Investigation of Neutrophil Swarming to *Mycobacterium marinum* Infection in Zebrafish

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Abstract

The neutrophil behaviour termed swarming is the coordinated recruitment of neutrophils towards a site of challenge where they form clusters around the injury or infection. Neutrophils are deployed against *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), but whether they employ swarming to fight this infection remains unclear. The alarming rise in TB antimicrobial resistance urgently necessitates development of new treatments. In this thesis, I investigate neutrophil swarming in response to mycobacteria in an *in vivo* zebrafish model and assess whether this neutrophil behaviour can be modulated for therapeutic benefit.

Localised infection with *Mycobacterium marinum* (*Mm*), a close relative of human *Mtb*, in a muscle block was used to investigate and characterise neutrophil swarming in the zebrafish model. Neutrophil swarms initiated rapidly in response to live and heat-killed *Mtb*¸ and were stable for at least 3 hours on average. Swarms did not appear to increase reductions of bacterial burden over time.

In other experimental models, such as mice and cell cultures, leukotriene B4 (LTB4) has been established as a crucial mediator in the swarming response. Signalling of LTB4 and other potential swarming mediators was modulated to investigate involvement in neutrophil swarming to *Mm*. Genetic knockdown of leukotriene A4 hydrolase (LTA4H) reduced swarm duration and area, but not neutrophil numbers within the somite, and inhibition of various other targets had no effect. These data show the swarming response in zebrafish can be modulated.

Stabilisation of hypoxia-inducible factor (HIF)-1α can be induced to boost neutrophil killing of *Mm* and thereby reduce bacterial burden, but its involvement in swarming is unclear. The host-protective effect of Hif-1α stabilisation was detectable in localised *Mm* infection and increased swarming prevalence. This research shows Hif-1α may exert its protective effect partially through neutrophil swarming.

In conclusion, this marks the first in-depth investigation into neutrophil swarming to *Mm* infection and lays the foundation for its modulation as a potential therapeutic strategy.

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List of Abbreviations

5-LO	5-lipoxygenase
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ASPA	Animals (Scientific Procedures) Act 1986
ATP	Adenosine triphosphate
BLT	Leukotriene B4 receptor
CCR	CC chemokine receptor
CCRL	CC receptor ligand
CFU	Colony-forming unit
CHT	Caudal hematopoietic tissue
COVID	Coronavirus disease
CRISPR-Cas9	Clustered regularly interspaced palindromic repeats-Cas9
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C motif receptor
DAF-FM-DA	4-amino-5-methylamino-2′-7′-difluorofluorescein diacetate
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMOG	Dimethyloxalylglycine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dpf	Days post fertilisation
ECM	Extracellular matrix
ESAT-6	early secretory antigenic target-6 (ESAT-6) secretion system-1
ESL-1	E-selectin ligand 1
ESX-1	ESAT-6 secretion system-1
FG4592	Roxadustat
FIJI	Fiji Is Just ImageJ
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine triphosphate
Hbi	Hours before infection

HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HK	Heat-killing
HMGB1	High mobility group box 1
Hpf	Hours post fertilisation
Hpi	Hours post infection
Hpw	Hours post wounding
HRE	Hormone responsive element
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KD	Knockdown
LMP	Low melting point
LPS	Lipopolysaccharide
LTA4H	Leukotriene A4 hydrolyse
LTB4	Leukotriene B4
LTB4R	Leukotriene B4 receptor
LTBI	Latent tuberculosis infection
MAC-1	Macrophage-1 antigen
MIF	Macrophage migration inhibitory factor
Mm	*Mycobacterium marinum*
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
Mtb	*Mycobacterium tuberculosis*
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OXPHOS	Oxidative phosphorylation
PAD	Protein arginine deiminase
PAM	Protospacer adjacent motif

PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimycocerosate
PD-L1	Programmed death-ligand 1
PFKL	phosphofructokinase-1 liver type
PHD	Prolyl hydroxylase
PI3K	Phosphoinositide 3-kinase
PR	Phenol red
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PVP	Polyvinylpyrrolidone
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1
SEM	Standard error of the mean
SFK	SRC family kinases
T7SS	Type VII secretion system
TB	Tuberculosis
TH cell	T-helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VHL	Von Hippel-Lindau
WHO	World Health Organisation

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1. General Introduction

Neutrophils are the most abundant phagocytes of the human immune system and make up around 50% to 70% of circulating leukocytes (Mayadas et al., 2014). They have a remarkably short turnover, with their release into circulation and elimination suggested to occur within the span of a single day (Rosales, 2018). However, more recent findings suggest the total lifespan of neutrophils is considerably higher at 5.4 days (Koenderman et al., 2022). Regardless, these short timespans requiretimely and abundant production of more neutrophils is to replenish the dead cells. This is done in the bone marrow, which produces around 10^{11} neutrophils per day, and up to 10^{12} in the presence of a bacterial infection (Mayadas et al., 2014). Neutrophil lifespan is increased considerably during immune challenge to allow them to perform their functions at sites of infection and injury, including clearance of bacteria and dead cells (Mayadas et al., 2014; McCracken & Allen, 2014). They also communicate with macrophages and cells of adaptive immunity, like dendritic cells, T cells, and B cells (Y. Li et al., 2019). After fulfilling their functions, neutrophils may die from apoptosis and are subsequently cleared through phagocytosis by macrophages, a process which is called efferocytosis (Rosales, 2020). Additionally, a recently-described neutrophil behaviour called reverse migration is the movement of neutrophils away from the site of inflammation, and is dependent on a variety of largely undefined signals, which may include important neutrophil signals like leukotriene B4 (LTB4) and the atypical chemokine receptor C-C motif chemokine receptor-like 2 (CCRL2) (Ji et al., 2024).

