan and Sun, 2009). Larvae showing developmental delay or severe morphological defects were excluded from experimentation.
Table 2.3. Details of morpholinos

<table>
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<th>Target</th>
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<th>Morpholino sequence (5’-3’)</th>
<th>Dose</th>
<th>Action</th>
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<td>Varied</td>
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<td>(Mostowy et al., 2013)</td>
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<td>SB</td>
<td>(Mazon-Moya et al., 2017)</td>
</tr>
<tr>
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<td>1-2 nl of</td>
<td>SB, TB</td>
<td>(Clay et al., 2007, Su et al., 2007)</td>
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<td>SB</td>
<td>(Hall et al., 2012)</td>
</tr>
</tbody>
</table>

SB, splice-blocking; TB, translation-blocking

2.3.2. Pharmacological manipulation of zebrafish larvae

To block IL-1 receptor signaling, Embryo Medium was supplemented with premade anakinra (Kineret®, Swedish Orphan Biovitrum, Stockholm, Sweden) to a final concentration of 10 µM and refreshed daily from 1 dpf until assay completion. To deplete macrophages in zebrafish...
larvae, a stock concentration of 1 M Metronidazole (Sigma-Aldrich) was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Larvae were bathed in 10 mM Metronidazole, 1 % DMSO in Embryo Medium from 1 dpf to assay completion; control larvae were exposed to 1 % DMSO alone.

2.3.3. Western blotting of zebrafish larvae

For extraction of zebrafish proteins, 5-10 embryos were mechanically homogenised in 200 µl Extraction Buffer (1 M Tris, 5 M NaCl, 0.5 M EDTA, 0.01% Triton X-100). Homogenates were centrifuged (15 min, 4°C, 16,000 x g) and supernatant transferred to a 1.5 microcentrifuge tube containing 50 µl Laemml Buffer (10 mM Tris-HCl pH 6.8, 2 % (w/v) sodium dodecyl sulfate (SDS), 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 0.01 % (w/v) Bromophenol Blue (Sigma-Aldrich)). Extracts were heated at 95°C for 15 min, loaded onto a 5 % polyacrylamide stacking gel (0.125 M Tris (pH 6.8), 0.1 % (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.01 % (v/v) TEMED), and electrophoresed on an 8 % polyacrylamide resolving gel (0.375 M Tris (pH 8.8), 0.1 % (w/v) SDS, 0.1 % (w/v) APS, 0.06 % (v/v) TEMED). Samples were run at ~100 V for 45 min alongside a protein marker. Proteins separated by electrophoresis were transferred to a nitrocellulose polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA) previously activated in 100 % ethanol and equilibrated in Transfer Buffer (192 mM glycine, 25 mM Tris, 10 % methanol). Transfer was conducted at 15 V, 2.5 mA for 15 min. Following transfer, membranes were incubated in Blocking Buffer (5 % (w/v) non-fat dried milk in PBST (PBS, 0.1 % (v/v) Tween®-20 (Sigma-Aldrich)) for 1 h at RT. Membranes were blotted with a 1:2000 dilution of primary rabbit anti-SEPT7 antibody (IBL, Hamburg, Germany), or anti-GAPDH (GeneTex, Irvine, CA, USA) as a loading control, in Blocking Buffer, overnight at 4°C. Membranes were washed 3x for 5 min in PBST, before final incubation with a 1:2000 dilution of HRP-conjugated anti-rabbit secondary antibody in Blocking Buffer for 1 h at RT. Membranes were washed 3x in PBST and proteins detected by chemiluminescence using
ECL Western Blotting Detection Reagents (GE Healthcare, Amersham UK) as per manufacturer’s instruction, and a ChemiDoc Touch Imager (Bio-Rad Laboratories, Hercules, CA, USA).

2.3.4. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was harvested from 5-10 snap-frozen and extracted using the RNAqueous®-Micro Kit (Thermo Fisher Scientific) as per manufacturer’s instruction. RNA was reverse transcribed using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) as per manufacturer’s instruction. Primers for il-1b, tnf-a and gcsfa are previously described (Stockhammer et al., 2009, Hall et al., 2012). Quantitative PCR reactions were performed in technical duplicate or triplicate on a Rotor GeneQ (Qiagen) thermocycler and using SYBR Green Reaction Power Mix (Thermo Fisher Scientific). Quantities of cDNA were normalised using the housekeeping gene ef1a1l1 (Mostowy et al., 2013) and the 2-ΔΔCT method (Livak and Schmittgen, 2001).

2.3.5. Acridine orange staining

For staining of dead and dying cells, larvae were bathed in Embryo Medium supplemented with 2 µg / ml acridine orange hemi(zinc chloride) salt (Sigma-Aldrich) from 20 hours post infection (hpi) for 30 min. Larvae were washed 3x in Embryo Medium and imaged immediately by confocal microscopy.

2.3.6. Caspase-1 activity assay

Caspase-1 activity was detected using the FAM-FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN, USA) as previously described (Progatzky et al., 2014). Briefly, a 150X stock concentration of FAM-YVAD-FMK was
prepared according to manufacturer’s instruction. For each experimental condition, 5-10 larvae were transferred to a 1.5 ml microcentrifuge tube and bathed in 60 µl of 1X FAM-YVAD-FMK reagent diluted in Embryo Medium. Larvae were treated with reagent from 4.5 hpi to 6 hpi and washed 3x in Embryo Medium immediately prior to confocal imaging.

2.3.7. Immunostaining of zebrafish larvae

For each experimental group, up to 10 larvae were transferred to a single 1.5 ml microcentrifuge tube and fixed overnight in PBS, 4 % (w/v) paraformaldehyde (PFA), 0.4 % (v/v) Triton™ X-100 at 4°C. Fixed larvae were washed 3x for 5 min in PBS, 0.4 % (v/v) Triton™ X-100 and then for 20 min in PBS, 1 % (v/v) Triton™ X-100 at RT. Larvae were bathed in Blocking Buffer II (PBST, 10 % (v/v) foetal bovine serum (FBS), 1 % (v/v) DMSO) for 1 h at RT before supplementing with a 1:1000 dilution of human anti-SEPT7 antibody (Immuno-Biological Laboratories, Gunma, Japan) and incubating overnight at 4°C. Larvae were washed 4x for 15 min in 0.1% PBST at RT before overnight incubation in Blocking Buffer II supplemented with a 1:500 dilution of secondary antibody at 4°C. Larvae were washed 4x for 15 min in 0.1% PBST at RT and cleared by incubation in a glycerol series diluted in 0.4 % PBST as follows: 15 % (v/v) glycerol (1 h, RT), 30% (v/v) glycerol (2-3 h, RT), 60 % (v/v) glycerol (overnight, 4°C), 80% (v/v) glycerol (overnight, 4°C). Larvae were stored at 4°C in 80% (v/v) glycerol prior to imaging.

2.4. Microscopy and image analysis

2.4.1. In vivo microscopy of zebrafish

To prevent melanin synthesis in larvae, Embryo Medium was supplemented with 0.003 % 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) from 24 hpi. For transmission and fluorescence stereomicroscopy, larvae were maintained in Petri dishes and imaged using a Leica M205FA
microscope (Leica Microsystems, Wetzlar, Germany) with a 10x (NA 0.5) dry objective. For confocal microscopy, larvae were maintained in glass-bottom 35 mM dishes (MatTek Corporation, Ashland, MA, USA) and imaged using an Inverted Zeiss LSM 710 microscope (Carl Zeiss AG, Oberkochen, Germany) with 10x, 20x, 40x oil, 63x oil and 100x oil immersion objectives. For single image capture, live anaesthetised larvae were maintained in Embryo Medium. For time-lapse imaging, live anaesthetised larvae were immobilised in 1% (w/v) TopVision Low Melting Point Agarose (Thermo Fisher Scientific) in Embryo Medium. Fixed, immunostained larvae were transferred to a drop of 80% (v/v) glycerol for imaging. Multiple position Z-stacks of up to 400 µm were acquired at regular intervals.

2.4.2. *In vitro* microscopy of bacteria

Bacteria were mounted on agarose pads and widefield microscopy was performed on an Axiovert 200 M microscope (Carl Zeiss AG) using a 63x oil immersion objective.

2.4.3. Image processing and image analysis

All .LSM .LIF, .TIF, .PNG and .AVI files were processed and analysed using ImageJ/FUJI software (Schneider et al., 2012). Assessment of bacterial burden by fluorescence stereomicroscopy is described in Section 2.2.3. Leukocyte and stem cell quantifications were performed manually from images of transgenic larvae taken by stereomicroscopy. Analysis of acridine orange staining was automated and performed on a hindbrain ‘region of interest’; maximally projected Z-stacks taken by confocal microscopy were subject to ‘threshold’ and ‘analyse particle’ functions on ImageJ. Analysis of Caspase-1 activity was automated and performed on a hindbrain ‘region of interest’; maximally projected Z-stacks taken by confocal microscopy were subject to ‘threshold’ and ‘measure area percentage’ functions on ImageJ. Confocal and widefield images represent maximum projections of a
series of Z-stacked images. Stereomicroscopy images represent single slices of a Z-stack image series.

### 2.5. Statistical analyses

Data are represented as Mean ± standard error of the mean (SEM). Statistical significance for: host cell quantifications, cell death counts, Caspase-1 assays, bacterial enumerations and gene expression data was determined by Student’s t-test. Student’s t-test was performed on raw data for host cell quantifications, cell death counts and Caspase-1 assays. Student's t-test was performed on Log$_{10}$ values for bacterial enumerations and Log$_2$ values for gene expression data. Where appropriate, Bonferroni post-test was used for multiple testing. Statistical significance for zebrafish survival assays and in vitro bacterial predation assays were determined by log rank Mantel-Cox test. Statistical analyses were conducted using Prism software (GraphPad Software Inc.).
CHAPTER 3. SEPTINS RESTRICT INFLAMMATION AND PROTECT ZEBRAFISH LARVAE FROM SHIGELLA INFECTION

3.1. Introduction

3.1.1. The cytoskeleton is a key determinant of innate immunity

The eukaryotic cytoskeleton is a structural network comprised of four main elements: actin, microtubules, intermediate filaments and septins (Fletcher and Mullins, 2010, Mostowy and Cossart, 2012). Bacterial pathogens commonly manipulate the cytoskeleton to enable host cell invasion and support intracellular survival (Haglund and Welch, 2011). Conversely, cytoskeletal components have been implicated in bacterial sensing and antibacterial functions including autophagy and inflammation control (Mostowy, 2014, Rogers et al., 1992). As such, the cytoskeleton is widely regarded as a key player in innate immunity and can represent a novel target for infection therapies (Mostowy and Shenoy, 2015).

3.1.2. A role for the cytoskeleton in inflammasome activation

It has recently emerged that cytoskeletal components play key roles in inflammasome regulation (Mostowy and Shenoy, 2015). Studies have shown that clustering of actin filaments blocks activation of the NLRP3 inflammasome, and defects in actin depolymerisation stimulate the pyrin inflammasome (Pelegrin and Surprenant, 2009, Johnson et al., 2013, Kim et al., 2015). Consistent with this, aberrant actin dynamics have now been linked to autoinflammatory disease in humans (Standing et al., 2017). Moreover, recent work has implicated microtubules in inflammasome assembly, and the intermediate filament vimentin in inflammasome activation (Misawa et al., 2013, dos Santos et al., 2015). Despite accumulating evidence for cytoskeleton-inflammasome interplay, a role for septins in inflammation control has not yet been tested.
3.1.3. Septins: the fourth component of the cytoskeleton

Septins are a highly conserved family of filament forming proteins (30-65 kDa) (Mostowy and Cossart, 2012, Nishihama et al., 2011); members are subdivided into four groups (called the SEPT2, SEPT3, SEPT6 and SEPT7 groups) based on amino acid sequence homology, with each group named after the founding member (Cao et al., 2007, Pan et al., 2007). Each individual septin possesses: (i) a polybasic region which directly binds phosphoinositides, (ii) a guanosine-triphosphate (GTP) -binding domain and (iii) a septin unique element (SUE) of unknown function (Zhang et al., 1999, Casamayor and Snyder, 2003, Leipe et al., 2002, Pan et al., 2007). These core elements are flanked by amino- (N) and carboxy- (C) termini of varying lengths (Pan et al., 2007). Septins of distinct groups interact via GTP-binding domains (G interface) and N- and C- termini (NC interface) to form symmetrical hetero-oligomeric complexes, such as hexamers and octomers (Fig. 3.1) (Sirajuddin et al., 2007, Sirajuddin et al., 2009). These complexes assemble end-to-end to form filaments and higher order structures, such as bundled filaments (up to 32-40 nm in length) and rings (~0.6 µm in diameter) (Fig. 3.2) (Bertin et al., 2008, Garcia et al., 2011, DeMay et al., 2011). Septin expression patterns vary between the cell types of a given organism, and thus the composition of septin filaments can differ between tissues. How different septin configurations affect the biochemical and physical properties of the septin filament is largely unknown. Using X-ray crystallography, the structure of a human SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7 complex was revealed, highlighting its non-polar assembly (Sirajuddin et al., 2007, John et al., 2007). Moreover, this analysis revealed that human SEPT7 (the only member of the SEPT7 group) is essential for filament assembly and septin function. Although poorly understood, interactions between septins are viewed to be regulated by conformational changes induced by GTP binding, hydrolysis and post-translational modifications (Sirajuddin et al., 2007, Sirajuddin et al., 2009, Garcia et al., 2011, Zhang et al., 2000, Johnson and Blobel, 1999, Dobbelae re et al., 2003). Septin function is further mediated by extensive interactions with membrane, actin and microtubules (Tanaka-

Figure 3.1. Molecular structure of septin filament

(A) Schematic of the common structures and classifications of the septin family. Septins are divided into four groups based on sequence homology. Members of the SEPT6 group lack a key threonine residue (Thr78; indicated by asterisk) and so are unable to hydrolyse GTP to GDP.

(B) The crystal structure of the SEPT2-SEPT6-SEPT7 hexameric complex. Complexes assemble to form septin filaments. Arrows indicate the position of septin coiled-coil domains, which are not represented in the crystal structure. Arrowheads indicate the location of septin polybasic regions.

Figure taken from (Mostowy and Cossart, 2012).
3.1.4. The roles of septins *in vivo*

Septins preferentially assemble at sites of micron-scale membrane curvature, and are found at the cleavage furrow (discussed further in Chapter 5), the base of phagocytic cups, dendritic spines and cilia, the annulus of sperm, and sites of mitochondrial fission (Bridges et al., 2016, Lobato-Marquez and Mostowy, 2016, Pagliuso et al., 2016, Sirianni et al., 2016, Ihara et al., 2005, Mostowy et al., 2009, Huang et al., 2008, Hu et al., 2010, Tada et al., 2007, Kinoshita and Noda, 2001) (**Fig. 3.3**). Here, septins function as scaffolds to recruit proteins or as barriers to limit diffusion for subcellular compartmentalisation (Caudron and Barral, 2009, Mostowy and Cossart, 2012, Kinoshita, 2006). As a consequence of their diverse biological functions, septins are implicated in a multitude of human diseases.
including cancer, neuropathies, ciliopathies, infertility and infection (Russell and Hall, 2005, Marttinen et al., 2015, Palander et al., 2017, Kuo et al., 2012, Torraca and Mostowy, 2016).

Figure 3.3. Roles for septins *in vivo*

**(A)** Septins form a ring at the cleavage furrow of dividing cells to recruit proteins for cytokinesis, and can act as a diffusion barrier to separate proteins between mother and daughter cells.

**(B)** A septin ring at the annulus of mammalian sperm forms a diffusion barrier to segregate the anterior and posterior tail.

**(C)** Septin rings at the base of cilia limit diffusion between ciliary and plasma membranes.

**(D)** In neurons, septins assemble at the neck of dendritic spines and act as diffusion barriers.

**(E)** Septins accumulate at the base of the phagocytic cup to regulate bacterial entry.

Figure adapted from (Mostowy and Cossart, 2012).
3.1.5. The role of septins in host-pathogen interactions

Septins are increasingly recognised for their roles in bacterial infection and host defence (Torraca and Mostowy, 2016). Septin polymerisation at the plasma membrane is proposed to remodel actin for host cell entry by invading enteropathogenic *E. coli*, *L. monocytogenes*, *S. Typhimurium* and *S. flexneri* (Scholz et al., 2015, Mostowy et al., 2009). Intracellularly, septins can form rings around cytosolic bacteria such as *L. monocytogenes*, *M. marinum* and *S. flexneri* that are polymerising actin (Mostowy et al., 2010, Mostowy et al., 2013). In the case of *S. flexneri*, septin assemblies can entrap bacteria in ‘cages’ via interactions with mitochondria, and these serve to promote autophagy and restrict bacterial replication (Sirianni et al., 2016, Mostowy et al., 2010). Initially characterised using human cell lines, septin-caging has also been described *in vivo* using *Shigella* infection of zebrafish larvae, highlighting the septin cage as an evolutionarily conserved assembly of host defence (Mostowy et al., 2013).

3.1.6. The study of septins in zebrafish

The zebrafish genome comprises 18 septins, including an orthologue for almost all of the 14 human septins, and at least 1 representative protein for each of the four human septin groups (*Table 3.1*) (Willis et al., 2016a). The zebrafish has contributed significantly to the field of developmental biology (Detrich et al., 2010). In the case of septins, *in vivo* studies using zebrafish have linked *Sept9a* and *Sept9b* to growth defects, demonstrated roles for multiple septins (*Sept8a*, *Sept8b*, *Sept3*, *Sept5a* and *Sept5b*) in the developing central nervous system, and shown the importance of *Sept15* in the regulation of actin for cardiac function (Landsverk et al., 2010, Berger et al., 2017, Helmpobst et al., 2017, Dash et al., 2017). For the study of disease, *Sept6-* and *Sept15-* depleted larvae display phenotypes resembling human ciliopathies (Zhai et al., 2014, Dash et al., 2014). Together, these studies well-demonstrate the power of the zebrafish model for translational septin research.
Table 3.1. Classification of zebrafish septins

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<th>PROTEIN ID</th>
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<th>Length (aa)</th>
<th>Position Septin-type G-domain</th>
<th>Septin group</th>
<th>Closest human homolog</th>
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3.1.7. Aims of this chapter

*Shigella* has significantly advanced our understanding of cellular microbiology and inflammation (Killackey et al., 2016). Using the transparent zebrafish larva, we have the unique opportunity to examine a complete innate immune response to *Shigella* infection in vivo. In this chapter, we establish a novel hindbrain *Shigella*-zebrafish model to study localised bacterial infection, and investigate the role of septins in innate immunity and inflammation control.
3.2. Results

3.2.1. Establishment of *Shigella* infection in the zebrafish hindbrain ventricle to study a localised infection

The hindbrain ventricle (HBV) of the zebrafish larvae is well-suited to study host pathogen interactions, as it is optically accessible and enables visualisation of leukocyte infiltration (Fig. 3.4A). To establish a *Shigella*-zebrafish HBV infection model, larvae were injected at 3 dpf with either a low dose (0.5-3.0 x 10^3 colony forming units (CFUs)) or high dose (1.0-2.0 x 10^4 CFUs) of *S. flexneri* M90T. Survival assays revealed that larval viability was unaffected by a low dose of *S. flexneri*, and high doses were associated with ~40% mortality within 48 hours post infection (hpi) (Fig. 3.4B). To quantify infection burden over time, homogenates of viable larvae were plated onto bacterial agar for enumeration of CFUs; larvae that had succumbed to infection were not included in analyses. Colony counts revealed a strong correlation between bacterial burden and survival. Larvae injected with a low dose of *Shigella* controlled bacterial replication and infection was largely eliminated by 48 hpi (Fig. 3.4C). In contrast, larvae inoculated with a high dose of *Shigella* showed increasing bacterial loads over time, indicative of uncontrolled bacterial replication. To follow the progression of infection *in vivo*, we performed time-lapse imaging of GFP-*Shigella* infected larvae using fluorescent stereomicroscopy. In agreement with CFU assays, larvae infected with a low dose of *Shigella* showed limited bacterial replication, whereas larvae infected with a high dose of *Shigella* showed increasing bacterial fluorescence at both 24 and 48 hpi (Fig. 3.4D). Surprisingly, even lethal doses of *Shigella* fail to disseminate outside the zebrafish hindbrain and forebrain. This is a fortunate aspect of our model, considering that a localised infection is ideal to study leukocyte interactions with bacteria over time. Pathogenesis of *S. flexneri* in humans is dependent on the T3SS (Schroeder et al., 2007, Mattock and Blocker, 2017). To determine the role of the T3SS in our *Shigella*-zebrafish infection model, we performed HBV infections using a T3SS-deficient (T3SS-) strain of *S. flexneri* (ΔmxiD strain). In agreement with human data, larvae infected with T3SS- *Shigella* presented ~100% survival and
controlled bacterial replication (Fig. 3.4E and 3.4F). Together, these results show that the zebrafish HBV can be used to study a dose-dependent response to *Shigella* infection *in vivo*.