1.1. Neutrophil production and release

Two-thirds of haematopoiesis in the bone marrow is dedicated to myelopoiesis, the production of monocytes and granulocytes, which include neutrophils (Borregaard, 2010). They originate from haematopoietic stem cells that eventually differentiate into granulocyte-monocyte progenitors through a cascade of stimuli (Rosales, 2018). Release of granulocyte colony-stimulating factor (G-CSF) results in the dedication of these cells to neutrophil generation (Rosales, 2018). Subsequent maturation into neutrophils is dependent on regulation by certain transcription factors, including STAT3, PU1, and C/EBP (Rosales, 2020). Mature neutrophils may be retained in the bone marrow in a population that is approximately 20 times the size of circulating neutrophils, and are ready for immediate release should the need arise (Bekkering & Torensma, 2013). Release of neutrophils into circulation eventually leads to initiation of negative feedback loops that curtail production of G-CSF (Borregaard, 2010; Mayadas et al., 2014). For instance, senescent neutrophils are engulfed by macrophages or dendritic cells, which induces release of anti-inflammatory signals that results in a reduction in interleukin (IL)-

23 production by macrophages (Borregaard, 2010; Mayadas et al., 2014). In turn, this causes a reduction in IL-17 production, which is induced by IL-23 release, subsequently leading to a decrease in production of G-CSF (Mayadas et al., 2014).

The release of neutrophils into circulation is determined by a variety of signals, with c-x-c motif receptor 4 (CXCR4), CXCR2, and G-CSF as some of the key regulators (Furze & Rankin, 2008). CXCR4 is expressed on the cell surface of mature neutrophils at low levels, and its primary ligand is stromal-derived factor 1 (SDF-1, also known as CXCL1), which is a chemokine that is constantly produced by stromal cells of the bone marrow (Summers et al., 2010). Expression of CXCR4 on bone marrow neutrophils is not able to initiate chemotaxis by itself, and is instead thought to act as a retention signal, as administration of a CXCR4 antagonist in mice and humans induces egress of neutrophils out of the bone marrow (Furze & Rankin, 2008). G-CSF may indirectly affect neutrophil release from the bone marrow through CXCR4 by downregulating stromal cell SDF-1 production and neutrophil expression of CXCR4 (Furze & Rankin, 2008; Rosales, 2018). G-CSF can also induce upregulation of CXCR2 ligands on megakaryocytes located within the bone marrow, resulting in neutrophil release (Rosales, 2018). Bone marrow endothelial cells also express ligands of CXCR2, such as CXCL1 and CXCL2, and endothelial cells outside of the bone marrow express CXCR2 ligands when neutrophils need to be released, including CXCL1, CXCL2, CXCL5, and CXCL8, indicating the involvement of CXCR2 signalling in mature neutrophil release (Borregaard, 2010; Rosales, 2018).

1.2. Neutrophil recruitment and mobilisation

Neutrophils are known as the first wave of response after tissue damage or infection (Fine et al., 2020). Recruitment of these cells towards the site of challenge is a complicated process that involves a complex web of signals, including damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), CXCR2, and more (de Oliveira et al., 2016). Additionally, the location, such as the heart, liver, or brain, may affect the stimuli involved in neutrophil recruitment (Margraf et al., 2019).

1.2.1. DAMPs

DAMPs are self-derived structures that are released when tissue is damaged or stressed and are recognised by the immune system (Pittman & Kubes, 2013). The immune system can recognise these structures as being altered or relocated form their original cellular habitat and can therefore distinguish between healthy and unhealthy tissue (Pittman & Kubes, 2013). For instance, extracellular ATP or mitochondrial DNA may escape into the extracellular

environment and induce immune cell activation (Patel, 2018). Stressed cells may also actively release DAMPs, like the release of the highly conserved DNA chaperone called high mobility group box 1 (HMGB1) through cytoplasmic vesicles (Denning et al., 2019; Zhou et al., 2022). Recognition of DAMPs by neutrophils is facilitated by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (de Oliveira et al., 2016). For instance, HMGB1 is recognised by TLR2, TLR4, and TLR9, in addition to the receptor for advanced glycation end products (RAGE), resulting in activation of the pro-inflammatory NF-κB pathway (Roh & Sohn, 2018). This pathway may aid in neutrophil recruitment by inducing differentiation of T-helper (Th) 1 cells into IL-17-producing Th17 cells (T. Liu et al., 2017), or by generation of CXCL2 and CXCL8 by activated monocytes (Q. Guo et al., 2024). Another DAMP, extracellular ATP, binds to the $P2X_7$ receptor on macrophages and endothelium, resulting in IL-1β production and, subsequently, recruitment of neutrophils through CXCL1 and CXCL2 production (Pittman & Kubes, 2013).