**Figure 3.4. Establishment of *Shigella* infection in the zebrafish hindbrain ventricle to study a localised infection**

(A) Cartoon of zebrafish larva (3 dpf) showing localization of neutrophils prior to infection (red) and the site of *S. flexneri* M90T (green) injection in the HBV.

(B) Survival curves of WT AB larvae injected in the HBV at 3 dpf with a low dose ($\leq 3.0 \times 10^3$ CFUs) or high dose ($\geq 1.0 \times 10^4$ CFUs) of *S. flexneri* M90T. Larval viability was scored for 48
hpi. Data are pooled from five independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. ***, p < 0.001.

(C) Time-course enumeration of live *Shigella* from homogenates of WT AB larvae injected with a low or high dose of *S. flexneri* M90T as in (B). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from five independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(D) Representative images of WT AB larvae infected with a low or high dose of GFP-*S. flexneri* M90T (green) as in (B). For each dose, the same larva was imaged by fluorescent stereomicroscopy over 48 hpi. Scale bars, 100 µm.

(E) Survival curves of WT AB larvae injected in the HBV at 3 dpf with a low or high dose of T3SS- *S. flexneri* M90T (ΔmxiD strain). Larval viability was scored for 48 hpi. Data are pooled from 2 independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05.

(F) Time-course enumeration of live *Shigella* from homogenates of WT AB larvae injected with a low or high dose of T3SS- *S. flexneri* M90T as in (E). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from 3 independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05. **, p < 0.01; ***, p < 0.001.
3.2.2. Neutrophils control hindbrain infections of *S. flexneri* in zebrafish larvae

We next used our *Shigella*-zebrafish model to study the role of leukocyte populations in the control of a localised *Shigella* infection *in vivo*. For this, we outcrossed Tg(*mpeg1*:Gal4-FF)225/Tg(UAS-E1b:nfsB.mCherry)c264 with Tg(*mpx*:GFP)c114 zebrafish (herein referred to as *mpeg1*:G/U:mCherry and *mpx*:GFP) to generate double transgenic larvae with mCherry-expressing macrophages and GFP-expressing neutrophils (Ellett et al., 2011, Renshaw et al., 2006). Larvae were infected with a low dose of Crimson-*S. flexneri* and imaged by high resolution confocal microscopy to capture leukocyte-*Shigella* interactions (*Fig. 3.5A*). Here, we observed aggregates of *S. flexneri* form on the epithelial walls of the hindbrain prior to bacterial clearance. Macrophages were first to arrive at the infection site (~20 mpi), however failed to control replication of engulfed bacteria and underwent cell death. By contrast, neutrophil recruitment to the infection site occurred hours following the infection, and was required to phagocytose and eliminate bacteria as well as debris from macrophages overwhelmed during the *Shigella* infection process.

Considering the crucial role for neutrophils in *Shigella* clearance, we infected the HBV of Tg(*lyz*:dsRed)250 zebrafish larvae (herein referred to as *lyz*:dsRed), which harbour dsRed-expressing neutrophils, and imaged neutrophil-*Shigella* interactions by confocal microscopy (Hall et al., 2007). Whilst larvae infected with a low dose of *S. flexneri* displayed neutrophil recruitment concomitant with bacterial clearance (*Fig. 3.5B*), high dose infections were associated with uncontrolled bacterial replication and neutrophil cell death (*Fig. 3.5C*). Consistent with data obtained using a zebrafish model of systemic *S. flexneri* infection (Mostowy et al., 2013), these results using HBV infection highlight the important role of neutrophils in *Shigella* clearance *in vivo*. 

Data obtained in collaboration with M. Mazon-Moya.
Figure 3.5. Neutrophils control hindbrain infections of *S. flexneri* in zebrafish larvae

(A) Frames extracted from time-lapse confocal imaging of *mpeg1:G/U:mCherry x mpx:GFP* zebrafish larvae (with red macrophages and green neutrophils) injected in the HBV at 3 dpf with a low dose (≤3.0 x 10³ CFUs) of Crimson-*S. flexneri* M90T (white). First frame 20 mpi, followed by frames at 1, 3, and 12 hpi. Maximum intensity Z-projection images (2 μm serial optical sections) are shown. Scale bars, 50 μm.

(B-C) Frames extracted from time-lapse confocal imaging of *lyz:dsRed* larvae (with red neutrophils) injected in the HBV at 3 dpf with a low dose (B) or high dose (≥1.0 x 10⁴ CFUs) (C) of GFP-*S. flexneri* M90T (green). First frame 20 mpi, followed by frames at 3, 7 and 11 hpi. Maximum intensity Z-projection images (2 μm serial optical sections) are shown. Scale bars, 50 μm. Data obtained in collaboration with L. Boucontet and E. Colucci-Guyon.
3.2.3. Sept15 is required to control *S. flexneri* infection in vivo

Humans have 14 septins and structural analyses have revealed that human SEPT7 is essential for septin filament formation and function (Sirajuddin et al., 2007, Peterson and Petty, 2010). Zebrafish have 3 orthologues of SEPT7: Sept7a, Sept7b and Sept15 (Willis et al., 2016a). Consistent with a conserved role for SEPT7 between species, zebrafish Sept7b and Sept15 share 88.7% and 92.5% protein identity with human SEPT7, respectively (Table 3.1) (Willis et al., 2016a). Such high homology enables antibodies raised against human SEPT7 to be used in zebrafish (Mostowy et al., 2013). To determine the localisation of SEPT7 homologues in the zebrafish, either WT or transgenic larvae with fluorescent neutrophils and macrophages were labelled with human anti-SEPT7. Confocal microscopy of stained larvae revealed SEPT7 orthologues in all cell types tested: epithelial cells, macrophages and neutrophils (Fig. 3.6A).

To test *in vivo* the role of septins in host defence, we focused primarily on Sept15 as it is highly homologous to human SEPT7. Here, 1-8 cell stage zebrafish embryos were microinjected with either an morpholino antisense oligonucleotide against Sept15 or a control morpholino (i.e. with no known target in the zebrafish genome). Depletion of Sept15 was confirmed by Western blotting of 3 dpf zebrafish lysates with anti-SEPT7 (Fig. 3.6B, Appendix 1A and 1B). Given that septins have important roles in vertebrate development, the dose of Sept15 morpholino was optimised to achieve partial Sept15 knockdown in larvae without inducing any major morphological abnormalities. Larvae regarded as abnormal (i.e. developmentally delayed or with curved trunk or heart oedema) were excluded from analyses. To test the role of septins in host defence against *Shigella*, Sept15 morphants were injected at 3 dpf with a low dose of *S. flexneri* in the HBV. Survival assays and plating of CFUs revealed that, in contrast to control morphants, Sept15 morphants presented significantly reduced survival and were unable to restrict bacterial replication (Fig. 3.6C and 3.6D). Imaging of GFP-*Shigella* infections *in vivo* showed that, unlike control morphants,
bacterial burden was significantly increased in Sept15-depleted larvae at both 24 and 48 hpi (Fig. 3.6E). Similar defects were observed in Sept15 morphants infected with S. flexneri systemically via the caudal vein, and also in larvae depleted of Sept7b, an alternative SEPT7 homologue (Appendix 1C-1H). To assess the role of Shigella virulence in the susceptibility of Sept15 morphants, control or Sept15 morphants were infected with avirulent T3SS- S. flexneri in the HBV. Survival and CFU assays showed that Sept15 morphants do not have impaired survival upon T3SS- infection and are able to clear bacterial burden effectively (Fig. 3.6F and 3.6G). Collectively, these results show that Sept15 morphants are susceptible to S. flexneri infection, and that susceptibility is mediated by the Shigella T3SS.
Figure 3.6. Sept15 is required to control *S. flexneri* infection *in vivo*

**(A)** Immunostaining of zebrafish larvae at 3 dpf with antibody against SEPT7 (red). Images of (i) WT AB caudal fin epithelium (ii) *mpeg1*:YFP (with green macrophages) (iii) *mpx*:GFP (with green neutrophils). Scale bars, 10 µm. Data obtained by V. Torraca.

**(B)** Representative western blot of extracts from 3 dpf larvae injected with control (Ctrl) or Sept15 morpholino (Mo). Antibodies used against SEPT7 or GAPDH (as a control). Full blot shown in Appendix 1A.
(C) WT AB larvae were treated with Ctrl or Sept15 Mo. Morphant larvae were injected in the HBV at 3 dpf with a low dose (≤3.0 x 10^3 CFUs) of *S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. **, p < 0.01.

(D) Time-course enumeration of live *Shigella* from homogenates of WT AB Ctrl and Sept15 morphants injected with a low dose of *S. flexneri* M90T as in (C). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as p < 0.05. *, p < 0.05; ***, p < 0.001.

(E) Representative images of WT AB Ctrl and Sept15 morphants injected with a low dose of GFP-*S. flexneri* M90T (green) as in (C). For each condition, the same larva was imaged by fluorescent stereomicroscopy over 48 hpi. Scale bars, 100 µm.

(F) WT AB larvae were treated with Ctrl or Sept15 Mo. Morphant larvae were injected in the HBV at 3 dpf with a low dose of T3SS- *S. flexneri* M90T (ΔmxID strain). Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05.

(G) Time-course enumeration of live *Shigella* from homogenates of WT AB Ctrl and Sept15 morphants injected with a low dose of T3SS- *S. flexneri* M90T as in (F). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled
circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05.

Data for (B-G) obtained in collaboration with M. Mazon-Moya.

3.2.4. S. flexneri induces neutrophil death in Sept15 morphants

Neutrophils control Shigella infection in vivo (Mostowy et al., 2013, Phalipon and Sansonetti, 2007). To examine neutrophil function upon Sept15 depletion, transgenic morphants with dsRed-labelled neutrophils were infected with a low dose of GFP-Shigella in the HBV. Imaging by time lapse confocal microscopy revealed massive neutrophil recruitment to infection in both control and Sept15 morphants (Fig. 3.7A and 3.7B). However, unlike control morphants, neutrophils in Sept15-depleted larvae could not control infection and underwent cell death. To quantify neutrophil killing, we imaged fluorescent neutrophils in control and Sept15 morphants following HBV infection with S. flexneri. Counts in control morphants at 6 hpi showed that, although neutrophil number was unaffected by a low dose of S. flexneri, the neutrophil population was significantly reduced following a high dose infection (Fig. 3.7C). In contrast, quantifications of infected Sept15 morphants showed that neutrophil number was significantly reduced upon infection with either a low or high dose of Shigella infection (Fig. 3.7D). Importantly, infection with T3SS- S. flexneri did not deplete neutrophil populations in Sept15 morphants, indicating that Sept15-depleted neutrophils can control avirulent bacteria similar to control larvae (Fig. 3.7E and 3.7F).

Interestingly, uninfected Sept15 morphants comprise ~50% fewer neutrophils at 3 dpf (Fig. 3.7D, this will be more closely examined in Chapter 5). Irf8 is a lineage commitment factor
which boosts neutrophil number and suppresses monocyte production (Li et al., 2011). To
determine whether increased mortality in Sept15 morphants is a result of reduced neutrophil
populations in the developing larvae, we co-injected Sept15 and Irf8 morpholino
oligonucleotides to recover neutrophil number, and performed hindbrain infections (Fig.
3.7G). Increasing neutrophil numbers in Sept15 morphants did not significantly improve
larval survival or reduce bacterial burden (Fig. 3.7H and 3.7I). These data indicate that the
susceptibility of Sept15 morphants to Shigella infection is not due to fewer neutrophils per
se. Instead, these results suggest that the neutrophils of septin-depleted larvae are unable to
cope with bacterial challenge. As Irf8 depletion also leads to macrophage depletion, we
reasoned this may also enhance the susceptibility of Sept15 morphants to infection (Li et al.,
2011). To test this, macrophages in mpeg1:G/U:mCherry were ablated by exposure to
metronidazole, and 3 dpf larvae infected in the HBV with a high dose of S. flexneri. In line
with a role for macrophages in host defence against Shigella, larval mortality was
significantly increased in macrophage-depleted larvae (Fig. 3.7J and 3.7K). As in humans,
zebrafish macrophages are unable to control Shigella burden and undergo cell death
(Mostowy et al., 2013, Zychlinsky et al., 1992). We therefore propose that macrophages play
a key role in the initial phagocytosis of Shigella, and that bacterial containment aids efficient
scavenging by neutrophils; however, this hypothesis awaits investigation. In summary, these
data strongly suggest that neutrophil death is responsible for the susceptibility of septin
morphants to Shigella infection.
Chapter 3. Results

A. Neutrophils

B. Neutrophils

C. # neutrophils (Ctrl Mo)

D. # neutrophils (Sept15 Mo)

E. # neutrophils (Sept15 Mo)

F. # neutrophils (Sept15 Mo)

G. # neutrophils (Sept15 Mo)

H. % Survival

I. Log10 CFU

J. % Survival

K. Log10 CFU
Figure 3.7. S. flexneri induces neutrophil death in Sept15 morphants

(A-B) Lyz:dsRed zebrafish larvae (with red neutrophils) were treated with control (Ctrl) (A) or Sept15 (B) morpholino (Mo). Morphant larvae were injected in the HBV at 3 dpf with a low dose ($\leq 3.0 \times 10^3$ CFUs) of GFP-S. flexneri M90T (green). Frames extracted from time-lapse confocal imaging. First frame 20 mpi, followed by frames at 6, 15, and 20 hpi. Maximum intensity Z-projection images (2 µm serial optical sections) are shown. Scale bars, 50 µm. 

Data obtained in collaboration with L. Boucontet and E. Colucci-Guyon.

(C-D) Lyz:dsRed Ctrl (C) and Sept15 (D) morphants were not injected or injected in the HBV at 3 dpf with a low dose or high dose ($\geq 1.0 \times 10^4$ CFUs) of S. flexneri M90T. Neutrophils were quantified 6 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 4 larvae per time point per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance with Bonferroni correction defined as: p < 0.025. **, p < 0.005; ***, p < 0.0005.

(E-F) Lyz:dsRed Ctrl (E) and Sept15 (F) morphants were not injected or injected in the HBV at 3 dpf with a low or high dose of T3SS- S. flexneri M90T ($\Delta mxiD$ strain). Neutrophils quantified 6 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (n ≥ 4 larvae per time point per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance with Bonferroni correction defined as: p < 0.025.

(G) Quantification of neutrophils in lyz:dsRed larvae injected with Ctrl, Sept15 or Sept15+Irf8 Mo. Neutrophils quantified 3 dpf. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 5 larvae per condition per experiment). p values between conditions
determined by unpaired one-tailed Student’s t test. Significance with Bonferroni correction defined as: \( p < 0.025 \). ***, \( p < 0.0005 \).

(H) WT AB larvae were treated with Sept15 or Sept15+Irf8 Mo. Morphant larvae were injected in the HBV at 3 dpf with a low dose of *S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The \( p \) value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: \( p < 0.05 \).

(I) Time-course enumeration of live *Shigella* from homogenates of WT AB Sept15 or Sept15+Irf8 morphants injected with a low dose of *S. flexneri* M90T as in (H). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). \( p \) values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: \( p < 0.05 \).

(J) Survival curves of Ctrl or macrophage ablated (i.e. metronidazole treated) *mpeg1:G/U:mCherry* larvae injected in the HBV at 3 dpf with a high dose of *S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The \( p \) value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: \( p < 0.05 \). ***, \( p < 0.001 \).

(K) Time-course enumeration of live *Shigella* from homogenates of Ctrl or macrophage ablated *mpeg1:G/U:mCherry* larvae injected with a high dose of *S. flexneri* M90T as in (J). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are
representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05.

Data (C-K) obtained in collaboration with M. Mazon-Moya and V. Torraca.

3.2.5. Sept15 restricts the inflammatory response in vivo

Neutrophils are key for the control of acute inflammation (Jones et al., 2016, Kolaczkowska and Kubes, 2013). S. flexneri is a highly inflammatory pathogen (Mostowy et al., 2013, Schnupf and Sansonetti, 2012, Yang et al., 2014, Sansonetti, 2006). We therefore hypothesised that Sept15 morphants may be more susceptible to Shigella due to infection-induced inflammation. To visualise the spatiotemporal expression of key pro-inflammatory marker il-1b upon infection in vivo, we outcrossed Tg(il-1b:GFP-F)zf550 larvae (herein referred to as il-1b:GFP-F) which expresses farnesylated (i.e. membrane-targeted) GFP under the control of the il-1b promoter, with lyz:dsRed larvae with dsRed-labelled neutrophils (Nguyen-Chi et al., 2014). Double transgenic control or Sept15 morphants were infected in the HBV with a low dose of Crimson-Shigella and imaged by high resolution confocal microscopy. In both morphant types, we observed induction of il-1b in neutrophil, macrophage and epithelial cell populations at the infection site (Fig. 3.8A and 3.8B). Interestingly, we observed more obvious il-1b reporter activity occurring alongside increasing bacterial burden and host cell death in Sept15 morphants; however, total il-1b was not quantifiably different between control and Sept15 morphants because much of the il-1b derived fluorescence in Sept15 morphants dissipated upon neutrophil cell death. Caspase-1 activity is important for the processing and maturation of IL-1β (Kostura et al., 1989, Black et al., 1989, Thornberry et al., 1992, Sollberger et al., 2014). To test whether Caspase-1 activity is affected by the depletion of Sept15, we used a fluorochrome-labelled inhibitor of Caspase-1 (FLICA) assay
and the fluorescent probe FAM-YVAD-FMK, which irreversibly binds and inhibits active Caspase-1 (Progatzky et al., 2014). Using this assay, we could detect significantly more Caspase-1 activity in infected Sept15 morphants by 6 hpi (Fig. 3.8C). Caspase-1 is heavily implicated in cell death pathways (Miao et al., 2011, Jorgensen and Miao, 2015). To test whether Caspase-1 activity is associated with increased host cell death and larval mortality in Sept15 morphants, we quantified dying cells using acridine orange (AO). AO is a stain which binds nucleic acids and fluorescently labels dying cells (Tucker and Lardelli, 2007). Control and Sept15 morphants were infected with a low dose of *Shigella* in the HBV, and AO-positive cells quantified at 6 hpi. Analyses of images taken by confocal microscopy revealed significantly more dying cells (1.7 ± 0.3-fold) in infected Sept15 morphants, as compared to infected controls (Fig. 3.8D and 3.8E). Taken together, these data demonstrate that cell death induced by hyperinflammation is responsible, at least in part, for the susceptibility of Sept15 morphants to *Shigella* infection. Previous work has linked increases in TNF-mediated inflammation to defects in infection control (Tobin et al., 2010). We thus tested the role of *tnf*-a in susceptibility of Sept15 morphants to *Shigella* infection. For this, we injected *lta4h* mRNA into the 1-8 cell stage zebrafish larvae to increase Leukotriene A4 hydrolase protein and *tnf*-a expression without inducing *il-1b* (Appendix 2A and 2B). Increased levels of *tnf*-a were not associated with enhanced susceptibility to *Shigella* infection (Appendix 2C and 2D). These data indicate that the susceptibility of Sept15 morphants to *Shigella* infection is mediated specifically by *il-1b*- and not *tnf*-a- induced signalling pathways.
Figure 3.8. Sept15 restricts the inflammatory response in vivo

**Figure 3.8.** Sept15 restricts the inflammatory response in vivo

(A-B) *Il-1b*:GFP-F *x* *lyz:*dsRed zebrafish larvae (with red neutrophils, and green *il-1b*-expressing cells [false coloured white]) were treated with control (Ctrl) (A) or Sept15 (B) morpholino (Mo). Morphant larvae were injected in the HBV at 3 dpf with a low dose (≤3.0 × 10^3 CFUs) of Crimson-*S. flexneri* M90T (false coloured green). Frames extracted from time-lapse confocal imaging at 19 hpi. Maximum intensity Z-projection images (2 µm serial optical sections) are shown. Scale bars, 50 µm. Data obtained in collaboration with L. Boucontet and E. Colucci-Guyon.

(C) Relative % of Caspase-1 activity levels measured in Ctrl and Sept15 morphants injected in the HBV at 3 dpf with ~5.0 × 10^3 CFUs of *S. flexneri* M90T. Quantifications performed at 25 hpi. Each circle represents a count from an individual larva. Mean ± SEM (horizontal
bars) is shown. Data are pooled from three independent experiments (n ≥ 5 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. **; p < 0.01. Data obtained in collaboration with V. Torraca.

(D) Representative images of Ctrl and Sept15 morphants injected in the HBV at 3 dpf with a low dose of S. flexneri M90T and stained for acridine orange (AO; green). Images taken 20 hpi. Dotted line shows the area where cells were quantified in (D) (i.e. the infected HBV). Scale bars, 100 µm.

(E) Number of AO positive cells counted in the HBV of Ctrl or Sept15 morphants 20 hpi with a low dose of S. flexneri M90T as in (C). Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 6 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance testing performed by Student’s t test. Significance defined as: p < 0.05. **, p < 0.01.

3.2.6. Treatment with anakinra reduces inflammation and rescues Sept15 deficiency in vivo

Anakinra is a synthetic antagonist of the human interleukin 1 receptor (IL-1R) and pharmacological agent used to dampen inflammation in cases of autoimmune disease (Aksentijevich et al., 2009, Mertens and Singh, 2009, Kone-Paut and Galeotti, 2014). Competitive binding of anakinra to IL-1R blocks IL-1β signalling in humans and although not previously tested in fish, anakinra presents comparable homology to both human IL-1β (31.0%) and zebrafish Il-1β (29.7%) (Arend et al., 1998). Using our Shigella-zebrafish model we found that anakinra treatment reduced neutrophil death, as well as larval mortality in response to a high dose Shigella infection (Appendix 3A and 3B). Interestingly, anakinra did not significantly reduce bacterial burden, indicating that hyperinflammation is responsible for
zebrafish mortality upon \textit{Shigella} infection \textbf{(Appendix 3C)}. To determine whether suppression of II-1\(\beta\)-signalling could rescue Sept15 deficiency \textit{in vivo}, Sept15 morphants were treated with anakinra prior to infection with a low dose of \textit{Shigella} in the HBV. Remarkably, anakinra was able to prevent neutrophil cell death and reduce larval mortality in response to \textit{Shigella} infection whilst not impacting on bacterial burden \textbf{(Appendix 3D-3F)}. These results indicate that pharmacological suppression of inflammation can protect neutrophils and larvae against \textit{Shigella} infection, and that hyperinflammation is a crucial factor in susceptibility of Sept15 morphants to \textit{Shigella} infection.

\section*{3.3. Discussion}

\subsection*{3.3.1. Overview}

The zebrafish is a valuable vertebrate model to visualise the cell biology of infection \textit{in vivo} \textbf{(Kanther and Rawls, 2010, Renshaw and Trede, 2012, Meijer et al., 2014)}. A zebrafish model of systemic \textit{Shigella} infection model has previously been used to study bacterial autophagy \textit{in vivo} \textbf{(Mostowy et al., 2013)}. In this chapter, we develop a hindbrain \textit{Shigella}-zebrafish infection model and discover a new role for septins in restriction of inflammation and host defence \textit{in vivo}.

\subsection*{3.3.2. A zebrafish model of localised \textit{S. flexneri} infection}

The zebrafish hindbrain has previously been used to visualise infection by a variety of important human pathogens including \textit{S. Typhimurium}, \textit{M. marinum}, \textit{P. aeruginosa} and \textit{L. monocytogenes} \textbf{(Hall et al., 2012, Clay et al., 2007, Takaki et al., 2013, Phennicie et al., 2010, McCarthy et al., 2017, Vincent et al., 2016, Mesquita et al., 2017)}. In this study, we visualise the dose-dependent control of \textit{S. flexneri} infection, whereby the immune system successfully eliminates low doses of \textit{Shigella} but is unable to control higher inocula, resulting
in larval mortality (Fig. 3.4B-3.4D). Secretion of bacterial effectors by the T3SS is essential for *Shigella* virulence in humans (Schroeder et al., 2007, Mattock and Blocker, 2017). This aspect of pathogenesis is faithfully replicated in the zebrafish, and infections with T3SS- *S. flexneri* are rapidly cleared and do not compromise larval survival (Fig. 3.4E and 3.4F). Together, these data support the use of zebrafish infection to investigate mechanisms of *Shigella* pathogenesis *in vivo*.

### 3.3.3. Neutrophils control *S. flexneri* infection in zebrafish

Strikingly, despite similarities in the intracellular lifestyle of *L. monocytogenes, M. marinum* and *S. flexneri* (i.e. actin based motility and cell-cell spread) (Stevens et al., 2006, Welch and Way, 2013), and unlike what has been observed for *Listeria* (Vincent et al., 2016) or *M. marinum* (Clay et al., 2007), even lethal hindbrain infections of invasive *S. flexneri* fail to disseminate outside the forebrain and hindbrain of the zebrafish (Fig. 3.4C). As such, our model enables detailed investigation of *Shigella*-leukocyte interactions within a compartmentalised site *in vivo*. In this case, we observe robust and rapid neutrophil recruitment effectively eliminates a low dose of *Shigella*, and that high doses lead to both neutrophil and larval death (Fig. 3.5A-3.5C). Work in the zebrafish has shown that, whereas macrophages engulf fluid-borne bacteria, neutrophils preferentially phagocytose surface-associated pathogens (Colucci-Guyon et al., 2011). Considering this, although much of the hindbrain comprises a fluid-filled cavity, it is likely that *Shigella* adhesion to the neuroepithelium and expression of immune-stimulatory virulence factors act to recruit neutrophils *in vivo*.

### 3.3.4. Morpholino treatment to study Sept15 in the zebrafish

The zebrafish model has provided key insights into the role of septins in human development and disease (Willis et al., 2016a). Human SEPT7 is crucial for septin function; we therefore
focused our studies on the role of the closely-related zebrafish Sept15, which we find to be expressed ubiquitously (in both non-immune and immune cells) in zebrafish (Sirajuddin et al., 2007) (Fig. 3.6A). Reverse genetic approaches are commonly used to infer gene function in vivo, by identifying phenotypes arising from disruption of a target gene. Genetic mutants, i.e. knockouts, can be generated using programmable site-specific nucleases, such as CRISPR/Cas9, or genes may be knocked down transiently using antisense oligonucleotides, such as morpholinos. Genetic compensation allows an organism to maintain fitness despite genetic perturbation, by modulating the expression of redundant genes with similar function or genes in a particular network. Recent work has shown that compensation differs depending on whether a given gene is knocked down or knocked out, which can explain failure of knockdowns to phenocopy corresponding mutants. To date, attempts to engineer a Sept15 knockout zebrafish using CRISPR/Cas9 technologies have been unsuccessful. Although CRISPR targeting is able to induce frame shifts in the sept15 gene, we have observed that mutations in this gene are embryonar lethal and therefore incompatible with the generation of a stable knockout line (data not shown). These findings are in agreement with studies showing that Sept7 knockout is embryonic lethal in mice (Menon et al., 2014). By contrast, using a dose-dependent morpholino treatment we could achieve partial knockdown of Sept15 (Fig. 3.6B). Morpholino antisense oligonucleotides are commonly used for gene knockdown studies in zebrafish, but can induce off-target effects. To reliably distinguish between specific and non-specific phenotypes, published guidelines advise: (i) minimising the quantity of morpholino injected, (ii) using a control morpholino with no known target in the zebrafish genome, (iii) repeating experiments using a morpholino targeting a distinct portion of the target gene or a target gene expected to yield a similar phenotype, and (iv) performing rescue experiments by co-injecting target-encoding RNA that is not recognised by the morpholino (i.e. without introns if using a splice-inhibiting morpholino or without key regions of the 5’ untranslated region if using a translation-blocking morpholino). Therefore, to best support the conclusions drawn in this study, RNA rescue
experiments should be performed, in addition to analysis of the effect of splice-targeting morpholinos using RT-PCR.

3.3.5. Sept15 is required for host defence against S. flexneri infection in vivo

In this chapter, we study the role of septins in host defence against bacterial infection. Infection experiments in Sept15 morphants showed that in contrast to control larvae, Sept15-depleted larvae are unable to control bacterial replication and succumb to a low dose *Shigella* infection (Fig. 3.6C-3.6E). Host defence was also compromised in Sept15-depleted larvae systemically infected with *S. flexneri*, indicating that the role of septins in host defence is independent of infection site (Appendix 1A and 1B). Furthermore, depletion of an alternative septin, Sept7b showed that the role of septin during infection was not specific to a single septin (Appendix 1C-1E). Instead, function is likely dependent on the coordinated actions of multiple septins existing in hetero-oligomeric structures (Mostowy and Cossart, 2012). Septins are important for ciliary function (Palander et al., 2017). During zebrafish larval development, cilia direct the movement of cerebrospinal fluid (CSF), and septin morphants have been shown to accumulate CSF in the hindbrain (Dash et al., 2014). If severe, hydrocephaly might be predicted to compromise the hindbrain and contribute to larval mortality upon HBV infection. However, given that we see little evidence of fluid accumulation in the HBV of our Sept15 morphants (Appendix 1B), and that *Shigella* burden is significantly increased in septin-depleted larvae (Fig. 3.6D), we do not believe ciliary dysfunction is contributing to zebrafish death in this study. Importantly, despite clear defects in host defence, Sept15 morphant larvae survive and can successfully eliminate T3SS-*S. flexneri* (Fig. 3.6F and 3.6G). These data show that Sept15 morphants are not wholly immune-deficient, but face specific challenges when responding to infection with pathogenic bacteria.
3.3.6. *S. flexneri* infection causes neutrophil death in Sept15 morphants

Neutrophils control *Shigella* infection *in vivo* (Mostowy et al., 2013, Phalipon and Sansonetti, 2007). Examination of Sept15 morphants by confocal microscopy highlighted a failure of neutrophils to control a low dose *Shigella* infection and this is associated with neutrophil cell death (Fig. 3.7A and 3.7B). Quantifications revealed that, in contrast to control larvae, low doses of infection induced neutropenia in Sept15 morphants (Fig. 3.7C and 3.7D). Interestingly, neutropenia in Sept15-depleted larvae is a result of bacterial virulence, as infection with T3SS- *S. flexneri* does not deplete neutrophil numbers (Fig. 3.7E and 3.7F).

Although fewer neutrophils are present in developing Sept15 morphants than control larvae, experiments using the Irf8 morpholino (to boost neutrophil number) revealed that neutrophil reduction per se was not responsible for increased susceptibility to *Shigella* infection (Fig. 3.7G-3.7I). Mechanisms underlying reduced neutrophil numbers in uninfected Sept15 morphants are explored in depth in Chapter 5.

3.3.7. Sept15 restricts infection-mediated inflammation *in vivo*

*S. flexneri* induces expression of proinflammatory cytokines, such as *il-1b* and *tnf-a*, in humans and zebrafish, as well as in rabbit ileal loop and mouse intraperitoneal infection models (Mostowy et al., 2013, Schnupf and Sansonetti, 2012, Raqib et al., 1995, Yang et al., 2014). Although mammalian models are not yet amenable to high-resolution *in vivo* imaging, the optically accessible zebrafish larva presents a unique opportunity to spatiotemporally examine the molecular and inflammatory response to *Shigella* infection *in vivo* (Meijer et al., 2014). Using time-lapse confocal microscopy and transgenic zebrafish lines, we observed the induction of *il-1b* expression in epithelial cells, as well as in neutrophils and macrophages (Fig. 3.8A). These data indicate that both non-immune and immune cell types can be sources of inflammation during *Shigella* infection. Expression of *il-1b* was particularly striking at later stages of Sept15 morphant infection, suggesting uncontrolled inflammation in
these larvae (Fig. 3.8B). Given the tools currently available, the direct detection of mature IL-1β in zebrafish is not currently possible. Instead, we assessed Caspase-1 activity, crucial for the maturation of IL-1β protein, and found this to be significantly increased in Sept15 morphants (Fig. 3.8C). To examine the downstream physiological effects of increased IL-1β signalling and Caspase-1 activity, we used a cell death staining assay and saw increased host cell death in the HBV of infected Sept15 morphants (Fig. 3.8D and 3.8E). Considering the depletion of neutrophils observed upon infection of Sept15 morphants (Fig. 3.7B and 3.7D), it is tempting to speculate that the cell death we observe here (i.e. cells labelled by AO) represents hyperinflamed neutrophils that are dead or dying (Fig. 3.8D and 3.8E).

Previous studies have demonstrated that increased expression of lta4h induces tnf-a in zebrafish larvae and increases host susceptibility to M. marinum infection (Tobin et al., 2012). Interestingly, the upregulation of tnf-a did not increase larval susceptibility to Shigella infection using our model (Appendix 2A-2D). These data indicate that host defence in Sept15 morphants is compromised specifically by activation of the IL-1β signalling cascade. These results are in line with studies that show pyroptosis (i.e. IL-1β-mediated cell death) favours Shigella infection (Hermansson et al., 2016).

### 3.3.8. Anakinra reduces inflammation and rescues Sept15 deficiency in vivo

Dysregulation of IL-1 signalling is often implicated in human diseases with an inflammatory component (Schett et al., 2016, Dinarello, 2011). Suppression of inflammation is important to relieve disease symptoms, and blocking of IL-1R using anakinra has been used to treat rheumatoid arthritis, chronic granulomatous disease and cryopyrin-associated periodic syndromes in humans (Mertens and Singh, 2009, Kone-Paut and Galeotti, 2014, de Luca et al., 2014, Goldbach-Mansky et al., 2006). In this study, we discovered that anakinra rescues neutrophil and larval death following a high dose Shigella infection, without reducing bacterial burden (Appendix 3A-3C). These findings are similar to those recently obtained using a Burkholderia-zebrafish infection model (Mesureur et al., 2017), and show that
hyperinflammation is a significant determinant of larval death in response to different bacterial infections. In the case of hyperinflammation during *Shigella* infection of Sept15 morphants, blocking IL-1β signalling using anakinra rescued zebrafish survival, without significantly affecting bacterial burden (*Appendix 3D-3F*). Taken together, our results show that pharmacological suppression of inflammation can rescue septin-deficiency, and highlight the use of *Shigella* as a paradigm to discover novel mechanisms of inflammation control.

### 3.3.9. A role for septins in inflammation control

Multiple studies have implicated actin in the restriction of inflammasome activity (Pelegrin and Surprenant, 2009, Jin et al., 2013, Johnson et al., 2013, Kim et al., 2015, Standing et al., 2017). Considering that septins filaments interact with actin (Kinoshita et al., 2002, Schmidt and Nichols, 2004b), it is possible that septins block inflammation via direct interaction with inflammatory components, or indirectly through the regulation of actin dynamics. In future studies, we aim to precisely define the role of septins in the assembly and function of inflammasome complexes. An advanced understanding of the role of the cytoskeleton in mediating the inflammasome will be central to its therapeutic manipulation *in vivo*.

### 3.3.10. Conclusions

In conclusion, we have developed a *Shigella*-zebrafish model to study localised *S. flexneri* infection and host-leukocyte interactions *in vivo*. This model will help to reveal novel aspects of neutrophil biology and inform therapies for diseases with a central neutrophil component. Using this model, we identify an important role for Sept15 in neutrophil-mediated control of *S. flexneri* infection, and provide the first evidence for a role of septins in inflammation control. This study adds weight to a growing literature linking the cytoskeleton to
inflammation, and further encourages the development of therapeutics to target the cytoskeleton for the resolution of inflammatory disease.
4.1. Introduction

4.1.1. Antibiotic resistance: a global health threat

Resistance to antimicrobials increases mortality and burdens healthcare systems (Taubes, 2008, Boucher et al., 2009, Rice, 2010). Antibiotic resistance is rising at an alarming rate amongst *Shigella* spp., and similar trends have been seen for many other Gram-negative bacterial species (Sivapalasingam et al., 2006, Slama, 2008, Khaghani et al., 2014, Harrington, 2015). Such infections are endemic to developing countries with inadequate sanitation and poor healthcare systems, which augment the spread of resistance (Ayukekbong et al., 2017).

4.1.2. Live therapies as a strategy to overcome antibiotic resistance

Traditional and modern high-throughput approaches to antibiotic discovery have largely failed to counter bacterial resistance (Taubes, 2008, Lyddiard et al., 2016). Efforts are now being made to (i) restore the efficacy of existing antibiotics, and (ii) explore creative solutions to overcome antibiotic resistance (Williamson et al., 2017, Reardon, 2015, Spellberg et al., 2013, Peters et al., 2008, Xu et al., 2014). Live therapies can evolve alongside a bacterial pathogen and therefore minimise the chance of inducing resistance. Interspecies bacterial interactions are common in nature and often antagonistic (Hibbing et al., 2010). Indeed, many of the antibiotics used today are derived from natural compounds identified in bacteria; but such drugs often only have a single mutable target (Clardy et al., 2009).
4.1.3. Predatory bacteria

Predatory bacteria are Gram-negative species which invade and consume other Gram-negatives, such as *Shigella*, and represent a novel candidate for antibacterial therapy (Reardon, 2015, Sockett and Lambert, 2004, Kadouri et al., 2013, Shatzkes et al., 2017a). Different bacterial predators adopt distinct strategies to target prey and enable predator replication (Perez et al., 2016). Depending on the predator, prey may be consumed either (i) externally, following attachment to the prey cell envelope (i.e. epibiotic predation), or (ii) internally, following invasion of the periplasm or cytoplasm (i.e. endobiotic predation) (Pasternak et al., 2014). Collectively, predatory bacteria are referred to as ‘*Bdellovibrio* and like organisms’ (BALOs) (Davidov and Jurkevitch, 2004, Jurkevitch, 2012).

4.1.4. The predatory lifestyle of *Bdellovibrio*

*Bdellovibrio* are small (0.5 x 1.0 µm) predatory bacteria discovered in the 1960s (Stolp and Starr, 1963, Sockett, 2009). Following decades of neglect resulting from practical difficulties in predator culture and manipulation, the field of *Bdellovibrio* has experienced a resurgence due to advances in genetic methodologies and a complete genome sequence (Rendulic et al., 2004, Cotter and Thomashow, 1992, Medina et al., 2008, Steyert and Pineiro, 2007). Predator motility is mediated by a single flagellum and enables prey location via chemotaxis (Seidler and Starr, 1968, Lambert et al., 2003). Predator-prey interactions are mediated by Type IV pili, but the mechanism by which *Bdellovibrio* invade prey is largely unknown (Evans et al., 2007, Medina et al., 2008). In brief, *Bdellovibrio* attached to the prey outer membrane secrete hydrolytic enzymes which compromise the prey outer cell membrane in a localised manner, enabling entry into the prey periplasm (Burnham et al., 1968, Rendulic et al., 2004). During invasion, modification of the prey cell wall rounds typically rod-shaped bacteria to form a stable ‘bdelloplast’ in which the predator can grow (Thomashow and Rittenberg, 1978, Lerner et al., 2012, Starr and Baigent, 1966). Inside the periplasm, *Bdellovibrio* digest
the prey and use this biomass for growth (Seidler and Starr, 1969a). The massive array of hydrolytic enzymes encoded by the *Bdellovibrio* genome (including 20 DNases, 15 lipases, 10 glycanases, 150 proteases) highlights this bacterium’s capacity to destruct prey cells (Rendulic et al., 2004). Inside the bdelloplast, prey replicate by elongating bidirectionally and septating to produce ∼4-6 bacterial progeny which exit through pores in the outer membrane following prey lysis (Fenton et al., 2010, Lambert et al., 2016). A full predatory cycle typically lasts 3-4 h (Fig. 4.1) (Sockett, 2009, Starr and Baigent, 1966). *Bdellovibrio* are unable to undergo binary fission (Fenton et al., 2010), and it is likely that this compromises host-independent growth as only ∼1 in $10^7$ bacteria can enter a slow axenic growth phase (i.e. replicate in the absence of prey) (Seidler and Starr, 1969b).