1.2.2. PAMPs

PAMPs are derived from non-self, microbial products, are commonly recognised by PRRs, and activate the same transcription machinery as DAMPs (Denning et al., 2019). Lipopolysaccharides (LPS), a common bacterial surface molecule (Bertani & Ruiz, 2018), and unmethylated cytosine phosphate guanosine motifs, prevalent in bacteria (Krieg, 2002), are some of the most well-known PAMPs. PAMPs and DAMPs can interact with each other and certain PAMPs, like CBM and PreSET, can induce DAMP generation (Patel, 2018). For instance, HMGB1 has been found to bind to PAMPs such as LPS to increase the inflammatory response (Denning et al., 2019). The ways in which PAMPs affect neutrophil recruitment are similar to those of DAMPs, as both DAMPs and PAMPs signal through many of the same receptors despite their structural differences (Pittman & Kubes, 2013).

1.2.3. Chemokine signalling

CXCR1 and CXCR2 are two of the most important receptors involved in neutrophil trafficking, and is the target for migration chemokines like CXCL1-3, and CXCL5-8 (de Oliveira et al., 2016). All of these chemokines can bind to CXCR2, but only CXCL6 and CXCL8 (also known as IL-8) bind to CXCR1, despite the high sequence homology between the receptors (Capucetti et al., 2020). Activation of these receptors further direct neutrophil migration through, for instance, phosphoinositide 3-kinase (PI3K) and SRC family kinases (SFKs) (de Oliveira et al., 2016). Signalling through CXCR1 and CXCR2 may activate the NF-κB pathway, which may then induce cytokine transcription to further contribute to neutrophil recruitment (Capucetti et al., 2020). CXCR2 can also heterodimerise with a 7-transmembrane receptor called CCRL2, which is able to mediate neutrophil recruitment by affecting CXCR2 expression

and function (S. Cambier et al., 2023). For example, CCRL2 does not bind CXCL8 unlike CXCR2, and CCRL2-deficient neutrophils in mice showed reduced velocity towards a CXCL8 gradient, indicating heterodimerisation aids CXCR2-mediated signalling of CXCL8 (Del Prete et al., 2017). The importance of CXCR1 and CXCR2 signalling in neutrophil trafficking is evident from studies in a variety of diseases, where targeting of these receptors is associated with a reduction in neutrophil-mediated disease progression (Cheng et al., 2021; Del Prete et al., 2017; Mattos et al., 2020; Nywening et al., 2018; Ritzman et al., 2010; W. Zhuang et al., 2023).

1.2.4. Calcium flux

Calcium signalling in humans is required in the activation neutrophils by linking of chemokines to GPCRs (Dixit & Simon, 2012). This activation results in a rapid increase in calcium levels within the cytosol either from calcium release from intracellular storage, or by calcium influx from the extracellular milieu, which contains significantly higher levels of calcium during resting conditions (Immler et al., 2018). For example, extracellular calcium influx in zebrafish was partially required for calcium fluxes in neutrophils present at a wound site (Poplimont et al., 2020). Furthermore, connexin43 was shown to be required for calcium fluxes in neutrophils at the wound site, and its inhibition reduced neutrophil accumulation at the wound (Poplimont et al., 2020). In other work, treatment of zebrafish with a calcium channel antagonist caused a significant loss of neutrophil recruitment and directionality towards a tail fin wound (Beerman et al., 2015). In this same model, calcium was also found to be enriched at the leading edge of migrating neutrophils (Beerman et al., 2015). Calcium signalling is also important for neutrophil extravasation, described further below, which is the migration of neutrophils from the bloodstream to surrounding tissue (discussed below) (Dixit & Simon, 2012). Adhesion of neutrophils during the extravasation process has been shown to be dependent on calcium signalling, which upregulates β2 integrins responsible for neutrophil arrest (Schaff et al., 2008).

1.2.5. LTB4

Leukotriene B4 is a leukotriene that has been revealed to be of importance in recruitment of distant neutrophils towards a site of challenge (Kienle & Lämmermann, 2016). In response to an ear dermis wound in a mouse model, recruitment of relatively distant neutrophils from the wound site was stifled in neutrophils containing a knockout for one of the receptors of LTB4: LTB4 receptor 1 (LTB4R1) (Lämmermann et al., 2013). At the basis of this is an autocrine and paracrine signalling cascade, in which neighbouring neutrophils respond to LTB4 secretion of neutrophils and initiate directional migration towards the chemoattractant (Afonso et al., 2012).

More will be discussed about LTB4 and its involvement in a specific neutrophil behaviour further below.