*Figure 4.1. Schematic of Bdellovibrio life cycle*

(I-II) Motile predatory *Bdellovibrio* locate and attach to prey Gram-negative bacteria. Attachment lasts ∼15 mins. (III) Predatory bacteria invade the prey periplasm and prey are
rounded by DD-endopeptidase action on the cell wall peptidoglycan. Invasion lasts ~15 mins. (IV) Prey bacteria are killed within 30 min and kept intact as *Bdellovibrio* consume their contents and grow. (V and VI) Following replication, *Bdellovibrio* lyse prey 180–240 min after invasion, releasing further predators. *Bdellovibrio* progeny can then repeat the predatory cycle. OM, outer membrane; CW, cell wall peptidoglycan; CM, cytoplasmic membrane. Figure taken from (Willis et al., 2016b).

### 4.1.5. Considerations for the medical application of *Bdellovibrio*

As a live therapy, there are unique considerations for the use of *Bdellovibrio* in a clinical setting (Sockett and Lambert, 2004). In contrast to bacteriophage, there are no reports of prey having acquired genetic resistance to *Bdellovibrio* (Labrie et al., 2010). Furthermore, there is little evidence of horizontal DNA transfer between predator and prey species, nullifying any concerns that *Bdellovibrio* may themselves become pathogenic to a human host (Rendulic et al., 2004). Although *Bdellovibrio* has no known association with human disease, it is crucial to fully evaluate the risk of its introduction into a human patient.

### 4.1.6. The immune response to *Bdellovibrio*

Considering that *Bdellovibrio* is a Gram-negative bacterium, it can be expected to induce a degree of immune stimulation. To this end, studies conducted *ex vivo* have shown that exposure of mouse or human macrophages to predator LPS or intact *Bdellovibrio* is not cytotoxic and elicits only a weak cytokine response as compared to pathogenic bacteria (Monnappa et al., 2016, Schwudke et al., 2003, Gupta et al., 2016). Similar results were seen using epithelial and colorectal cell lines (Gupta et al., 2016, Monnappa et al., 2016, Shanks et al., 2013). *In vivo*, *Bdellovibrio* has been detected among the intestinal flora of horses, chickens and healthy humans suggesting that this bacterium is not pathogenic to a vertebrate host (Schwudke et al., 2001, Iebba et al., 2013). Moreover, recent studies in mice
and rats have shown that *Bdellovibrio* administered to the respiratory tract, gut or bloodstream does not compromise animal wellbeing and induces only a modest inflammatory response which resolves within days following inoculation (Shatzkes et al., 2017b, Shatzkes et al., 2017c, Shatzkes et al., 2016, Shatzkes et al., 2015). Despite these observations, very little is known about the interactions between *Bdellovibrio* and immune cells, and this must be more fully characterised before predatory bacteria can be used therapeutically.

### 4.1.7. Predation of human bacterial pathogens by *Bdellovibrio in vivo*

*In vitro*, *Bdellovibrio* can target a broad range of drug-resistant and medically relevant Gram-negative bacteria in both planktonic cultures and polymicrobial biofilms (Sun et al., 2017, Dashiff et al., 2011). However, only a handful of studies have tested the ability of *Bdellovibrio* to predare *in vivo* using an animal model. Oral dosing of *Bdellovibrio* has been shown to reduce numbers of pathogenic *Salmonella enterica* serovar Enteritidis in the gut of chickens (Atterbury et al., 2011). Similarly, bacterial predators reduced pathogenic *Vibrio* spp. in the intestines of shrimp, and induced changes in the gut microbiome of rats which were predicted to be beneficial (Li et al., 2014a, Shatzkes et al., 2017c). Finally, despite promising *in vitro* data, topical application of *Bdellovibrio* failed to treat ocular infection in cattle (Shanks et al., 2013, Boileau et al., 2016, Boileau et al., 2011). Collectively, these studies highlight the importance of testing predation *in vivo*, and in testing the role a functional immune system in mediating predator-prey interactions.

### 4.1.8. Aims of this chapter

The transparent zebrafish larva is uniquely suited to study bacterial infection and to visualise the efficiency of novel therapeutics *in vivo* (Kanther and Rawls, 2010, Renshaw and Trede, 2012, Meijer et al., 2014, Gibert et al., 2013). In this chapter, we use the zebrafish larva as
an in vivo model to study *Bdellovibrio* interactions with host innate immune cells, and to explore bacterial predation of *Shigella* by *Bdellovibrio* in the context of innate immunity.

4.2. Results

4.2.1. *Bdellovibrio* are non-pathogenic and viable in zebrafish larvae

To develop *Bdellovibrio* as an in vivo therapeutic agent, we tested the pathogenicity of *Bdellovibrio* using zebrafish larvae. For this, we employed fluorescently-tagged variants of the reference strain *B. bacteriovorus* HD100 for which the complete genome sequence is available (Rendulic et al., 2004). Infections were performed in the zebrafish HBV, as this site is highly amenable to imaging *Bdellovibrio*-leukocyte interactions. At 3 dpf, WT AB zebrafish larvae were injected in the HBV with 1.0-2.0 x 10^5 plaque forming units (PFUs) of mCherry-*Bdellovibrio*. Examination by light microscopy failed to highlight any developmental or morphological abnormalities in *Bdellovibrio*-injected larvae (Fig. 4.2A). Locomotor activity is a common behavioural readout of substance toxicity in the zebrafish larva (Padilla et al., 2011), and was not affected by inoculation with predatory bacteria (unpublished observations). Moreover, survival assays of *Bdellovibrio*-injected larvae showed that larval viability was ~100\% at 48 hpi (Fig. 4.2B). Collectively, these results indicate that *Bdellovibrio* are non-pathogenic in zebrafish larvae.

We next assessed survival of *Bdellovibrio* within the zebrafish larva. For this, larvae were injected at 3 dpf with 1.0-10.0 x 10^4 PFUs of mCherry-*Bdellovibrio* in the HBV and larval homogenates plated on lawns of *E. coli* at time points following infection. Enumeration of PFUs showed that larval bacterial burden was significantly reduced from 24 hpi and largely eliminated by 72 hpi (Fig. 4.2C). Larvae infected at 2 dpf or inoculated with a higher dose of *Bdellovibrio* (1.0-10.0 x 10^5 PFUs) demonstrated similar declines in bacterial load (Fig. 4.2D-4.2F). In agreement with quantifications of live bacteria, imaging of larvae by fluorescent
stereomicroscopy revealed a gradual decrease in mCherry-fluorescence over time, and low but detectable levels of bacterial fluorescence at 24 hpi (Fig. 4.2G). Similar observations were made following systemic injection of *Bdellovibrio* via the zebrafish caudal vein (Fig. 4.2H). Although *Bdellovibrio* are cleared over the days following injection in vivo, predators require only 30 min to invade a prey cell in vitro (Starr and Baigent, 1966). We therefore concluded that *Bdellovibrio* are sufficiently viable in the zebrafish to predate on Gram-negative bacteria.
Chapter 4. Results

A

Log10 PFU

B
dellovibrio

D

Log10 PFU

C

Shigella

E

High dose, 2 dpf

F

High dose, 3 dpf

G

Bdellovibrio

H

Bdellovibrio

Survival proportions: Survival of collated at 72 h
Figure 4.2. *Bdellovibrio* are non-pathogenic and viable within zebrafish larvae

(A) WT AB zebrafish larvae were injected in the HBV at 3 dpf with PBS or 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio*, and imaged by stereomicroscopy at 48 hpi (i.e. 5 dpf). Representative images of larval morphology are shown. Scale bars, 0.5 mm.

(B) Survival curve of WT AB larvae injected in the HBV at 3 dpf with 1.0-10.0 x 10^4 PFUs of mCherry-*Bdellovibrio*. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 22 larvae per experiment).

(C-F) Time-course enumeration of live *Bdellovibrio* from homogenates of WT AB larvae injected in the HBV at 2 or 3 dpf with a dose range of 1.0-100.0 x 10^4 PFUs of mCherry *Bdellovibrio*. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. (C) Data are representative of one experiment. (D-F) Data are pooled from two independent experiments (using up to 8 larvae per time point per experiment). p values (versus 0 hpi) determined by unpaired two-tailed Student’s t test. Significance with Bonferroni correction defined as: (C, E) p < 0.01. **, p < 0.002; ***, p < 0.0003. (D) p < 0.0125. **, p < 0.0025; ***, p < 0.0002. (F) p < 0.0166. ***, p < 0.0003. Data in (C, E and F) obtained by C. Moore.

(G) WT AB larvae injected at 3 dpf in the HBV with 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio* (red) were imaged by fluorescent stereomicroscopy over 24 hpi. Representative images from a single larva are shown. Scale bar, 100 µm.

(H) 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio* (red) were injected systemically into 3 dpf WT AB larvae via the caudal vein and larvae imaged by fluorescent stereomicroscopy over 24 hpi. Representative images from a single larva are shown. The area inside the white dashed line indicates the blood stream. CV, caudal vein.

Data obtained in collaboration with L. Sockett, C. Moore and C. Lambert.
4.2.2. *Bdellovibrio* treatment protects against *Shigella* infection *in vivo*

To study *Bdellovibrio* as an injectable therapy, we employed the hindbrain *Shigella*-zebrafish infection model, developed in Chapter 3. The M90T strain of *S. flexneri* used in this study is both streptomycin- and carbenicillin resistant. To test bacterial predation *in vivo*, WT AB larvae were injected in the HBV at 3 dpf with a typically lethal dose of GFP-*Shigella* (>5.0 x 10^3 CFUs). *Shigella*-infected larvae were treated 30-90 min later with a secondary hindbrain injection of either PBS or 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio*. Remarkably, survival assays showed a 35% reduction in mortality in *Bdellovibrio*-treated larvae at 72 hpi (Fig. 4.3A). To quantify pathogen load over the course of infection in PBS- or *Bdellovibrio*-treated zebrafish, viable larvae were homogenised at chosen time points and homogenates plated on LB-agar. Enumeration of bacterial colonies showed increasing numbers of *Shigella* in PBS-treated larvae (Fig. 4.3B). By contrast, *Bdellovibrio*-treated larvae were able to control bacterial replication and a significant reduction in *Shigella* burden was detectable already at 2 hpi (Fig. 4.3B). In agreement with previous data (Chapter 3, Fig. 3.4D), stereomicroscopy of *Shigella* infected larvae treated with PBS revealed increasing fluorescence of GFP-*Shigella* over time (Fig. 4.3C). In contrast, larvae treated with *Bdellovibrio* showed diminishing fluorescence from GFP-*Shigella* over the 24 h following infection (Fig. 4.3C). Reduction of GFP-fluorescence occurred in regions of contact with mCherry-*Bdellovibrio*. Together, these results support a strong therapeutic effect of *Bdellovibrio* against multidrug resistant *Shigella* infection *in vivo*. 
Figure 4.3. *Bdellovibrio* treatment protects against *Shigella* infection in vivo

(A) Survival curve of WT AB zebrafish larvae injected in the HBV at 3 dpf with >5.0 x 10^3 CFUs of GFP- *S. flexneri* M90T and treated with an injection of either PBS or 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio* 30-90 min later. Larval viability was scored for 72 hpi. Data are pooled from three independent experiments (n ≥ 22 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 2, 24, 48, and 72 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. ***, p < 0.001.

(B) Time-course enumeration of live *Shigella* from homogenates of WT AB larvae injected with GFP- *S. flexneri* M90T and treated with either PBS or mCherry-*Bdellovibrio* as in (A). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from four independent experiments (using up to 3 larvae per time point per
experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(C) WT AB larvae were injected with GFP- S. flexneri M90T (green) and treated with either PBS or mCherry-Bdellovibrio (red) as in (A). Representative images from single larva are shown. Dotted square shows region of interaction between Shigella and Bdellovibrio. For each treatment, the same larva was imaged over time. Scale bars, 100 µm.

Data obtained in collaboration with L. Sockett, C. Moore and C. Lambert.

4.2.3. Bdellovibrio predate on Shigella in vitro and in vivo

Bdellovibrio prey on a diverse array of Gram-negative bacteria (Dashiff et al., 2011, Sun et al., 2017). To test the susceptibility of S. flexneri M90T to predation by B. bacteriovorus, predator-prey assays were conducted in vitro. Predatory mCherry-Bdellovibrio were co-incubated with GFP-Shigella. At time points following co-incubation, samples of bacterial co-culture were taken, plated on LB agar and Shigella CFUs enumerated. Strikingly enumerations revealed a >4,000-fold reduction in the Shigella population following 21 h of co-incubation with Bdellovibrio (Fig. 4.4A). Successful predation enables Bdellovibrio replication (Sockett, 2009). To correlate trends in predator and prey populations, automated plate-reader assays were performed. From co-cultures, measurements of optical density (OD$_{600}$, as a readout of Shigella lysis by Bdellovibrio) and Bdellovibrio mCherry-fluorescence were taken. Here, significant decreases in OD$_{600}$ accompanied increases in mCherry fluorescence, indicative of Shigella-killing and growth of Bdellovibrio (Fig. 4.4B and 4.4C). Additional evidence of in vitro predation was seen in samples of co-culture analysed by widefield microscopy (Fig. 4.4D). Shigella are rod-shaped bacteria measuring ~0.5 (width) x 2.0 (length) µM. However, in the presence of Bdellovibrio, most Shigella displayed a rounded morphology (~1.0 x 1.0 µM), and GFP-Shigella comprised a single mCherry-
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*Bdellovibrio*. These observations clearly describe the formation of bdelloplasts (i.e. parasitised prey cells), and show that *Bdellovibrio* predate *Shigella in vitro*.

Active predation by *Bdellovibrio* has not yet been visualised in any animal model. To investigate predation of *Shigella* by *Bdellovibrio in vivo*, we performed sequential tail muscle injections of GFP-*Shigella* and mCherry-*Bdellovibrio* in 3 dpf zebrafish larvae. The tail best supports simple, high resolution imaging because it is the thinnest portion of the larvae. Larvae were imaged by confocal microscopy to visualise predator-prey interactions at the level of the single bacterial cell. Here, we observed a large number of rounded *Shigella* cells, suggestive of bdelloplast formation (Fig. 4.4E). Remarkably, using time-lapse microscopy at higher magnifications, we captured invasion of GFP-*Shigella* by a single mCherry-*Bdellovibrio* inside zebrafish larvae (Fig. 4.4F). To determine whether replication of *Bdellovibrio* was occurring in vivo, zebrafish larvae were injected with mCherry-*Bdellovibrio* alone or following a dose of GFP-*Shigella*. At 5 and 24 hpi, larval homogenates were plated on *E. coli* lawns for counting of live *Bdellovibrio* PFUs. As shown in previous experiments (Fig. 4.2C and 4.2G), *Bdellovibrio* injected alone were slowly eliminated from the larvae and showed no evidence of replication (Fig. 4.4G). Conversely, when injected 60-90 minutes after *Shigella* prey, *Bdellovibrio* numbers increased significantly (Fig. 4.4G). Collectively, results obtained using high resolution microscopy and bacterial counts provide the first direct evidence of bacterial predation by *Bdellovibrio in vivo*. 
Figure 4.4. *Bdellovibrio* predate on *Shigella in vitro* and *in vivo*

(A) GFP-*S. flexneri* M90T was incubated alone or with mCherry-*Bdellovibrio* at an MOI of ~100 in buffer. Live *Shigella* were enumerated over 21 h following incubation. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments. The p value between conditions was determined by paired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001. Data obtained in collaboration with S. Krokowski.

(B-C) GFP-*S. flexneri* M90T was incubated alone or with mCherry-*Bdellovibrio* at an MOI of ~100-1000 in buffer. Measurements of (B) OD$_{600}$ (to represent *Shigella* numbers) and (C)
mCherry fluorescence intensity (to represent *Bdellovibrio* numbers), were taken over 6 h following incubation. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments. p values between conditions was determined by paired one-tailed Student’s t test. Significance defined as: p < 0.05. **, p < 0.01; ***, p < 0.001. Data obtained in collaboration with S. Krokowski.

**D** GFP-*S. flexneri* M90T (green) was incubated alone or with mCherry-*Bdellovibrio* (red) as in **A**. DAPI (blue) was used to stain DNA. Bacteria were visualised by wide-field fluorescent microscopy. Representative images of cultures 1 h post incubation show rod-shaped *Shigella* (left) and rounded *Shigella* invaded by smaller *Bdellovibrio* (right). Scale bars, 1 μm. Data obtained in collaboration with S. Krokowski.

**E-F** WT AB zebrafish larvae were injected at 3 dpf in the tail muscle with 1.0 x 10^3 CFUs of GFP-*S. flexneri* M90T (green) followed by an injection of 1-2 x 10^5 mCherry-*Bdellovibrio* (red) 30-90 min later. Larvae were imaged by confocal microscopy at **E** 20x or **F** 63x magnification, 1 h post injection with *Bdellovibrio*. **E** A representative image shows the different morphologies of *Shigella in vivo*, including rod-shaped *Shigella* (arrow) and a high proportion of rounded *Shigella* (arrowheads) at regions of contact with *Bdellovibrio*. Scale bar, 10 μm. **F** Representative images of invasive predation of *Shigella* by *Bdellovibrio* inside a larva, captured by time lapse microscopy. Scale bar, 2.5 μm. mpi, minutes post-infection.

**G** WT AB larvae were injected in the HBV at 3 dpf with 2.0-6.0 x 10^5 CFUs of GFP-*S. flexneri* M90T (green) and treated with an injection of either PBS or 1.0-30.0 x 10^2 PFUs of mCherry-*Bdellovibrio* (red) 30-90 min later. Live *Bdellovibrio* were enumerated from larval homogenates over time. Only viable were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (using up to 3 larvae used per time point
per experiment). p values (versus 0 hpi) determined by unpaired two-tailed Student’s t test. Significance with Bonferroni correction defined as: $p < 0.0125$. **, $p < 0.005$; ***, $p < 0.0005$.

Data obtained in collaboration with L. Sockett, C. Moore and C. Lambert.

### 4.2.4. Zebrafish leukocytes engulf and eliminate *Bdellovibrio in vivo*

Although safe in zebrafish larvae, it is important to fully understand the host response to *Bdellovibrio* treatment. In previous imaging of mCherry-*Bdellovibrio* in WT AB larvae, we saw the initial dispersed mass of fluorescence condense and form dynamic punctae (~10 μM in diameter) prior to clearance (Fig. 4.2G). Similar observations were made upon systemic injection of mCherry-*Bdellovibrio* via a caudal vein infection route (Fig. 4.2H). These observations suggest the recognition and engulfment of *Bdellovibrio* by host immune cells. Interactions between *Bdellovibrio* and immune cells are mostly unknown. To test the neutrophil response to infection, we injected a low dose of mCherry-*Bdellovibrio* into the HBV of 3 day old *mpx*:GFP larvae, with GFP-expressing neutrophils (Renshaw et al., 2006).