1.3. Extravasation

Once neutrophils arrive near sites of inflammation or infection, they leave the blood stream to enter neighbouring inflamed or infected tissue in a process called extravasation (Schnoor et al., 2021). Endothelial cells are activated by stimuli like TNFα, IL-1β, and IL-17 that originate from sites of inflammation or infection, and they start production of P-selectin, E-selectin, and integrin members intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (Borregaard, 2010). P-selectin glycoprotein ligand-1 (PSGL-1) is constitutively expressed around the tips of microvilli neutrophils and facilitates neutrophil rolling along the endothelial wall (Sundd et al., 2013). Subsequently, E-selectin ligand 1 (ESL-1) present on the sides of neutrophil microvilli bind to E-selectin, which slows down rolling of the cell along the endothelial wall (Borregaard, 2010). P-selectin rolling velocity is close to 40 μm/s, and E-selectin rolling velocity is around 3-7 μm/s (Zarbock & Ley, 2009). Combined expression of P- and E-selectin is thought to be synergistic, with P-selectin being mainly responsible for the number of responding neutrophils and E-selectin mediating reduction of rolling velocity (Zarbock & Ley, 2009). In addition to E-selectin, rolling velocity is further reduced by a range of signalling proteins and actin-binding proteins, including Skap2 and Myosin-1e (Schnoor et al., 2021). Accumulation of chemokines, most notably of which being IL-8, results in the activation of the rolling neutrophil, which leads to upregulation of integrin molecules that firmly adhere to the endothelial cells (Mortaz et al., 2018). Other chemokines that initiate neutrophil activation are CXCL1, CXCL2, and CXCL5, which all interact with the CXCR2 receptor (Kolaczkowska & Kubes, 2013). After activation, neutrophils may either enter the surrounding tissue in a process called transmigration, or they may adapt elongated morphology and slowly move along the endothelial cell wall in a process called crawling (Kolaczkowska & Kubes, 2013). This process is largely dependent on macrophage-1 antigen (Mac-1), but may also be influenced by other mediators, like CXCL1 (Schnoor et al., 2021). Afterwards, neutrophils may enter surrounding tissue either by migrating through an endothelial cell, called the transcellular process, or by squeezing through junctions between neighbouring endothelial cells, termed the paracellular process (Borregaard, 2010; Kolaczkowska & Kubes, 2013; Schnoor et al., 2021). The latter is the route that is most frequently used by neutrophils and is relatively faster compared to the former (Kolaczkowska & Kubes, 2013). Following transmigration, neutrophils follow gradients of chemoattractants such as CXCL2 and N-formylmethionine-leucyl-phenylalanine (fMLP) to initiate interaction with pathogenic threats or cell debris (Mortaz et al., 2018; Rosales, 2018; Schnoor et al., 2021).

1.4. Basic neutrophil functions

Once arrived at the site of inflammation or injury, neutrophils employ a number of functions that aid in clearance of dead or dying cells (Ramos & Oehler, 2024) or clearance of pathogenic threats (Rosales, 2018).

1.4.1. Degranulation

Degranulation is the release of pro-inflammatory substances that are contained within granules (Lacy, 2006). Granules can be arranged in four different groups: primary granules, also known as azurophil granules, secondary granules, also known as specific granules, tertiary granules, also known as gelatinase granules, and secretory vesicles (Liew & Kubes, 2019). Key components of primary granules are myeloperoxidase (MPO) and neutrophil elastase (NE) (Chistiakov et al., 2015), and these components are relatively the most toxic mediators found in neutrophil granules (Lacy, 2006). MPO is responsible for killing of pathogenic bacteria by inducing production of cytotoxic agents and it inactivates proteins through irreversible modification of structures (Chistiakov et al., 2015). NE degrades important extracellular matrix proteins such as elastin, and degrades virulence factors of a number of well-known pathogens, including *Shigella* and *Salmonella* (Chistiakov et al., 2015). Secondary granules contain, most notably, lactoferrin, which offers widespread microbicidal activity and is able to suppress virus replication by RNA hydrolysation of viruses (Chistiakov et al., 2015). Both secondary and, particularly, tertiary granules have contents that degrade the extracellular matrix (ECM) (Mollinedo, 2019). Matrix metalloproteinase-9 (MMP-9) present in tertiary granules is indeed known to break down the ECM, but also activates neutrophil stimulant IL-1β (Liew & Kubes, 2019). Tertiary granules have many such important effects of neutrophil behaviour, including cell adhesion through adhesion molecules such as β_2 integrin MAC-1 and neutrophil extravasation through MMP-9 and Rap1 (Mollinedo, 2019). Finally, secretory vesicles govern the delivery of membrane-associated receptors to the cell surface (Rawat et al., 2021). They have also been found to contain pre-formed cytokines, including IL-6, IL-12, and CXCL2 (Sheshachalam et al., 2014). The four groups of granules described above are arranged in order of formation during neutrophil maturation, but they are mobilised in reverse order (Rawat et al., 2021). This stepwise release is tightly regulated, in part by calcium signalling, to prevent excessive toxicity to the host while combating infection (Liew & Kubes, 2019).

1.4.2. Phagocytosis

Phagocytosis is the engulfment of a particle into a phagosome, a vacuole derived from the plasma membrane, after which it further acquires particle-degrading properties through a process called maturation (W. L. Lee et al., 2003). A complicated pathway of signalling cascades and cytoskeletal rearrangements are required for engulfment of particles (Liew & Kubes, 2019). Phagocytosis of neutrophils differs from that of macrophages, as internalisation of particles in neutrophils is generally faster than in macrophages, and neutrophils rapidly fuse granules with the phagosome instead of the fusion process during macrophage phagosome maturation, in which the phagosome transforms into a phagolysosome (Nordenfelt & Tapper, 2011). In addition to granules, reactive oxygen species (ROS) are inserted into the phagocytic vacuole and aid in killing phagocytosed microbes (Liew & Kubes, 2019). These ROS are a direct result of the production of a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of the NADPH oxidase (NOX) family in a process called respiratory burst, which deficiency is associated with compromised bacterial killing in anaerobic situations (W. L. Lee et al., 2003; Liew & Kubes, 2019).