To visualise macrophage interactions, we performed infections with mTeal-*Bdellovibrio* in *mpeg1*:G/U:mCherry larvae, with mCherry-expressing macrophages (Ellett et al., 2011). Time lapse imaging by fluorescent stereomicroscopy revealed clear recruitment of both neutrophils and macrophages to the HBV by 6 hpi (Fig. 4.5A and 4.5B). Quantifications revealed significant neutrophil recruitment (1.5 ± 0.1-fold) and more macrophages (1.2 ± 0.1-fold) in the hindbrain upon *Bdellovibrio* injection, as compared to PBS-injected controls (Fig. 4.5C and 4.5D). Moreover, colocalisation of *Bdellovibrio* and leukocyte fluorescence strongly suggested bacterial engulfment by both neutrophils and macrophages. To visualise leukocyte interactions at higher resolution, zebrafish larvae with fluorescent neutrophils or macrophages were injected in the tail muscle with *Bdellovibrio* and imaged by confocal microscopy. High resolution microscopy confirmed engulfment of *Bdellovibrio* by both neutrophils and macrophages (Fig. 4.5E and 4.5F). Surprisingly, time-lapse imaging
captured persistence of mTeal-\textit{Bdellovibrio} fluorescence within macrophages for up to 6 h. Together, these results show that zebrafish immune cells recognise and engulf \textit{Bdellovibrio} \textit{in vivo}.

To assess the role of leukocytes in clearance of \textit{Bdellovibrio} from the host, we performed infections in immune-compromised zebrafish larvae. Pu.1 is a transcription factor driving myeloid gene expression in zebrafish (Hsu et al., 2004). We injected a morpholino antisense oligonucleotide against \textit{pu.1} into the 1-8 cell stage embryo to deplete leukocytes, and infected 3 dpf larvae with $1.0-2.0 \times 10^5$ PFUs of mCherry-\textit{Bdellovibrio}. Similar infections were performed on larvae treated with a control morpholino. Larval survival did not differ significantly between control and Pu.1 morphants exposed to \textit{Bdellovibrio} (Fig. 4.5G). At 6, 24, and 48 hpi, control and Pu.1 morphants were homogenised and live bacteria enumerated by plating of PFUs. In support of a role for leukocytes in bacterial clearance, significantly more \textit{Bdellovibrio} were recovered from leukocyte-depleted larvae (Fig. 4.5H). Together, our results show active killing of \textit{Bdellovibrio} by host innate immune cells in the zebrafish larva.
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A. Neutrophils  Bdellovibrio  Merge

B. Macrophages  Bdellovibrio  Merge

C. # neutrophils in hindbrain

D. # macrophages in hindbrain

E. Neutrophil  Bdellovibrio  Merge

F. Macrophage  Bdellovibrio  Merge

G. % Survival

H. i. Log 10 PFU

H. ii. Log 10 PFU

H. iii. Log 10 PFU
Figure 4.5. Zebrafish leukocytes engulf and eliminate *Bdellovibrio in vivo*

(A) Mpx:GFP zebrafish larvae (with green neutrophils) were injected in the HBV at 3 dpf with 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio* (red). Time lapse imaging by fluorescent stereomicroscopy captured interactions between neutrophils and *Bdellovibrio*. Representative images from a single larva are shown. Scale bar, 100 µm.

(B) Mpeg1:G/U:mCherry larvae (with red macrophages, false coloured green) were injected in the HBV at 3 dpf with 1.0-2.0 x 10^5 PFUs of mTeal-*Bdellovibrio* (green, false coloured red). Time lapse imaging by fluorescent stereomicroscopy captured interactions between macrophages and *Bdellovibrio*. Representative images from a single larva are shown. Scale bar, 100 µm.

(C) Mpx:GFP larvae were injected with PBS or mCherry-*Bdellovibrio* as in (A), and GFP-expressing neutrophils in the head region quantified at 6 hpi. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (using up to 7 larvae per condition per experiment). The p value between conditions was determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(D) Mpeg1:G/U::mCherry larvae were injected with PBS or mTeal-*Bdellovibrio* as in (B), and mCherry-expressing macrophages in the head region quantified at 6 hpi. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (using up to 7 larvae per condition per experiment). The p value between conditions was determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05.

(E) Mpx:GFP larvae (with green neutrophils) were injected in the tail muscle at 3 dpf with 1.0-2.0 x 10^5 PFUs of mCherry-*Bdellovibrio* (red). Imaging by confocal microscopy captured single-cell interactions between neutrophils and *Bdellovibrio* at 100x magnification. A representative image is shown. Scale bar, 5 µm.
(F) Mpeg1:G/U:mCherry larvae (with red macrophages, false coloured green) were injected in the tail muscle at 3 dpf with 1.0-2.0 x 10^5 PFUs of mTeal-Bdelovibrio (green, false coloured red). Imaging by confocal microscopy captured single-cell interactions between macrophages and Bdelovibrio at 63x magnification. A representative image is shown. Scale bar, 10 µm.

(G) Survival curve of mpx:GFP larvae treated with control (Ctrl) or Pu.1 morpholino (Mo) to deplete leukocytes. Morphant larvae were injected in the hindbrain at 3 dpf with 0.1-10.0 x 10^5 PFUs of mCherry-Bdelovibrio. Larval viability was scored for 72 hpi. Data are pooled from two independent experiments (n ≥ 21 larvae per condition per experiment.). Up to three larvae per condition were taken for CFUs at 24, 48 and 72 h time points. p value between conditions determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05.

(H) Time-course enumeration of live Bdelovibrio from homogenates of mpx:GFP Ctrl and Pu.1 morphants injected with mCherry-Bdelovibrio as in (G). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. As inoculums from independent experiments were variable up to 2 log-fold, data from three independent experiments (using up to 3 larvae per time point) are shown separately. p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Data obtained in collaboration with L. Sockett, C. Moore and C. Lambert.

4.2.5. The cytokine response to Bdelovibrio injection in vivo

To understand the recognition of predatory bacteria by the innate immune system, we used our Shigella-zebrafish model to measure the cytokine response to Bdelovibrio injection. For
this, zebrafish larvae were injected with either GFP-Shigella, mCherry-Bdellovibrio or both Shigella + Bdellovibrio combined. Of note, to mimic the host cytokine response to a therapeutic dose of Bdellovibrio, we injected up to 100-fold more Bdellovibrio than Shigella, and therefore responses to the different bacteria are not directly comparable. At 6 hpi, larvae were sacrificed and RT-qPCR was used to detect the expression of key pro-inflammatory cytokines il-1b and tnf-a. Relative to PBS-injected controls, increased expression of both il-1b and tnf-a was detected following infections with either Shigella, Bdellovibrio or Shigella + Bdellovibrio combined (Fig. 4.6A and 4.6B). Importantly, cytokine expression following infection with Shigella + Bdellovibrio is not increased compared to infection with Shigella alone. Together, these data show that Bdellovibrio-treated larvae are not inducing a hyperinflammatory immune state to enable Shigella clearance.

Figure 4.6. The cytokine response to Bdellovibrio injection in vivo

(A-B) WT AB zebrafish larvae were injected in the HBV at 3 dpf with either: PBS, >5.0 x 10^3 CFUs of GFP-Shigella, 1.0-2.0 x 10^5 PFUs of mCherry-Bdellovibrio or sequentially with the above doses of Shigella and Bdellovibrio. RNA was extracted from pools of 5 larvae and expression of (A) il-1b and (B) tnf-a mRNA transcripts determined by RT-qPCR. Data are pooled from (A) three or (B) four, independent experiments. p values (versus PBS controls)
determined by unpaired two-tailed Student’s t-test. Significance with Bonferroni correction defined as: $p < 0.0166$. $***$, $p < 0.0003$. Data obtained in collaboration with M. Mazon-Moya, L. Sockett, C. Moore and C. Lambert.

4.2.6. *Bdellovibrio* work alongside leukocytes to protect against *Shigella* infection *in vivo*

To investigate the interplay between *Bdellovibrio* and host innate immunity, we performed predation experiments in leukocyte-depleted larvae. Control and immune-compromised Pu.1 morphants were injected with a lethal dose of *Shigella* in the HBV, followed by a second injection of either PBS or *Bdellovibrio*. Survival assays revealed that although predatory bacteria could rescue the survival of immune-compromised larvae infected with *Shigella*, *Bdellovibrio* therapy was most effective in the presence of the host innate immune system (Fig. 4.7A). To relate zebrafish survival to pathogen load, viable larvae were plated at 6, 24 and 48 hpi, and CFUs were quantified. Pu.1 morphants displayed a significant reduction in pathogen load following *Bdellovibrio* treatment highlighting predation in immune compromised larvae *in vivo*. Remarkably, the most dramatic pathogen control was observed using *Bdellovibrio* treatment in immune-competent larvae (Fig. 4.7B). These results reveal that the maximum benefit of *Bdellovibrio* therapy to enable *Shigella* clearance is achieved together with host innate immune cells.
Figure 4.7. *Bdellovibrio* work alongside leukocytes to protect against *Shigella* infection *in vivo*

(A) *Mpx*:GFP zebrafish larvae were treated with control (Ctrl) or Pu.1 morpholino (Mo). Morphants larvae were injected in the HBV at 3 dpf with >5.0 x 10³ CFUs of GFP-*S. flexneri* M90T and treated with an injection of either PBS or 1.0-2.0 x 10⁵ PFUs of mCherry-*Bdellovibrio* 30-90 min later. Larval viability was scored for 72 hpi. Data are pooled from three independent experiments (n ≥ 12 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 6, 24, and 48 h time points. Top graph represents collated data, bottom graph represents *Bdellovibrio*-treated larvae only i.e. a subset of the data above. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. ***, p < 0.001.
(B) Time-course enumeration of live *Shigella* from homogenates of Mpx:GFP Ctrl and Pu.1 morphants injected with GFP-S. *flexneri* M90T and treated with either PBS or mCherry-*Bdellovibrio* as in (A). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). Top graph represents collated data, bottom graph represents *Bdellovibrio*-treated larvae only i.e. a subset of the data above. *p* values between conditions at cognate time points determined by unpaired two-tailed Student’s *t* test. Significance defined as: *p* < 0.05. *, *p* < 0.05; **, *p* < 0.01.

Data obtained in collaboration with M. Mazon-Moya, L. Sockett, C. Moore and C. Lambert.

4.3. Discussion

4.3.1. Overview

The rise in antibiotic resistance is a global threat to human health and alternative therapies are urgently needed (Taubes, 2008, Rice, 2010, Slama, 2008, Boucher et al., 2009). The use of predatory bacteria for the treatment of antibiotic resistant bacterial infection *in vivo* is largely untested (Sockett and Lambert, 2004, Kadouri et al., 2013, Shatzkes et al., 2017a). In this chapter, a *Shigella*-zebrafish infection model is used to explore the therapeutic potential of *Bdellovibrio* in the context of a functional innate immune system.

4.3.2. *Bdellovibrio* are non-pathogenic and survive in zebrafish larvae

*Bdellovibrio* are ubiquitous in the environment and it is likely that these predatory bacteria are ingested alongside organic foods and water (Davidov and Jurkevitch, 2004). Indeed, *Bdellovibrio* have been detected in the guts of healthy humans, and to date there has been
no association between these bacteria and disease (Iebba et al., 2013, Schwudke et al., 2001). To test for pathogenicity in vivo, we performed infections with *Bdellovibrio* in the zebrafish HBV. Similar to results obtained using rat and mouse models, we found that localised injection of *Bdellovibrio* is non-pathogenic to zebrafish larvae (Fig. 4.2A and 4.2B) (Shatzkes et al., 2017b, Shatzkes et al., 2015, Shatzkes et al., 2016, Shatzkes et al., 2017c). Together, these data are promising for the future use of *Bdellovibrio* in higher vertebrates.

Further experiments in the zebrafish showed that *B. bacteriovorus* is cleared from the host in the days following infection (Fig. 4.2C-4.2H). These results reflect the lifestyle of *Bdellovibrio* in which bacteria are largely unable to replicate in the absence of prey (Sockett, 2009). Despite eventual clearance of *Bdellovibrio* by the zebrafish immune system, *Bdellovibrio* was considered to survive long enough in vivo to undergo effective predation.

### 4.3.3. *Bdellovibrio* injections protect zebrafish larvae from *Shigella* infection

Although previous work has shown that application of *Bdellovibrio* can reduce numbers of pathogenic bacteria in the gut of chickens and shrimp, in vivo studies have been limited (Atterbury et al., 2011, Li et al., 2014a). No study has yet been able to provide direct evidence of bacterial predation in vivo, in part because model organisms have not been amenable to in vivo microscopy. Here, we tested the predatory behaviour of *Bdellovibrio* in our *Shigella*-zebrafish infection model. The zebrafish HBV is a compartmentalised infection site accessible to immune cell populations, allowing us to study predator prey interactions in the context of a host innate immune system. Data presented in Chapter 3 shows that the zebrafish immune system alone is insufficient to control the replication of a high HBV dose of *Shigella*, resulting in larval death. Strikingly, results in this chapter reveal that treatment with *Bdellovibrio* can significantly reduce pathogen burden and rescue zebrafish mortality (Fig. 4.3A-4.3C). Lethal HBV *Shigella* infections remain within the hindbrain and forebrain of the larva, enabling us to evaluate *Bdellovibrio* as a therapeutic against infection in a wound or body compartment. Interestingly, a study recently published has shown that *B. bacteriovorus*
can attenuate *Klebsiella pneumoniae* infection in the rat lung (Shatzkes et al., 2016). However, in contrast to the results we obtained studying a compartmentalised *Shigella* infection, the efficacy of *Bdellovibrio* for the treatment of systemic bacterial infection is less promising. Recent studies have shown that *B. bacteriovorus* is not therapeutic to *K. pneumoniae* blood infection in rats (Shatzkes et al., 2017b), and that bacterial predation is largely inhibited by human serum (Im et al., 2017). Therefore, further testing in systemic models of infection is required before assumptions can be made as to the efficacy of *Bdellovibrio* as a treatment for bacteraemia or disseminated infection.

### 4.3.4. *Bdellovibrio* predate *S. flexneri in vivo*

Consistent with previous studies studying *Bdellovibrio* predation *in vitro* (Gillis and Nakamura, 1970, Dashiff et al., 2011), we showed the *in vitro* predation of *Shigella* by *Bdellovibrio* using the multidrug resistant strain M90T (Fig. 4.4A-4.4D). However, prior to our study, bacterial predation had not been directly tested *in vivo*. Using high resolution confocal microscopy, we captured active predation of *Shigella* by *Bdellovibrio* within the zebrafish larva and saw that invasion and rounding of prey occurred over 1 h, i.e. similar time frames to those observed *in vitro* (Fig. 4.4E and 4.4F) (Starr and Baigent, 1966). Although many Gram-negative pathogens including *S. flexneri* have an intracellular lifestyle, bacterial predation captured in this study appear to largely occur extracellularly. Crucial evidence of bacterial predation *in vivo* was obtained using bacterial enumerations, which revealed prey-dependent replication of *Bdellovibrio* (Fig. 4.4G). The predatory cycle of *Bdellovibrio* lasts 3-4 h (Sockett, 2009), and by 24 hpi, we recovered up to 57.3-fold more predatory bacteria than originally injected. As *Bdellovibrio* typically produce ~6 progeny per prey cell, these data indicate that *Bdellovibrio* can undergo multiple rounds of predation *in vivo*. Together, these data provide the first direct evidence of bacterial killing by a predatory bacterium *in vivo*. For further proof of bacterial predation *in vivo*, future studies may use serial section electron microscopy to look for bdelloplasts in *Shigella* and *Bdellovibrio* injected zebrafish
larvae, or use fluorescent-activated cell sorting to isolate bdelloplasts comprised of fluorescently-tagged predator and prey from larval homogenates.

4.3.5. Zebrafish innate immune cells eliminate *Bdellovibrio in vivo*

We have discovered that the host immune response is a crucial aspect of therapy using living antibiotics. At 3 dpf, larvae comprise a fully functional innate immune system which is highly homologous to that of humans and is mediated by neutrophils and macrophages (Renshaw and Trede, 2012). Little is known of the interactions between immune cells and *Bdellovibrio in vivo*, however features of these bacteria may be predicted to minimise host detection. *Bdellovibrio* comprise a modified mannosylated LPS outer membrane, a single sheathed flagellum, and relatively low gene expression when outside a bacterial prey cell (Schwudke et al., 2003, Seidler and Starr, 1968, Lambert et al., 2010, Karunker et al., 2013).

In this study, imaging of fluorescent transgenic larvae demonstrates the recruitment of both neutrophils and macrophages to *Bdellovibrio in vivo* (Fig. 4.5A-4.5D). Phagocytic engulfment by both cell types was visualised by confocal microscopy (Fig. 4.5E and 4.5F). Surprisingly, real time microscopy revealed that the mCherry fluorescence of *Bdellovibrio* can last for hours within macrophages. These observations suggest that *Bdellovibrio* can survive intracellularly for a limited time; it is therefore tempting to speculate that predation could also occur inside a host cell.

4.3.6. The cytokine response to *Bdellovibrio* injection in vivo

Testing of immune-compromised zebrafish larvae highlighted a role for leukocytes in clearance of *Bdellovibrio* by the host (Fig. 4.5H). Leukocyte clearance of predatory bacteria may be considered a beneficial property of a limited treatment required to treat acute infection. Importantly, the viability of immune-compromised larvae was not significantly
affected by injection of *Bdellovibrio* (Fig. 4.5G). Bacterial infections often present alongside immunodeficiencies, and the pathogenicity of live therapies in patients with weakened immunity is an important consideration. Innate immune responses to infection are orchestrated by cytokine signalling (Lacy, 2015). Using our zebrafish infection model, we found that *Bdellovibrio* treatment did not augment the proinflammatory response to *Shigella* infection (Fig. 4.6A and 4.6B). Previous data obtained using other animal models (Shatzkes et al., 2017b, Shatzkes et al., 2017c, Shatzkes et al., 2016, Shatzkes et al., 2015), and the very high doses of predators used to provoke an inflammatory response in this study, indicate that *Bdellovibrio* are only weakly immunogenic. These results show that the benefits of *Bdellovibrio* treatment are unlikely due to immune-stimulation of the host, and instead due to bacterial predation *in vivo*.

### 4.3.7. *Bdellovibrio* work with host leukocytes to protect against *S. flexneri* infection

To dissect the relative contributions of *Bdellovibrio* and host innate immunity to *Shigella* clearance, we performed predation experiments in leukocyte-depleted larvae. Remarkably, although bacterial predation could alleviate some infection burden in an immune-compromised host, we found that maximum pathogen clearance was dependent upon the cooperative actions of *Bdellovibrio* and innate immune cells (Fig. 4.7A and 4.7B). These findings highlight the strength of considering eukaryotic-prokaryotic partnerships in the development of novel antimicrobial strategies.

### 4.3.8. A model for the antibacterial activity of *Bdellovibrio* against *S. flexneri in vivo*

Based on the data collected for this chapter, we propose the following model (Fig. 4.8). Injected *Bdellovibrio* act immediately to predate on a lethal dose of *Shigella* and reduce pathogen load. This treatment alleviates bacterial burden on the host immune system, allowing leukocytes to better control the remaining infection. As mature *Bdellovibrio* emerge
from lysed bdelloplasts, these bacteria may continue their predatory cycle until the prey is largely exhausted. It is likely that any remaining *Shigella* and *Bdellovibrio* will be ultimately cleared by the host innate immune cells. This model describes an additive effect of bacterial predation and the host innate immune system to improve survival in larvae challenged with a life-threatening, multidrug resistant, Gram-negative bacterial infection.

![Figure 4.8. Model of the therapeutic benefit of *Bdellovibrio* as an antibacterial agent against *S. flexneri* in vivo](image)

The zebrafish immune system alone is unable to control high doses of *Shigella* (green) in the HBV; without treatment, bacterial replication results in death of the larva. Injection of predatory *Bdellovibrio* (red) 30–90 min after *Shigella* infection is therapeutically beneficial to the host. Here, live invasive predation of *Shigella* by *Bdellovibrio* kills *Shigella*, significantly reducing host bacterial burden. Remaining *Shigella* and *Bdellovibrio* are ultimately cleared.
by host processes, including leukocyte action. Together, the immune system cooperates with predation to clear bacterial infection and promote larval survival.