Generally, phagocytosis by neutrophils is described in an antimicrobial context, but they are also involved in efferocytosis of apoptotic or dead cells (Ramos & Oehler, 2024). Apoptotic cells release signals including CCL3, CXCL1, CXCL5, and CXCL8, which attract neutrophils, and express signals on their cell surface associated with eliciting efferocytosis, like annexin A1 and thrombospondin 1, which are recognised by neutrophil surface receptors (Ramos & Oehler, 2024). Neutrophils have been shown to phagocytose apoptotic cells and, in cancer, dead tumour cells (Ramos & Oehler, 2024). They have also been shown to efferocytose their own, described as neutrophil cannibalism (Rydell-Törmänen et al., 2006). Efferocytosis of apoptotic cells by neutrophils pushes them towards an anti-inflammatory phenotype (Ramos & Oehler, 2024). For instance, apoptosis of neutrophils by other neutrophils reduced the release of pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α) and interferon-inducible protein-10 (Esmann et al., 2010). However, apoptosis also increased secretion of neutrophil-recruiting IL-8 and oncogene-α (Esmann et al., 2010). The authors suggested this chemokine secretion may further recruit neutrophils and contribute to inflammation downregulation through their apoptosis, or that it recruits keratinocytes for rapid wound healing (Esmann et al., 2010).

1.4.3. NETosis

Neutrophil extracellular traps (NETs) are webs of extracellular DNA that contain microbicidal proteins originating from granules and are released in a process of neutrophil pro-inflammatory cell death called NETosis (de Bont et al., 2019; Jaboury et al., 2023). Bacteria, viruses, fungi,

and parasites can be captured in these web-like structures and are killed by the antimicrobial activity of DNA, histones, and granular proteins found in NETs (Stephenson et al., 2016). A variety of pro-inflammatory signals can trigger the release of NETs, including LPS, IL-8, TNF, fMLP, and calcium (Remijsen et al., 2011). ROS has also been described to initiate NETosis by initiating NE translocation from primary granules into the cytosol, and then into the nucleus (Poli & Zanoni, 2023). In the nucleus, NE degrades chromatin through cleavage of histones (Yipp & Kubes, 2013). Similarly, MPO breaks down chromatin and is also stimulated by ROS (Poli & Zanoni, 2023; Yipp & Kubes, 2013). NETs are able to directly kill pathogens by ensnaring them and neutralising them through their antimicrobial properties, but there is also evidence for indirect killing by NETs (Yipp & Kubes, 2013). For example, trapping of *Staphylococcus aureus* by NETs did not affect bacterial killing in isolation, but instead aided macrophage-mediated killing of the captured pathogen by inducing phagocytosis and thereby transferring neutrophil antimicrobial peptides to the macrophage phagosome (Monteith et al., 2021).

1.4.4. Oxidative burst

Oxidative burst, also referred to as respiratory burst, is the production of ROS by NADPH oxidase (Nguyen et al., 2017). It can be directly triggered through activation of neutrophils by a variety of signals, including binding of fMLP to G protein-coupled receptors (GPCRs), binding of degraded bacterial products to Fc receptors after phagocytosis, and binding of bacterial ligands to integrin receptors (Nguyen et al., 2017). ROS production can also be primed by cytokines, meaning these cytokines do not elicit a ROS response by themselves, and instead boost the response initiated by a secondary response (El-Benna et al., 2016). Some of these priming agents can also directly induce ROS production if their concentration reaches a certain threshold, including fMLP and LTB4 (El-Benna et al., 2016). Other priming agents include TNFα, GM-CSF, IL-1β, IL-8, LPS, and zymosan (El-Benna et al., 2016). Priming of human neutrophils with TNFα or GM-CSF initiated a significantly higher ROS production response after secondary stimulation to fMLP compared to fMLP alone (El-Benna et al., 2016). Production of ROS is key in fighting bacterial and fungal infections due to their antimicrobial properties (Nguyen et al., 2017). They may both directly and indirectly aid in killing of pathogens, for instance by causing oxidative damage to biocompounds or by inducing pathogen-eliminating pathways such as NET formation (Paiva & Bozza, 2014). High concentrations of ROS are cell-deleterious, but at lower concentrations they may modulate cell growth, adhesion, differentiation, adhesion, senescence, apoptosis (Mittal et al., 2014), and migration (Hurd et al., 2012). Despite these important functions of ROS, their uncontrolled release can cause tissue damage which plays a role in many diseases, including rheumatoid arthritis (Mittal et al., 2014) and tuberculosis (Paiva & Bozza, 2014).

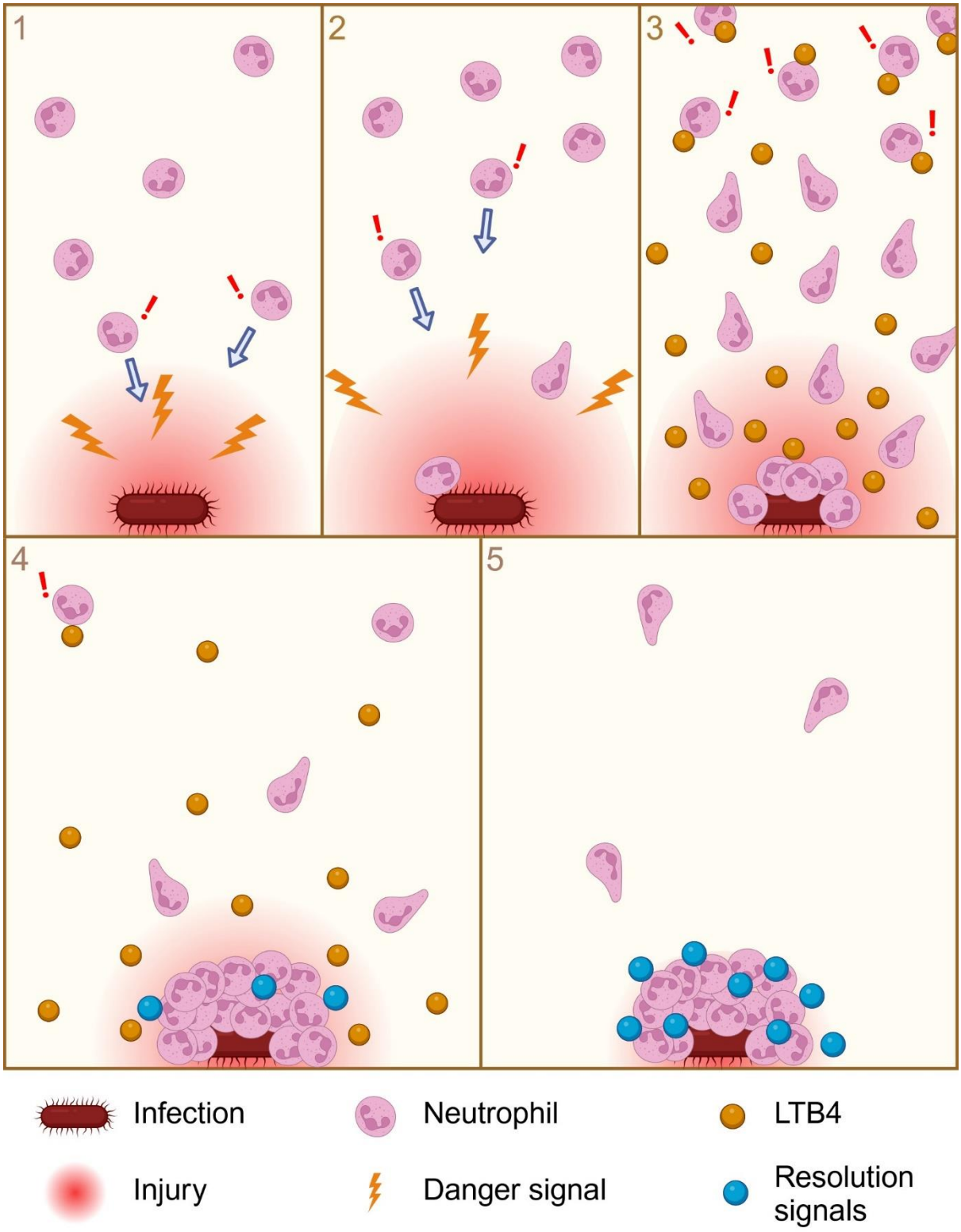


Figure 1.1 | The 5 steps of the neutrophil swarming response. 1) Danger signals originate from the site of tissue injury or infection. Nearby, randomly migrating neutrophils respond to these signals and migrate towards the source. 2) Neutrophils from more distal tissues are recruited to the site of challenge in an integrin-dependent manner. Recruitment is further amplified by release of unidentified chemoattractants originating from the developing swarm

centre. 3) Intracellular signal relay, largely mediated by an LTB4 gradient, recruits neutrophils from more distal tissues. 4) Neutrophils cluster in a process that is partially mediated by LTB4, remodel the ECM, and clear cell debris and pathogens. The LTB4 gradient decreases steadily. 5) Swarm resolution occurs due to largely unknown mediators, and neutrophils migrate away (Lämmermann, 2016). Other mediators responsible for resolution may be lipoxin A4 and resolvin E3, two mediators of inflammation resolution involved in swarming (Reátegui et al., 2017). Created using Biorender.com, adapted from (Lämmermann, 2016).