### 4.3.9. Conclusions

In conclusion, these data raise no concerns for the use of *Bdellovibrio* as an injectable therapy *in vivo* and results obtained using our *Shigella*-zebrafish infection model are encouraging for the development of future predatory bacterial therapies in higher vertebrates, including humans. It is hoped that results from this study will inspire further research into the use of *Bdellovibrio* as a treatment for multidrug resistant bacterial infections.
CHAPTER 5. SEPTIN-MEDIATED EMERGENCY GRANULOPOIESIS RESCUES ZEBRAFISH LARVAE FROM SECONDARY INFECTION

5.1. Introduction

5.1.1. Overview of the haematopoietic system

Haematopoiesis is the hierarchical process by which haematopoietic stem and progenitor cells (HSPCs) produce mature blood cells (Fig. 5.1) (Orkin, 2000). In vertebrates, haematopoiesis occurs in two successive waves (Jagannathan-Bogdan and Zon, 2013). The initial and transient primitive wave takes place during early development (from 15 to 25 dpf in humans) and gives rise to limited cell types derived from haematopoietic progenitor cells (HPCs) with little self-renewal capacity (Tavian et al., 2001). In contrast, the definitive wave (from 25 dpf onwards in humans) is driven by self-renewing haematopoietic stem cells (HSCs) and continues to produce the full complement of blood cells throughout adult life (Tavian et al., 2010, Tavian et al., 2001).
Chapter 5. Results

Figure 5.1. Overview of the haematopoietic system

Self-renewing, multipotent HSCs produce HPCs with high proliferative potential but no self-renewal capacity. Progenitors display bias towards the production of certain blood cell lineages e.g. common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs). Mature blood cells are largely post-mitotic and must be regenerated from HSPC pools. Figure adapted from (Khwaja et al., 2016).

5.1.2. The zebrafish as a model to study haematopoiesis

Blood cell production is highly complex and regulated by a multitude of factors whose levels fluctuate over time within the haematopoietic microenvironment or ‘niche’ (Lo Celso and Scadden, 2011). Many of the factors governing blood cell development and HSC activity are unknown, and studies must therefore be conducted in vivo. Though murine models have made significant contributions to the field of haematopoiesis, direct visualisation of the haematopoietic niche is limited and the mutation of many key haematopoietic genes is embryonic lethal in mice (Sykes and Scadden, 2013, Schmitt et al., 2014). By contrast, transgenic zebrafish larvae with fluorescent blood cells are highly amenable to imaging, and mutations in the haematopoietic system are well-tolerated as oxygen can reach tissues via passive diffusion, temporarily bypassing the requirement for circulation (Berman et al., 2012, Jagannathan-Bogdan and Zon, 2013). Studies in the zebrafish can therefore provide a comprehensive understanding of blood cell development.

5.1.3. HSCs during development

HSCs are a rare population of cells, functionally defined by their ability to repopulate the entire haematopoietic system upon transplantation into a lethally irradiated host (Eaves, 2015). In vertebrates such as humans and zebrafish, HSCs are generated by endothelial
haematopoietic transition (Gritz and Hirschi, 2016). During this process, HSCs emerge from the floor of the dorsal aorta and are born in the aorta-gonad-mesonephros (AGM) (Ivanovs et al., 2011, Kissa and Herbomel, 2010). Nascent HSCs enter the circulation and are transported to temporary haematopoietic organs, i.e. the foetal liver in humans and the caudal haematopoietic tissue (CHT) in zebrafish (Al-Drees et al., 2015, Paik and Zon, 2010). Following organ colonisation, HSC populations expand and differentiate to produce HPCs and mature blood cells. In a final step, HSCs travel to the adult haematopoietic organs i.e. the thymus and bone marrow in humans and the thymus and kidney in fish.

5.1.4. Lessons in HSC biology from the zebrafish

High-resolution imaging of zebrafish larvae with fluorescently-labelled HSCs has enabled the visualisation of many fundamental aspects of haematopoietic development for the first time (Martin et al., 2011). In particular, microscopy studies have provided direct evidence of stem cell emergence from the haemogenic dorsal aorta, and revealed remodelling of the perivascular niche upon HSC colonisation (Kissa and Herbomel, 2010, Tamplin et al., 2015). Experiments in the zebrafish were also amongst the first to link ‘sterile’ pro-inflammatory signalling cascades to HSC development; here, in the absence of infection or pathological inflammation, neutrophil-released TNF-α, as well as NF-κB- and interferon (IFN) signalling pathways are required for stem cell generation (Espin-Palazon et al., 2014, Li et al., 2014b, He et al., 2015). Considering these observations, it is unsurprising that the typically quiescent HSC population has emerged as a key player in the host response to infection (Pietras, 2017).

5.1.5. Haematopoiesis in the context of infection

Inflammatory pathogens cause immune cell death and drain leukocytes from the circulation via recruitment to the infection site (Bergsbaken et al., 2009). The maintenance of leukocyte
populations *in vivo* is crucial for host defence, and haematopoietic precursors are required to proliferate and differentiate to replenish exhausted immune effectors, often at the expense of other blood cell lineages. Whilst this was previously considered the task of the haematopoietic progenitors alone, studies have shown that the HSC compartment also responds to infection (King and Goodell, 2011, Prendergast and Essers, 2014). Experiments in mice have described expansion of the stem cell population upon *E. coli* and *P. aeruginosa* infection (Zhang et al., 2008, Rodriguez et al., 2009). Similarly, experiments in zebrafish have revealed HSC-driven increases in neutrophil production i.e. emergency granulopoiesis, following *S. Typhimurium* infection (Hall et al., 2012, Manz and Boettcher, 2014). Using infection to stress the haematopoietic system can therefore be used to stimulate HSPC activity and reveal important aspects of haematopoietic biology (King and Goodell, 2011, Prendergast and Essers, 2014).

5.1.6. Emergency granulopoiesis: HSPC activation during infection

In this case of emergency granulopoiesis, different mechanisms have been proposed to stimulate HSPC activity, and these are classified as ‘indirect’ or ‘direct’ (Fig. 5.2) (Manz and Boettcher, 2014, Boettcher and Manz, 2016). Indirect stimulation describes how immune cells (e.g. macrophages, neutrophils) or non-immune cells (e.g. epithelial cells) detect bacteria via PRRs and release granulopoietic cytokines such as granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Bendall and Bradstock, 2014, Ushach and Zlotnik, 2016). Systemic cytokines are then detected by receptors on HSPCs to stimulate proliferation and differentiation (McKinstry et al., 1997). Differently, direct activation depends upon pathogen detection by receptors, such as Toll-like receptors (TLRs), present on the stem and progenitor cells themselves (Nagai et al., 2006, Boiko and Borghesi, 2012). Here, activation may occur both in a cytokine-independent manner or via autocrine granulopoietic signalling. Suppression of stem cell
activity via negative feedback signalling pathways is also crucial to prevent exhaustion of the HSC population (Croker et al., 2004, King et al., 2011).

Figure 5.2. Emergency granulopoiesis: direct and indirect signalling pathways

Both indirect and direct activation of HSPCs is dependent upon sensing of PAMPs via PRRs such as TLRs. Indirect mechanisms require pathogen-sensing by receptors on mature immune cells or non-immune cells; here, detection induces the release of granulopoietic cytokines, such as G-CSF, and these signals stimulate HSPC proliferation and differentiation. Alternatively, PAMPs may bind receptors on the HSPCs themselves for direct activation. In this case, granulopoiesis may be initiated in a cytokine-independent or an autocrine cytokine-dependent manner. Figure adapted from (Manz and Boettcher, 2014).

5.1.7. HSC division

The proliferation and differentiation of HSCs is tightly regulated (Nakamura-Ishizu et al., 2014). Failure to control these processes can exhaust progenitor pools and drive haematological malignancies (Sato et al., 2009, King et al., 2011, Zagozdzon and Golab, 2015). At its most fundamental level, the balance between stem cell and daughter cell
populations is maintained by the number of symmetric versus asymmetric cell divisions (Beckmann et al., 2007, Ho and Wagner, 2007, Knoblich, 2008). Whereas symmetrical divisions are required to expand the stem cell population, asymmetrical divisions drive differentiation whilst ensuring HSCs are not depleted (Fig. 5.3).

![Figure 5.3. Symmetric and asymmetric division of HSCs](image)

**Figure 5.3. Symmetric and asymmetric division of HSCs**

Activation of quiescent HSCs promotes symmetric or asymmetric division. Symmetrical division produces two stem cells, expanding the HSC population. Asymmetric division gives rise to one stem cell for the maintenance of the HSC population, and one progenitor cell which can continue along the path of differentiation. Figure adapted from (Nakamura-Ishizu et al., 2014).

### 5.1.8. Roles for septins in cell division

Septins are a family of cytoskeletal proteins first identified in 1971 by Nobel Laureate Lee Hartwell in the budding yeast *Saccharomyces cerevisiae*, during mutagenesis screens to discover genes crucial for cell division (Hartwell, 1971, Mostowy and Cossart, 2012). Studies here revealed that these ‘cell division cycle’ (CDC) proteins (now called septins) form rings localised to the mother-bud neck during septation to enable cytokinesis (Byers and Goetsch, 1976, Haarer and Pringle, 1987, Kim et al., 1991). Since then, a conserved role for septins in division has been confirmed across eukaryotes, including humans, where septins are...
recognised to function in two ways (Kinoshita and Noda, 2001, Joo et al., 2005). Firstly, septins act as a scaffold at the division site to recruit proteins and enable formation of the contractile actin-myosin ring (Kinoshita, 2006, Joo et al., 2007, Founounou et al., 2013). Secondly, septins form diffusion barriers for subcellular compartmentalisation (Caudron and Barral, 2009). In the case of yeast, septins have been shown to asymmetrically segregate proteins between mother and daughter cells (Barral et al., 2000, Takizawa et al., 2000, Luedeke et al., 2005, Shcheprova et al., 2008). Although diffusion barriers exist at the mid-body of dividing mammalian cells, a functional role for septins in this process has not yet been tested (Schmidt and Nichols, 2004a). Interestingly, recent studies have indicated that certain haematopoietic cell types may undergo septin-independent division (Menon and Gaestel, 2015). Specifically, experiments using mice have shown that Sept7 is not essential for the cytokinesis of T lymphocytes in vivo, nor for the proliferation and differentiation of haematopoietic progenitors into myeloid and lymphoid cells in vitro (Menon et al., 2014, Mujal et al., 2016).

5.1.9. Aims of this chapter

In Chapter 3, we established a zebrafish model of Shigella infection to study neutrophil-mediated infection control and the role of septins in inflammation. In this chapter, we develop our Shigella-zebrafish model to study emergency granulopoiesis in vivo, and use infection as a stimulus to investigate the role of septins in haematopoiesis and stem cell biology.

5.2. Results

5.2.1. Development of a Shigella-zebrafish model to study emergency granulopoiesis

In both humans and zebrafish, HSCs emerge from the AGM during early embryonic development (~30 hours post fertilisation in zebrafish) (Kissa and Herbomel, 2010, Ivanovs
et al., 2011). Zebrafish HSCs migrate to the CHT from 2 dpf, and from 3 dpf to the kidney and thymus (i.e. optically inaccessible adult organs) (Paik and Zon, 2010). To capture HSC-driven haematopoiesis at the microscope, we therefore employ a 2 dpf Shigella-zebrafish infection model. In a first step, larvae were infected at 2 dpf with a low dose (0.5-2.0 x 10^3 CFUs) of S. flexneri M90T in the HBV, and assayed for survival and bacterial burden over 48 hpi. Importantly, infection did not impact larval survival, and CFU assays revealed that bacteria were mostly cleared within 24 hpi (Fig. 5.4A and 5.4B). Previous work has shown that neutrophils are essential for S. flexneri control in vivo (Chapter 3, Fig. 3.5) (Phalipon and Sansonetti, 2007, Mostowy et al., 2013). We therefore tested the neutrophil response in our Shigella-2 dpf zebrafish infection model. For this, lyz:dsRed larvae with fluorescently-labelled neutrophils were injected in the HBV with GFP-S. flexneri (Hall et al., 2007). Time-lapse imaging of larvae by fluorescent stereomicroscopy showed neutrophil recruitment to the infection site from 1 hpi, engulfment of Shigella and an obvious reduction in bacterial burden (Fig. 5.4C). Neutrophil quantifications in infected larvae revealed a slight but significant reduction in neutrophil numbers 24 h following a low dose Shigella infection in the HBV (Fig. 5.4D). Consistent with previous work presented in Chapter 3, these new data show that neutrophils are important to control Shigella in vivo and indicate that the host response to infection is likely to require emergency granulopoiesis.

To capture emergency granulopoiesis, 2 dpf transgenic larvae with fluorescent neutrophils were injected with either PBS or Shigella and imaged by fluorescent stereomicroscopy. As granulopoiesis primarily occurs in the CHT during normal development (Paik and Zon, 2010), we imaged the AGM to visualise production of de novo neutrophils induced by infection (Fig. 5.4E). Strikingly, we observed significantly more neutrophils (2.4-fold ± 0.5) in the AGM of infected larvae from 48 hpi (Fig. 5.4F). Similar increases in neutrophil production were observed following clearance of systemically injected Shigella, administered via the caudal vein (Fig. 5.4G). Shigella virulence is mediated by the T3SS (Schroeder et al., 2007,
Mattock and Blocker, 2017). To assess the contribution of the T3SS to emergency granulopoiesis, we infected lyz:dsRed larvae in the HBV with a low dose of T3SS- Shigella M90T (ΔmxiD strain). In support of a role for Shigella virulence in the initiation of emergency granulopoiesis, we detected only a small increase in neutrophil production in larvae infected with T3SS- bacteria, as compared to WT bacteria (Fig. 5.4H). To assess macrophage production upon infection, we infected mpeg1:G/U:mCherry larvae with mCherry-expressing macrophages with S. flexneri (Ellett et al., 2011). Quantification of macrophages in the AGM failed to show a significant increase in macrophage production upon infection (Fig. 5.4I). Collectively, these data show that S. flexneri drives a neutrophil-specific haematopoietic response, and that the Shigella-zebrafish infection model offers the potential to study mechanisms underlying emergency granulopoiesis in vivo.
Figure 5.4. Development of the *Shigella*-zebrafish model to study emergency granulopoiesis

(A) Survival curves of WT AB zebrafish larvae injected in the HBV at 2 dpf with a low dose (≤3.0 x 10^3 CFUs) of GFP-*S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are
pooled from three independent experiments (n ≥ 10 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05.

(B) Time-course enumeration of live *Shigella* from homogenates of WT AB larvae injected with a low dose of GFP-*S. flexneri* M90T as in (A). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). p values (versus 0 hpi) determined by unpaired two-tailed Student’s t test. Significance with Bonferroni correction defined as p < 0.025. ***, p < 0.0005.

(C) Frames extracted from time-lapse fluorescent stereomicroscopy imaging of a single *lyz*:dsRed larva (with red neutrophils) injected with a low dose of GFP-*S. flexneri* M90T (green) as in (A). First frame of infection site at 1 hpi, followed by frames at 6 and 12 hpi. Scale bar, 100 µm.

(D) *Lyz*:dsRed larvae were injected with PBS or a low dose of GFP-*S. flexneri* as in (A). Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the whole larva quantified 24 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 4 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. **, p < 0.01. Data obtained by J. Shelley.

(E) Representative images of whole larvae (above, dashed outline) and the AGM (below, i.e. site of the dashed box) of *lyz*:dsRed larvae (with red neutrophils) injected with PBS or a low dose of GFP-*S. flexneri* M90T as in (A). Images taken by fluorescent stereomicroscopy at 48 hpi.
(F) Quantifications of neutrophils in the AGM of *lyz*::*dsRed* larvae injected with PBS or a low dose of GFP-*S. flexneri* M90T as in (A), and imaged by fluorescent stereomicroscopy as in (E). Neutrophils in the AGM were quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 10 larvae per condition per experiment). p values between conditions at cognate time points determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(G) *Lyz*::*dsRed* larvae were injected in the caudal vein at 2 dpf with PBS or a low dose of GFP-*S. flexneri* M90T. Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (n ≥ 4 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(H) *Lyz*::*dsRed* larvae were injected in the HBV at 2 dpf with PBS or a low dose of T3SS-*S. flexneri* M90T. Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from four independent experiments (n ≥ 8 larvae per condition per experiment). p values between conditions at cognate time points determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(I) *Mpeg1*:G/U::mCherry larvae were injected in the HBV at 2 dpf with PBS or a low dose of GFP-*S. flexneri* M90T. Larvae were imaged by fluorescent stereomicroscopy and macrophages in the AGM quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (using up to 10 larvae per condition per experiment). p values
between conditions at cognate time points determined by unpaired one-tailed Student’s t-test. Significance defined as: p < 0.05. **, p < 0.01.

5.2.2. The granulopoietic response to Shigella infection is stem cell-driven

Unexpectedly, recent studies have shown that HSCs can directly respond to inflammatory cues (King and Goodell, 2011, Baldridge et al., 2011, Kovtonyuk et al., 2016). Runx1 is a transcription factor specific to HSC development (de Bruijn and Dzierzak, 2017, Kalev-Zylinska et al., 2002). To capture evidence of stem cell-driven neutrophil production, we outcrossed Tg(runx:mCherry) (herein referred to as runx:mCherry) and mpx:GFP zebrafish lines. Resulting larvae had mCherry-expressing stem cells and GFP-expressing neutrophils (Renshaw et al., 2006, Tamplin et al., 2015). In these larvae, photostability of the mCherry protein imparts a weak fluorescence onto stem cell progeny, i.e. haematopoietic progenitors. Confocal microscopy of double-transgenic larvae following Shigella infection showed bright-mCherry HSCs in the AGM, surrounded by dim-mCherry progenitor cells and adjacent to these progenitors, GFP neutrophils (Fig. 5.5A). By contrast, minimal granulopoiesis occurs within the AGM of uninfected larvae. These observations suggest that proliferation and differentiation of HSCs is driving emergency granulopoiesis upon Shigella infection. To test for infection-responsive HSC proliferation, 2 dpf Tg(runx:eGFP) larvae (herein referred to as runx:eGFP) with GFP-expressing HSCs were injected in the HBV with either PBS or Shigella, and imaged by fluorescent stereomicroscopy (Tamplin et al., 2015). Quantifications revealed a significant increase (5.3-fold ± 1.8) in the number of HSCs in the AGM of infected larvae at 48 hpi (Fig. 5.5B). Together, these results demonstrate that emergency granulopoiesis in response to Shigella infection is driven by HSC activity.
Figure 5.5. The granulopoietic response to *Shigella* infection is stem cell-driven

(A) *Runx*:mCherry x *mpx*:GFP zebrafish larvae (with red HSPCs and green neutrophils) were injected in the HBV at 2 dpf with a low dose ($\leq 3.0 \times 10^3$ CFUs) of *S. flexneri* M90T and the AGM imaged by confocal microscopy (x100 objective). Dashed lines represent vasculature. DA, dorsal aorta; CV, caudal vein. Cells expressing high levels of mCherry (*) were considered HSCs; cells expressing lower levels of mCherry were considered HPCs (i.e. HSC progeny, arrowheads). Maximum intensity z-projection images shown.

(B) *Runx*:eGFP larvae (with green HSCs) were injected with PBS or a low dose of mCherry-*S. flexneri* M90T as in (A). Larvae were imaged by fluorescent stereomicroscopy and HSCs in the AGM quantified 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments ($n \geq 6$ larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student's t test. Significance defined as: p < 0.05. **, p < 0.01.

5.2.3. Septins are required for stem cell-driven granulopoiesis

Recent work has shown that certain haematopoietic cell types may undergo septin-independent cell division (Menon and Gaestel, 2015, Menon et al., 2014, Mujal et al., 2016). To test the role of septins in zebrafish haematopoiesis *in vivo*, we depleted *lyz*:dsRed larvae
of Sept15, i.e. the zebrafish homologue of human SEPT7, using morpholino oligonucleotide as described previously (Chapter 3, Fig. 3.6B) and counted neutrophils over the course of development (Fig. 5.6A and 5.6B). To control for the effects of morpholino injection, a separate group of larvae were treated with a control morpholino (i.e. with no known target in the zebrafish genome). Neutrophil quantifications at 2 dpf showed similar numbers of neutrophils in control and Sept15 morphants, suggesting that neutrophil development during early, primitive haematopoiesis (i.e. not stem cell-driven) is not significantly affected by septin depletion (Fig. 5.6C). In contrast, quantifications at 4 dpf revealed significantly fewer neutrophils in Sept15 morphants (0.6-fold ± 0.0), suggesting that septins are important for neutrophil production in later, definitive waves of haematopoiesis (i.e. stem cell-driven) (Fig. 5.6D). These data are consistent with previous results which highlight neutropenia already in uninfected Sept15-depleted larvae at 3 dpf (Chapter 3, Fig. 3.7), and strongly suggest that septins play a role in stem cell-driven granulopoiesis.

In previous experiments we observed expansion of the HSC population alongside increased neutrophil production, indicating that Shigella-induced emergency granulopoiesis is driven by stem cells (Fig. 5.4F and 5.5B). To test the role of septins in stem cell-driven emergency granulopoiesis, we performed infection assays in control and Sept15 morphants with fluorescent neutrophils. Consistent with previous work (Chapter 3, Fig. 3.6), we observed that Sept15-depleted larvae are less able to control bacterial infection, and ~60% of Sept15 morphants are able to clear bacterial burden (as determined by fluorescent microscopy) and survive a low dose S. flexneri infection. Sept15 morphants having survived Shigella infection present a unique opportunity to study the role of septins in emergency granulopoiesis. Strikingly, Sept15 morphants that had cleared infection failed to undergo emergency granulopoiesis, and instead larvae showed a reduced number of neutrophils in the AGM at 48 hpi (0.6-fold ± 0.0) (Fig. 5.6E). Collectively, these data reveal a new role for septins in stem cell-driven haematopoiesis.
Figure 5.6. Septins are required for stem cell-driven granulopoiesis

(A) Schematic to show primitive (blue) and definitive (red) haematopoietic organs in the developing zebrafish larva. Primitive haematopoiesis is transient and driven by progenitors in the intermediate cell mass (ICM), anterior lateral mesoderm (ALM) and posterior blood island (PBI). During definitive haematopoiesis, HSCs emerge from the AGM and travel to the CHT. From 3 dpf, HSCs will migrate to ‘adult’ haematopoietic tissues, the thymus and kidney. Arrows represent the 2 and 4 dpf time points assessed in (C and D).

(B) Representative western blot of extracts from 2 dpf larvae injected with control (Ctrl) or Sept15 morpholino (Mo). Antibodies used against SEPT7 or GAPDH (as a control).

(C-D) Lyz:dsRed Ctrl or Sept15 morphants were imaged by fluorescent stereomicroscopy and neutrophils in the whole larva quantified at (C) 2 and (D) 4 dpf. Each circle represents a
count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from 3 independent experiments (n ≥ 4 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(E) Lyz:dsRed Ctrl or Sept15 morphants were injected in the HBV at 2 dpf with PBS or a low dose (≤3.0 x 10^3 CFUs) of GFP- S. flexneri M90T. Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. p values between conditions determined by unpaired one-tailed Student’s t test. Data are pooled from three independent experiments (n ≥ 3 larvae per condition per experiment). Significance defined as: ***, p < 0.001.

5.2.4. Sept15 is not required for granulopoietic cytokine signalling in vivo

Cytokine signalling is essential for the induction of emergency granulopoiesis (Manz and Boettcher, 2014, Boettcher and Manz, 2016). Macrophages have been described as a source of G-CSF, a growth factor required to stimulate neutrophil production by HSPCs (Hall et al., 2012, Manz and Boettcher, 2014). To assess the role of macrophages in the induction of emergency granulopoiesis by Shigella, we used a morpholino to target myeloid lineage-commitment factor Irf8 and deplete macrophages (Li et al., 2011). Quantifications showed significantly fewer macrophages in Irf8 morphants as compared to controls (Fig. 5.7A). Surprisingly, neutrophil counts in the AGM of Shigella-infected morphants revealed a robust granulopoietic response in macrophage-depleted larvae (Fig. 5.7B). In addition to macrophage depletion, Irf8 depletion can increase neutrophil numbers (Fig. 5.7C) (Li et al., 2011). To alternatively test a role for macrophages in increased neutrophil production upon infection, we used metronidazole to pharmacologically deplete macrophages in mpeg1;G/U;mCherry x mpk;GFP larvae (Gray et al., 2011) (Fig. 5.7D and 5.7E). Similar to
results observed using Irf8 morphants, pharmacological depletion of macrophages by metronidazole did not prevent emergency granulopoiesis (Fig. 5.7F). These data show that emergency granulopoiesis in response to *Shigella* can occur independently of macrophages.

To test whether G-CSF is important for emergency granulopoiesis in our *Shigella*-zebrafish model, we used a morpholino to target the G-CSF receptor (*gcsfr*) and block G-CSF signalling. In support of a role for G-CSF signalling in the induction of granulopoiesis by *Shigella* infection, Gcsfr morphants showed no significant increase in neutrophil production in the AGM upon infection (Fig. 5.7G). To determine whether impaired G-CSF signalling is responsible for the failure of septin-depleted larvae to undergo emergency granulopoiesis, we tested *gcsfa* expression in Sept15 morphants using RT-qPCR. Results here showed that *gcsfa* is induced in control larvae at 24 and 48 h (1.5-fold ± 0.0 and 1.8-fold ± 0.4, respectively) following infection, and that levels of *gcsfa* are not significantly different between infected control and Sept15 morphants (Fig. 5.7H). Taken together, these results exclude a role for Sept15 in G-CSF signalling crucial for emergency granulopoiesis. Instead, they suggest that septins act cell-autonomously, independent of exogenous signalling pathways, to support the proliferation and differentiation of HSPCs.
Figure 5.7. Sept15 is not required for granulopoietic cytokine signalling in vivo

(A) Mpeg1:G/U:mCherry zebrafish larvae were treated with control (Ctrl) or Irf8 morpholino (Mo). Larvae were imaged by fluorescent stereomicroscopy and macrophages in the whole larva quantified at 2 dpf. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are from one independent experiment (n ≥ 3 larvae per condition). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05, *; p < 0.01, **; p < 0.001, ***.

(B) Lyz:dsRed larvae were treated with Ctrl or Irf8 Mo. Morphants were injected in the HBV at 2 dpf with a low dose (≤3.0 x 10^3 CFUs) of GFP-S. flexneri M90T. Larvae were imaged by
fluorescent stereomicroscopy and neutrophils in the AGM quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 4 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05.

(C) Uninfected lyz:dsRed Ctrl or Irf8 morphants were imaged by fluorescent stereomicroscopy and neutrophils in the whole larva quantified at 2 dpf. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 8 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001. Data obtained in collaboration with V. Torraca.

(D-E) Mpeg1:G/U:mCherry x mpx:GFP larvae were untreated (Ctrl) or treated with metronidazole. Larvae were imaged by fluorescent stereomicroscopy and macrophages in the whole larva quantified at (D) 2 dpf and (E) 4 dpf. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 8 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001.

(F) Mpeg1:G/U:mCherry x mpx:GFP Ctrl or metronidazole-treated larvae were injected in the HBV at 2 dpf with a low dose of GFP-S. flexneri M90T. Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified up to 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 4 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05.
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(G) Lyz:dsRed larvae were treated with Ctrl or Gcsfr Mo. Morphants were injected in the HBV at 2 dpf with a low dose of GFP-S. flexneri M90T. Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified at 48 h following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 6 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. ***, p < 0.001. Data obtained in collaboration with V. Torraca.

(H) WT AB larvae were treated with Ctrl or Sept15 Mo. Morphants were injected in the HBV at 2 dpf with either PBS or a low dose (≤3.0 x 10³ CFUs) of GFP-S. flexneri M90T. RNA was extracted from pools of 5 larvae at 24 and 48 hpi and expression of gcsfa mRNA transcripts determined by RT-qPCR. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments. p values (versus 24 or 48 hpi Ctrl Mo PBS controls) determined by unpaired two-tailed Student’s t-test. Significance with Bonferroni correction defined as: p < 0.025. ***, p < 0.0005. Data obtained in collaboration with V. Torraca.

5.2.5. Septin-dependent emergency granulopoiesis mediates long-term host defence

To evaluate the duration of the emergency granulopoietic response, lyz:dsRed larvae infected in the HBV with a low dose of Shigella were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified up to 5 days post infection (dpi). Surprisingly, even though bacteria are mostly cleared within 24 hpi (Fig. 5.4B), quantifications revealed a significant increase in neutrophil numbers up to 4 dpi (Fig. 5.8A). To test whether emergency granulopoiesis can act as a host defence mechanism to actively boost innate immunity, we performed dual infection assays (Fig. 5.8B). Here, larvae were injected with PBS or ‘primed’ with a low dose of GFP-Shigella at 2 dpf, and subsequently injected with a lethal dose (>5.0 x 10⁴ CFUs) of mCherry-Shigella at 4 dpf. Consistent with
emergency granulopoiesis having a role in boosting host immunity, priming of larvae with Shigella rescued zebrafish survival and significantly improved bacterial clearance following secondary infection (Fig. 5.8C and 5.8D). Importantly, larvae do not present an adaptive immune system until 30 dpf, and thus the protection observed here is exclusively due to innate immunity (Renshaw and Trede, 2012).

In response to Shigella infection, Sept15 morphants show failures in emergency granulopoiesis (Fig. 5.6E). To determine whether septin-mediated emergency granulopoiesis is responsible for improved survival in primed morphants, we performed dual infection assays in control or Sept15-depleted larvae. Strikingly, in contrast to control morphants, the priming of Sept15 morphants failed to boost host innate immunity (Fig. 5.8E-5.8H). Instead, Sept15 morphants that had cleared a low dose of Shigella were more susceptible to a lethal infection than naïve Sept15 morphants. Together, these results show that emergency granulopoiesis can enhance host innate immunity to bacterial infection, and that this enhancement is coordinated by a septin-mediated stem cell response.
Figure 5.8. Septin-dependent emergency granulopoiesis mediates long-term host defence

(A) Lyz:dsRed zebrafish larvae were injected in the HBV at 2 dpf with PBS or a low dose (≤3.0 x 10^3 CFUs) of GFP-S. flexneri M90T. Larvae were imaged by fluorescent stereomicroscopy and neutrophils in the AGM quantified from 3-5 days following treatment. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (n ≥ 8 larvae per condition per experiment). p values between conditions at cognate time points determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. *, p < 0.05; ***, p < 0.001.
(B) Schematic of dual infection assays. At 2 dpf, WT AB larvae were injected in the HBV with PBS or a low dose of GFP-S. flexneri. At 4 dpf i.e. 48 hours post-primary injection (hp1i), larvae were injected with a high dose (>5.0 x 10^3 CFUs) of mCherry-S. flexneri. Analyses were performed on larvae up to 72 hours post-secondary infection (hp2i). Injection timepoints are indicated by arrows.

(C) Survival curves of WT AB larvae from dual infection assays as in (B). Larval viability was scored for 72 hpi. Data are pooled from two independent experiments (n ≥ 10 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. *, p < 0.05.

(D) Time-course enumeration of live Shigella from homogenates of WT AB larvae from dual infection assays as in (B). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (using up to 3 larvae per time point per experiment). p values between conditions determined by unpaired two-tailed Student's t test. Significance defined as: p < 0.05. **, p < 0.001.

(E) WT AB larvae were treated with control (Ctrl) morpholino (Mo) and morphants subjected to dual infection assays as in (B). Larval viability was scored for 72 hpi. Data are pooled from three independent experiments (n ≥ 10 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. **, p < 0.01.

(F) Ctrl morphants subjected to dual infection assays as in (B) were imaged by fluorescent stereomicroscopy over time and bacterial burden analysed to produce fluorescence intensity measurements. Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three
independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05. *, p < 0.05; ***, p < 0.001.

(G) WT AB larvae were treated with Sept15 Mo and morphants subjected to dual infection assays as in (B). Larval viability was scored for 72 hpi. Data are pooled from two independent experiments (n ≥ 9 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05.

(H) Sept15 morphants subjected to dual infection assays as in (B) were imaged by fluorescent stereomicroscopy over time and bacterial burden analysed to produce fluorescence intensity measurements. Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from two independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired one-tailed Student’s t test. Significance defined as: p < 0.05.

5.3. Discussion

5.3.1. Overview

The zebrafish larva is a valuable animal model to visualise haematopoiesis, infection and innate immunity in vivo (Kanther and Rawls, 2010, Renshaw and Trede, 2012, Meijer et al., 2014). Emergency granulopoiesis is a crucial aspect of the haematopoietic response to infection (Manz and Boettcher, 2014, Boettcher and Manz, 2016), and we have previously reported neutropenia in Sept15 morphants (Chapter 3, Fig. 3.7). In this chapter, we examine the role of Sept15 in haematopoietic cell development and stem cell-driven emergency granulopoiesis.
5.3.2. The *Shigella*-zebrafish model to study emergency granulopoiesis

*Shigella* is an inflammatory pathogen controlled by neutrophils (Mandic-Mulec et al., 1997, Mostowy et al., 2013, Phalipon and Sansonetti, 2007), and can therefore be used as a paradigm to study emergency granulopoiesis *in vivo*. In contrast to [Chapter 3](#) and [Chapter 4](#) in which larvae are infected at 3 dpf, we herein employ a 2 dpf *Shigella*-zebrafish infection model to enable visualisation of HSCs prior to their migration to optically inaccessible ‘adult’ haematopoietic organs. Importantly, both neutrophils and macrophages are present at 2 dpf in the zebrafish, and we first show that the innate immune system is sufficiently developed to control a low dose *Shigella* infection ([Fig. 5.4A-5.4C](#)). We could therefore use a low dose of *S. flexneri* in the HBV as a stimulus to investigate emergency granulopoiesis. Previous work using a *Salmonella*-zebrafish infection model has shown increased neutrophil production at 48 hpi (Hall et al., 2012). Consistent with this, neutrophil production following *Shigella* infection is increased from 48 hpi, i.e. 24 h after most bacteria are cleared ([Fig. 5.4E and 5.4F](#)). Most previous studies describe emergency granulopoiesis upon systemic infection (Manz and Boettcher, 2014, Boettcher and Manz, 2016). In contrast, we observe emergency granulopoiesis in response to both a localised hindbrain infection and systemic caudal vein infection ([Fig. 5.4F and 5.4G](#)). In agreement with previous studies that show emergency granulopoiesis following injections of LPS alone, neutrophil production increases in response to avirulent T3SS-*S. flexneri* (Nagai et al., 2006); however, induction is to a lesser degree than with WT *S. flexneri* ([Fig. 5.4H](#)). Interestingly, a recent study has shown that increased macrophage production occurs in zebrafish larvae following systemic *E. coli* infection (Hou et al., 2016). Results in this chapter, however, indicate that the haematopoietic response to *Shigella* infection is specific to neutrophils, and not the result of a global increase in myeloid cell production ([Fig. 5.4I](#)). Considering that neutrophils are important for the control of *Shigella* infection *in vivo* (Mostowy et al., 2013), it is tempting to speculate that the haematopoietic response is tailored to overcome the specific pathogen and its infection attributes.
5.3.3. *Shigella*-induced emergency granulopoiesis is stem cell-driven

HSCs are increasingly implicated in the immune response to infection (King and Goodell, 2011, Baldridge et al., 2011). Studies have shown that stem cell activity is important to stimulate granulopoiesis following infection by a number of human pathogens including *S. Typhimurium, E. coli, S. aureus, Mycobacterium avium* and *Candida albicans* (Hall et al., 2012, Zhang et al., 2008, Granick et al., 2013, Baldridge et al., 2010, Yanez et al., 2010). In agreement with current literature, and in support of a stem cell-driven granulopoietic response to *Shigella* infection, we observed HSC proliferation and differentiation in infected larvae (Fig. 5.5A and 5.5B). Using infection-driven neutrophil production as a read out of stem cell activity, we can now use our *in vivo* system to test the precise role of targeted molecular components in stem cell biology.

5.3.4. Septins are required for stem cell-driven granulopoiesis

Septins are recognised as essential for host cell division (Hartwell, 1971, Kinoshita and Noda, 2001, Mostowy and Cossart, 2012). However, recent studies have challenged this and reported septin-independent cytokinesis in certain haematopoietic cell types (Menon and Gaestel, 2015, Menon et al., 2014, Mujal et al., 2016). In our zebrafish model, we observe granulopoietic failures in Sept15-depleted larvae that become apparent only with the onset of stem cell-driven definitive haematopoiesis, and not during the earlier stem cell-independent forms of granulopoiesis (Fig. 5.6C and 5.6D). These data are in agreement with previous results showing significantly fewer neutrophils in uninfected Sept15 morphants at 3 dpi (Chapter 3, Fig. 3.7). Moreover, our work here reveals an inability of Sept15 morphants to undergo stem cell-driven emergency granulopoiesis in response to *Shigella* infection (Fig. 5.6E). In agreement with this, experiments using mice have shown that Sept7-depleted myeloid progenitors are able to undergo some proliferation and differentiation *in vitro*,
however the ability of these cell types to produce granulocytes is dramatically reduced (Menon et al., 2014).

5.3.5. Sept15 is not required for granulopoietic signalling in vivo

The haematopoietic response to infection is mediated by cytokine signalling, yet the cellular sources of cytokines are not fully known (Manz and Boettcher, 2014). A recent study using a Salmonella-zebrafish infection model proposed that macrophage-released G-CSF stimulates HSC proliferation and neutrophil production via a C/ebpβ-Nos2a pathway (Hall et al., 2012). Unlike S. Typhimurium, S. flexneri induces apoptosis in macrophages (Zychlinsky et al., 1992, Mostowy et al., 2013), and in our Shigella-zebrafish model we show that macrophage depletion does not compromise emergency granulopoiesis (Fig. 5.7A-5.7F). These data reveal that macrophages are dispensable for the induction of emergency granulopoiesis in response to Shigella infection, and we therefore propose that granulopoietic signalling is pathogen specific. Studies have shown that epithelial cells are crucial for the release of granulopoietic cytokines in response to uropathogenic E. coli infection (Ingersoll et al., 2008). In the case of Shigella infection of the hindbrain, the epithelial-lined cavity of the HBV may be a source of cytokines required to induce stem cell-driven granulopoiesis. While multiple cytokines have been implicated in HSPC proliferation and differentiation, G-CSF is widely regarded as the key signalling molecule in the context of emergency granulopoiesis (Manz and Boettcher, 2014, Boettcher and Manz, 2016, Bendall and Bradstock, 2014). In agreement with this, Gcsfr morphants failed to stimulate neutrophil production in response to Shigella infection (Fig. 5.7G). Previous work has revealed that induction of gcsfa is important for infection-induced emergency granulopoiesis in zebrafish (Hall et al., 2012). Importantly, we observe similar expression of gcsfa in both control and Sept15 morphants upon infection, and conclude that failures in G-CSF signalling are not responsible for defects in granulopoiesis upon septin depletion (Fig. 5.7H). We therefore propose that septin plays a cell autonomous role in HSPC biology.
5.3.6. A cell-autonomous role for septins in HSC biology

To respond to infection, HSPCs undergo rapid cell division and differentiation (Baldridge et al., 2011, King and Goodell, 2011). Importantly, HSCs couple these processes and undergo asymmetric division (Knoblich, 2008). Asymmetric division is an important biological feature of stem cells as it supports self-renewal and maintenance of a stem population (Ho and Wagner, 2007). In yeast, septins act as a diffusion barrier to segregate mother and daughter proteins during asymmetric division, and it is likely that they perform a similar function in mammalian cells (Caudron and Barral, 2009, Schmidt and Nichols, 2004a). Indeed, one study using zebrafish found that whilst suppression of Sept15 increased the number of pancreatic endocrine progenitors, it restricted differentiation (Dash et al., 2016). Therefore, in the absence of septins, HSC division may give rise to two phenotypically similar daughter cells which are unable to either self-propagate or differentiate, thereby preventing effective haematopoiesis. This precise role of septins in HSC biology awaits future investigation.