1.5. Neutrophil swarming

A neutrophil behaviour that has seen increasingly more attention in research over the last decade is neutrophil swarming (Brown & Yipp, 2023). Neutrophils can swarm around sites of tissue injury or infection in a behaviour resembling the swarming of insects, hence the term “neutrophil swarming” (Lämmermann, 2016). The swarming process involves coordinated migration of neutrophils towards a site of challenge that is mediated by an array of chemoattractants, such as chemokines and lipids, and intercellular signalling cascades, which results in the formation of stable neutrophil clusters over time (Lämmermann, 2016). Evolutionarily, neutrophil swarming is theorised to protect surrounding tissues by isolating the injury or infection, and is thought to increase effectiveness of antimicrobial activity by concentrating a collective neutrophil effort on neutralisation of the isolated microbes (Kienle & Lämmermann, 2016). There are subtle differences in phases involved in neutrophil swarming described in literature, but they generally describe recognition of individual neutrophils close to a site of challenge, further recruitment of neutrophils and initiation of swarming, followed by plateauing or stabilisation of swarming and, finally, resolution of swarming (Figure 1.1) (Isles et al., 2021, p. 20; Lämmermann, 2016; Lämmermann et al., 2013; Reátegui et al., 2017; Walters et al., 2019). The swarming process will be discussed in detail in accordance with work performed by Lämmermann and colleagues, who have pioneered and popularised investigation into the neutrophil swarming process (Lämmermann, 2016; Lämmermann et al., 2013).

1.5.1. Swarm initiation

Tissue damage through either injury or infection causes release of chemotactic signals that are recognised by neutrophils patrolling relatively near the site of challenge. These pioneer neutrophils respond to these signals and adjust their random migration to direct migration towards the site of challenge. Functional GPCR signalling is required for a proper neutrophil response to these initial signals, but the exact chemoattractants involved are unclear (Lämmermann, 2016). For example, knockout of common mediators of neutrophil migration,

such as *Ltb4r1*, *Cxcr2*, and *Ccr1*, did not affect the initial neutrophil recruitment in a mouse ear dermis wounding model (Lämmermann et al., 2013). DAMPs and PAMPs are likely to be involved in the initial recruitment and are capable of inducing neutrophil swarming (Song et al., 2023). Recognition of these signals may cause upregulation of adhesion molecules on endothelium which promotes neutrophil recruitment (Pittman & Kubes, 2013). These studies show that the initial recruitment of randomly migrating neutrophils is a complex process that cannot be attributed to a single signalling pathway and instead is mediated by recognition of a multitude of signalling molecules.

1.5.2. Swarm amplification through cell death

Once the initial neutrophils have reached the site of challenge, cell death of a few of these cells is enough to initiate neutrophil recruitment from relatively distant sites. Neutrophils migrate through the most direct way towards the site of challenge in an integrin-dependent migration process. Like with swarm initiation, the exact signalling mediators required for the distant recruitment of additional neutrophils are unclear (Lämmermann, 2016). Knockdown of common chemokine receptors involved in neutrophil migration, such as *Cxcr2* and *Ccr1* did not affect recruitment of these neutrophils (Lämmermann et al., 2013). DAMPs and PAMPs may again play a role in this step of neutrophil swarming. Both DAMPs and PAMPs act through largely the same transcription machinery (Denning et al., 2019), which includes TLR4 (K.-M. Lee & Seong, 2009). Platelets activated by TLR4 may bind to neutrophils which may lead to NET formation (Mussbacher et al., 2019). In a zebrafish tailfin injury model, pioneer neutrophils were shown to release NET-like structures, and general inhibition of NETosis and inhibition of NET-component NE inhibited swarm formation (Isles et al., 2021).

1.5.3. Swarm amplification through intercellular signalling

A key player in the recruitment of neutrophils towards the swarm after the initiation phase is LTB4, a signal-relay molecule and signal amplifier for neutrophils (Lämmermann, 2016). More specifically, LTB4 is crucial for recruitment of more distant neutrophils (200-300 µm) towards the site of challenge (Kienle & Lämmermann, 2016; Lämmermann, 2016). There are two receptors for LTB4 that are classified as GPCRs: LTB4R1 (or BLT1) and LTB4R2 (BLT2), of which LTB4R1 is the main receptor to interact with LTB4 (Saeki & Yokomizo, 2017). Knockout of *Ltb4r1* and disruption of GPCR signalling significantly impaired recruitment of more distant neutrophils towards the site of injury in a mouse ear dermis wounding model (Lämmermann et al., 2013). Neutrophils are the main producers of LTB4, as indicated by the lack of distant neutrophil recruitment after injection of *Alox5* knockout neutrophils into *Alox5* knockout mice, which are deficient in LTB4 production (Lämmermann et al., 2013). Injection of wildtype neutrophils into the same mouse line did result in functional distant neutrophil recruitment

(Lämmermann et al., 2013). This also indicates that neutrophils secrete LTB4, causing a signalling cascade that improves chemotaxis of a population of distant neutrophils (Lämmermann et al., 2013). This may in part be a result of calcium signalling, as activation of LTB4 biosynthesis was favoured in clustering, calcium-fluxing cells in a zebrafish wounding model (Poplimont et al., 2020). A study with mouse neutrophils showed similar results, as mouse pioneer neutrophils responding to zymosan particles displayed increased calcium levels (Khazen et al., 2022). These calcium signals triggered production of LTB4 and caused neutrophil clustering, which was abrogated after blocking of extracellular calcium entry or LTB4 signalling (Khazen et al., 2022).