5.3.7. Septin-mediated emergency granulopoiesis enhances host defence

Zebrafish do not develop adaptive immunity until 30 dpf, and therefore larvae provide a unique opportunity to examine the innate immune system in isolation (Renshaw and Trede, 2012). Historically, the capacity of an organism to respond to a secondary infection has been considered the task of the adaptive immune system. In contrast, emergency granulopoiesis is seen as a homeostatic mechanism to maintain leukocyte populations and enable the control of an existing bacterial infection (Manz and Boettcher, 2014, Boettcher and Manz, 2016). The ability of emergency granulopoiesis to augment the host response to bacterial infection is largely untested. Using our Shigella-zebrafish model, we observe increased neutrophil production for several days following the clearance of S. flexneri (Fig. 5.8A). These findings enabled us to investigate the role of emergency granulopoiesis in the enhancement of innate immunity using dual infection assays. Here, larvae pre-exposed to a
low dose of *Shigella* are significantly more protected against a secondary (typically lethal) *Shigella* infection (Fig. 5.8C and 5.8D). Remarkably, experiments in Sept15 morphants showed that ‘primed’ larva unable to undergo emergency granulopoiesis are not protected against secondary infection (Fig. 5.8E-5.8H). We therefore propose that septin-mediated emergency granulopoiesis functions beyond homeostasis to enhance innate immunity.

### 5.3.8. How does emergency granulopoiesis boost host defence?

Our results provide the first evidence that emergency granulopoiesis can boost host defence to secondary infection. Boosting of innate immunity may occur by brute force, whereby HSPCs are more populous and less quiescent owing to previous, rapid proliferation and are therefore more responsive to a secondary infection. In this case, enhanced neutrophil production would increase the number of neutrophils available to combat infection. On the other hand, boosting of innate immunity may occur by fine-tuning, whereby HSPCs produce neutrophils with an enhanced capacity to kill *Shigella*. In this case, ‘trained innate immunity’ can enable immune cells to exhibit characteristics typical of adaptive immunity via epigenetic reprogramming (Netea et al., 2016). The *in vivo* mechanisms underlying enhanced immunity in our *Shigella*-zebrafish model await investigation.

### 5.3.9. Conclusions

In summary, we have developed a *Shigella*-zebrafish model to study *in vivo* the mechanisms underlying emergency granulopoiesis and stem cell biology. Using this model, we have discovered a new role for septins in HSC function. Molecules important for the division and differentiation of HSCs will be of great interest to researchers of stem cells and haematological disease. Additionally, we show that emergency granulopoiesis is an important innate immune program required to enhance host defence. A complete understanding of the cellular and molecular mechanisms underlying HSC and immune cell
development will be important for the therapeutic manipulation of innate immunity in humans.
CHAPTER 6. CONCLUSIONS AND FUTURE WORK

6.1. Septins restrict inflammation and protect zebrafish larvae from *Shigella* infection

In Chapter 3, we develop a zebrafish model of localised *S. flexneri* infection, and use it to study the role of septins in inflammation control and host defence. We revealed that depletion of Sept15 dramatically increases host susceptibility to *Shigella* infection. Using live cell imaging we showed that failure of septin-depleted neutrophils to control bacterial load correlates with increased Caspase-1 activity and cell death at the site of infection. Strikingly, dampening of the IL-1-mediated inflammatory response using anakinra protects septin-depleted larvae from hyperinflammation, and rescues larval survival during *Shigella* infection. Based on these results, we propose that septin-depletion results in an excess of inflammation and increases neutrophil and host susceptibility to *Shigella* infection. This is in agreement with shigellosis in humans, which is a highly inflammatory disease whereby death is typically a consequence of inflammation-induced dysentery.

The relative contributions of inflammation and bacterial burden to neutrophil death have been difficult to distinguish in the zebrafish. Interestingly, time-lapse imaging of the recently published zebrafish ASC reporter line has been used to track spatio-temporal activation of the inflammasome *in vivo* (Kuri et al., 2017). ASC has been identified as an important mediator of inflammasome activation in response to *Shigella* infection (Suzuki et al., 2007), and thus live imaging of these larvae should provide insight into inflammasome activation and neutrophil death during *Shigella* infection and upon septin-depletion. Additionally, studies using other zebrafish infection models can help to reveal links between septins and host defence. For example, the natural fish pathogen *M. marinum* is largely controlled by macrophages (Clay et al., 2007), and work here can indicate whether septins also control inflammation in these cell types.
Work using the *Shigella*-zebrafish infection model provided the first evidence of bacterial septin caging *in vivo* (Mostowy et al., 2013), and it has recently been reported that failure to form septin cages in murine cell lines can impair bacterial autophagy, resulting in inflammasome activation (Lee et al., in press). It is therefore possible that failure of Sept15 morphants to entrap *Shigella* in septin cages may contribute to our hyperinflammatory phenotype. The precise means by which septins control inflammation are difficult to study *in vivo*. Therefore, future investigations will dissect the molecular mechanisms underlying septin-mediated immunity *ex vivo*, using mouse cells in which tools to study inflammation and in particular, the inflammasome, are better established. It is also important for studies linking septins and inflammation to consider the role of other cytoskeletal proteins (namely, actin), that have been shown to regulate inflammation and are known to interact extensively with septins (Kinoshita et al., 2002, Mostowy and Cossart, 2012, Mostowy and Shenoy, 2015).

The cytoskeleton is increasingly implicated in processes of cellular immunity (Mostowy and Shenoy, 2015). Our results obtained using the *Shigella*-zebrafish model reveal a novel role for septins in inflammation control. Factors found to be important in septin-mediated restriction of inflammation can represent novel drug targets for the control of bacterial infection and inflammatory disorders.

### 6.2. Injections of predatory bacteria work alongside host immune cells to treat *Shigella* infection in zebrafish larvae

In **Chapter 4**, we use the *Shigella*-zebrafish model to test *Bdellovibrio* as a living antibacterial therapy *in vivo*. We show that whilst zebrafish neutrophils and macrophages eliminate *Bdellovibrio* within days following injection, predatory bacteria survive for long enough in larvae to predate upon *S. flexneri* infection. Using confocal microscopy, we
provide the first direct evidence of bacterial predation in vivo, and show that *Bdellovibrio* treatment rescues zebrafish survival from an otherwise lethal infection. Finally, experiments in immune-compromised zebrafish reveal that the combined actions of both *Bdellovibrio* and host innate immunity are important for effective pathogen clearance.

Antibiotic resistant bacteria are an urgent health threat (Boucher et al., 2009), and we show that *Bdellovibrio* is effective in killing multi-drug resistant *Shigella*. Considering these results, researchers can next use zebrafish to test the susceptibility of other clinically relevant Gram-negative pathogens to predation by *Bdellovibrio*. Many important human pathogens are invasive, and it will therefore be interesting to determine, through studies in tissue culture, whether *Bdellovibrio* are able to predate intracellular pathogens inside host cells. Further studies should also explore ways to enhance predation efficiency. For example, modulation of predator surface proteins could be used to increase predator activity against a given pathogen, or even to target Gram-positive bacteria.

Emerging data suggests that blood-borne *Bdellovibrio* may be ineffective for the treatment of systemic bacterial infection (Im et al., 2017, Shatzkes et al., 2017b). In our study, we show predation in the zebrafish hindbrain, a compartmentalised infection site in which *Shigella* adhere to epithelial tissues. Given the slow gliding motility of *Bdellovibrio* (Lambert et al., 2011), and studies showing predation on Gram-negative biofilms (Kadouri and O'Toole, 2005, Iebba et al., 2014), we propose that predatory bacteria may be most beneficial for the treatment of topical wounds and compartmentalised infections. To bring *Bdellovibrio* therapy to a clinical setting, it is crucial to develop a practical therapeutic and this will require the refinement of bacterial preparations to eliminate potentially immunostimulatory moieties, and the exploration of different dosing strategies.
Together, these results establish the *Shigella*-zebrafish infection model as a platform to test novel antimicrobials *in vivo*. Ongoing studies in the zebrafish should continue to provide insight into the future of *Bdellovibrio* therapy, with the aim of using these data to combat multidrug resistant infection in mammalian models and humans.

### 6.3. Septin-mediated emergency granulopoiesis rescues zebrafish larvae from secondary infection

In **Chapter 5**, we use *Shigella* infection of zebrafish to study mechanisms underlying emergency granulopoiesis *in vivo*. We reveal that Sept15 is important for stem cell-driven neutrophil production, both during the definitive steady-state and in response to infection. We show that upstream granulopoietic signalling pathways are not disrupted by septin-depletion, and thus conclude that septins have a cell-autonomous function in stem and progenitor cell effectors. Finally, we use dual infection assays to demonstrate how septin-mediated emergency granulopoiesis can enhance host immunity, and rescue zebrafish from secondary infection.

The zebrafish larva provides a unique system in which to study HSC biology *in vivo* (Martin et al., 2011, Chen and Zon, 2009). Whilst we observe stem cell proliferation and differentiation concurrent with increases in neutrophil number in infected larvae (**Fig. 5.5A** and **5.5B**), in future studies, we aim to capture stem cell-mediated production of neutrophils in real time, using high-resolution real-time imaging of zebrafish larvae comprising fluorescent HSCs and neutrophils, e.g. *runx:mCherry & mpx:GFP* larvae. In this study, we also demonstrate that septins act cell-autonomously in HSCs to mediate neutrophil production. To further support a role for septins in stem cell driven granulopoiesis, we will use cell-type-specific CRISPR/Cas9 genome editing to generate transgenic zebrafish lines in which Sept15 is knocked out specifically in the HSC compartment e.g. by driving Cas9
expression from the *runx1* promoter, and test for emergency granulopoiesis (Ablain et al., 2015). Based on results here, we propose that Sept15 may have a role in symmetric and/or asymmetric stem cell division, and thereby mediate haematopoietic cell proliferation and differentiation. Previous studies in yeast have highlighted a role for septins in asymmetric division using advanced fluorescence microscopy techniques e.g. fluorescence-recovery after photobleaching (FRAP) (Shcheprova et al., 2008). Similar methods can be applied *in vivo* in the zebrafish, to determine a function for septins in forming a diffusion barrier at the stem cell division site. Colony-formation assays conducted *ex vivo* using septin-knockout HSCs can also shed light on a specific role for septins in proliferation and differentiation.

HSCs are the founding cells of the immune system (King and Goodell, 2011), and their manipulation can represent a novel way to control the host response to infection. In next steps, we will explore the mechanisms underlying enhanced immunity from stem cell-driven granulopoiesis. Dual infection assays using distinct pathogens (e.g. priming with *Shigella*, challenging with *Salmonella*) will reveal whether enhanced immunity is the result of memory to a specific pathogen (i.e. trained innate immunity). Furthermore, challenging larvae at later time points following infection priming (e.g. 7 dpi, as opposed to the 2 dpi time point used here) will inform us on the extent of protection that emergency granulopoiesis affords. Given that larvae do not comprise an adaptive immune system, we believe that the zebrafish represents a powerful new model for the study trained innate immunity *in vivo*.

A recent study has shown that acute inflammation primes epithelial stem cells and increases the rate of skin healing to secondary tissue damage (Naik et al., 2017). In this case, ‘inflammatory memory’ results from enhanced chromosomal accessibility and thus accelerated transcription of stress response genes. In future studies, it will be interesting to consider whether this phenomenon is common to other stem cell types, such as HSCs. To
determine whether similar changes to the chromatin underlie enhanced immunity in *Shigella*-primed zebrafish larvae, we can perform an assay for transposase-accessible chromatin using sequencing (ATAC-seq) to determine the chromatin accessibility states of key stress genes. Inflammatory memory may enable the HSPC compartment to react more efficiently to infection, by producing greater quantities of neutrophils or producing neutrophils with enhanced killing capacity. Such lasting epigenetic changes to the HSCs may also increase the risk of cancer and autoimmune disease.

Collectively, results here describe how infection-induced emergency granulopoiesis in the *Shigella*-zebrafish model can be used as a readout of HSPC function. An in-depth understanding of the cellular and molecular mechanisms governing immune cell development will be important for the therapeutic manipulation of innate immunity in humans.

6.4. Potential and limitations of the *Shigella*-zebrafish model system

As demonstrated in this thesis, our *Shigella*-zebrafish model supports: characterisation of host proteins in infection and inflammation (Chapter 3), testing of antibacterial agents (Chapter 4), and the discovery of novel host cell biology (Chapter 5). Zebrafish larvae are highly suited to high-throughput screening as they are low-cost and can be maintained in multi-well plates. There is therefore potential to integrate our infection model into an automated fluorescent imaging platform to screen either (i) a library of compounds for antibacterial action against *Shigella*, or (ii) a pool of mutant zebrafish to determine host genes that modulate immunity to *Shigella* infection. Although powerful for basic research, our model also carries several noteworthy limitations. *Shigella* infection naturally occurs at 37°C in the human gut and this temperature may be required for expression of some bacterial proteins important for disease; in this study, zebrafish are maintained at 28°C.
Development of the model may therefore include testing of infection at increased temperatures still compatible with zebrafish survival. Furthermore, whilst *Shigella*-phagocyte interactions are abundant in the infected zebrafish hindbrain, our localised infection model does not provide an opportunity to study interactions between *Shigella* and cells of the intestinal epithelium, and these are crucial for human shigellosis. As such, future studies may benefit from a zebrafish gut infection model.

6.5. Summary

In summary, the work presented in this thesis demonstrates the versatility of the *Shigella*-zebrafish infection model, and the breadth of innate immune understanding it can contribute. It is anticipated that the translatability of our system, combined with the optical accessibility and genetic tractability of the zebrafish larva will enable further contributions to the fields of innate immunity and host cell biology.


peptidoglycan deacetylation during *Bdellovibrio* predator-prey interaction prevents ultimate destruction of prey wall, liberating bacterial-ghosts. Sci Rep, 6, 26010.


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of shigellosis in the adult rabbit by colonic infection with *Shigella flexneri* 2a. *Infect Immun*, 63, 4350-7.


Figure 1. Sept15 and Sept7b are required to control *S. flexneri* infection in vivo

(A) WT AB zebrafish larvae were treated with control (Ctrl) or Sept15 morpholino (Mo). Representative western blot of extracts from 3 dpf morphant larvae. Antibodies used against SEPT7 or GAPDH (as a control). Related to Fig. 3.6B.

(B) WT AB Ctrl and Sept15 morphants were imaged by stereomicroscopy at 3 dpf. Representative images of larval morphology are shown. Scale bars, 0.5 mm.

(C) WT AB Ctrl and Sept15 morphants were injected in the caudal vein at 3 dpf with a low dose (≤3.0 x 10³ CFUs) of *S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. **, p < 0.01.

(D) Time-course enumeration of live *Shigella* from homogenates of WT AB Ctrl and Sept15 morphants injected with a low dose of *S. flexneri* M90T as in (A). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05. **, p < 0.01.

(E) WT AB zebrafish larvae were treated with control (Ctrl) or Sept7b Mo. Representative western blot of extracts from 3 dpf morphant larvae. Antibodies used against SEPT7 or EF1α (as a control).

(F) WT AB Ctrl and Sept7b morphants were imaged by stereomicroscopy at 3 dpf. Representative images of larval morphology are shown. Scale bars, 0.5 mm.
(G) WT AB Ctrl and Sept7b morphants injected in the HBV at 3 dpf with a low dose of *S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as p < 0.05. ***, p < 0.001.

(H) Time-course enumeration of live *Shigella* from homogenates of WT AB Ctrl and Sept7b morphants injected with a low dose of *S. flexneri* M90T as in (C). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05. *, p < 0.05.

Data obtained by M. Mazon-Moya.
Figure 2. Upregulation of *tnf-a* does not increase susceptibility to *S. flexneri* infection

(A-B) WT AB zebrafish larvae were treated with control (Ctrl) or *lta4h* RNA. At 3 dpf, RNA was extracted from pools of 5 larvae and expression of (A) *tnf-a* and (B) *il-1b* mRNA transcripts determined by qRT-PCR. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments. *p* values (versus Ctrl) determined by unpaired two-tailed Student’s t-test. Significance defined as: *p* < 0.05. *, *p* < 0.05.

(C) Survival curves of Ctrl and *lta4h* injected larvae, injected in the HBV at 3 dpf with a low dose of *S. flexneri* M90T. Larval viability was scored for 48 hpi. Data are pooled from three independent experiments (*n* ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The *p* value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: *p* < 0.05.

(D) Time-course enumeration of live *Shigella* from homogenates of Ctrl or *lta4h* injected larvae infected with a low dose of *S. flexneri* M90T as in (C). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per time point per experiment). *p* values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: *p* < 0.05. Data obtained by M. Mazon-Moya and V. Torraca.
Figure 3. Treatment with anakinra reduces inflammation and rescues Sept15 deficiency in vivo

(A) Lyz:dsRed zebrafish larvae were injected with control (Ctrl) morpholino (Mo). Morphant larvae were either not injected, or injected in the HBV at 3 dpf with a high dose (≥1.0 x 10^4 CFUs) of S. flexneri M90T. Infected larvae were not treated or treated with anakinra. Neutrophil quantifications were performed at 6 hpi. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 5 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance with Bonferroni correction defined as: p < 0.025. **, p < 0.005; ***, p < 0.0005.

(B) Survival curves of Ctrl morphants, injected in the HBV at 3 dpf with a high dose of S. flexneri M90T and either not treated or treated with anakinra. Larval viability was scored for

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**Note:** The text and diagrams are not fully transcribed due to limitations in the text extraction process. The full content is available in the raw text provided.
48 hpi. Data are pooled from four independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. ***, p < 0.001.

(C) Time-course enumeration of live Shigella from homogenates of Ctrl morphants infected with a high dose of S. flexneri M90T and not treated or treated with anakinra as in (B). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from four independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05.

(D) Lyz:dsRed larvae were injected with Sept15 Mo. Morphant larvae were either not injected, or injected in the HBV at 3 dpf with a low dose (≤3.0 x 10^3 CFUs) of S. flexneri M90T. Infected larvae were not treated or treated with anakinra. Neutrophil quantifications were performed at 6 hpi. Each circle represents a count from an individual larva. Mean ± SEM (horizontal bars) is shown. Data are pooled from three independent experiments (using up to 3 larvae per condition per experiment). p values between conditions determined by unpaired one-tailed Student’s t test. Significance with Bonferroni correction defined as: p < 0.025. ***, p < 0.0005.

(E) Survival curves of Sept15 morphants, injected in the HBV at 3 dpf with a low dose of S. flexneri M90T and either not treated or treated with anakinra. Larval viability was scored for 48 hpi. Data are pooled from five independent experiments (n ≥ 15 larvae per condition per experiment). Up to three larvae per condition were taken for CFUs at 24 and 48 h time points. The p value between conditions was determined by log-rank Mantel-Cox test. Significance defined as: p < 0.05. *, p < 0.05.
(F) Time-course enumeration of live *Shigella* from homogenates of Sept15 morphants infected with a low dose of *S. flexneri* M90T and not treated or treated with anakinra as in (E). Only viable larvae were included in analyses. Each circle represents a count from an individual larva. Half-filled circles are enumerations from larvae at 0 hpi and are representative of inocula for both conditions. Mean ± SEM (horizontal bars) is shown. Data are pooled from seven independent experiments (using up to 3 larvae per time point per experiment). p values between conditions at cognate time points determined by unpaired two-tailed Student’s t test. Significance defined as: p < 0.05.

Data obtained by **M. Mazon-Moya**.