LTB4R may work in tandem with CXCR1 and CXCR2 in the recruitment of distant neutrophils as shown in microarray experiments with zymosan-induced neutrophil swarming (Reátegui et al., 2017). Antibody blocking of CXCR1 and CXCR2 alone did not affect neutrophil recruitment to zymosan particles, but when CXCR1 and CXCR2 were blocked in addition to antagonistic blocking of LTB4R1 and LTB4R2 there was an even bigger reduction in neutrophil recruitment compared to that seen with LTB4R1 and LTB4R2 blocking alone (Reátegui et al., 2017). Such a dependency of LTB4R4 signalling on CXCR2 has been displayed in an earlier study in a mouse model for rheumatoid arthritis (Grespan et al., 2008). Here, pharmacological inhibition of LTB4 production significantly reduced CXCL1- and CXCL-5-induced neutrophil recruitment (Grespan et al., 2008). Additionally, stimulation of neutrophils with these chemokines significantly increased LTB4 release, and pharmacological inhibition with a CXCR1/CXCR2 inhibitor significantly decreased LTB4 levels (Grespan et al., 2008).

1.5.4. Swarm aggregation and tissue remodelling

The continued recruitment of neutrophils results in the formation of cell clusters at the site of challenge. Here, neutrophils slow down migration in favour of aggregation and initiate remodelling of the ECM (Lämmermann, 2016). Work in a mouse ear dermis wounding model suggests that a certain threshold in neutrophil numbers needs to be achieved for these cells to aggregate and further recruit other neutrophils, and that the cluster rapidly dissociates if this threshold is not met (Park, Choe, Park, et al., 2018). Collagen, which plays key roles in wound healing (Mathew-Steiner et al., 2021), is removed from the swarm centre, resulting in a collagen-free zone (Lämmermann et al., 2013). Deletion of talin and β2 integrins by targeting *Tln1* and *Itgb2* in neutrophils rendered them completely unable to enter into the collagen-free swarm centre, stressing the importance of integrins in neutrophil motility within this zone (Lämmermann et al., 2013).

GPCR signalling again plays a role in aggregation, as neutrophils with dysfunctional GPCR signalling are eventually excluded from neutrophil clusters (Lämmermann et al., 2013). Similarly, knockout of *Ltb4r1* in neutrophils participating in early swarming eventually results in the exclusion of these cells from a neutrophil cluster in favour of wildtype neutrophils (Lämmermann et al., 2013). Genetic deletion of *Cxcr2* and formyl peptide receptor 2 (*Fpr2*), which is important in host defence during inflammation and infection as well as neutrophil recruitment (C. Lee et al., 2023), resulted in impaired clustering as well (Lämmermann et al., 2013). Further evidence for CXCR2-involvement in neutrophil aggregation comes from a study that investigated neutrophil recruitment and aggregation in response to fat-associated lymphoid clusters, which were both significantly diminished in response to blocking of CXCR2 chemokine CXCL1 (Jackson-Jones et al., 2020). This study also implicated NET formation in neutrophil aggregation, as inhibition of protein arginine deiminase 4 (PAD4), important for NET release, abrogated neutrophil aggregation (Jackson-Jones et al., 2020). Finally, genetic knockout of GPCR-kinase 2 (*Grk2*), which is responsible for GPCR desensitisation, in mouse neutrophils significantly increased neutrophil aggregation and knockout cells showed dominance over control cells in neutrophil clusters with direct competition (Kienle et al., 2021).

1.5.5. Swarm resolution

The stop signals that result in the resolution of swarming have not been studied as extensively as neutrophil recruitment or aggregation, and are therefore less well-understood (Lämmermann, 2016). Due to the heavy involvement of GPCR signalling (Lämmermann et al., 2013), it stands to reason that GPCR desensitisation may play an important role in swarm resolution. Desensitisation of GPCRs is a negative feedback mechanisms that prevents excessive neutrophil stimulation and thereby can prevent neutrophils from entering a host-destructive instead of host-protective state (Y. Wang et al., 2023). The GPCR-kinase GRK2 appears to be a key player in GPCR desensitisation during the swarming response (Kienle et al., 2021). Knockout of GRK2, and not GRK3, GRK5, and GRK6, in mouse neutrophils resulted in significantly higher neutrophil displacement towards a CXCL2/LTB4 gradient, indicating GRK2 is the main GRK responsible for desensitisation (Kienle et al., 2021). It is important to note that knockout of GRK2 did not affect the initial recruitment of neutrophils towards the wound *in vivo* (Kienle et al., 2021), and GRK2 was therefore not discussed in the recruitment sections above. *Grk2* knockout significantly increased neutrophil aggregation behaviour, but also left these cells responsive to signals from outside the swarm, which resulted in them rapidly leaving the neutrophil swarms (Kienle et al., 2021). Interestingly, persistent migratory behaviour of *Grk2* knockout cells impaired their ability to ingest microbes and allowed bacteria to breach swarm barriers and subsequently escape (Kienle et al., 2021). No effects of GRK2 knockout on full swarm resolution were discussed (Kienle et al., 2021),

but it is clear that partial attenuation of certain swarming responses are imperative for a functional swarming response.

LTB4 signalling may be an important target for stop signals due to its heavy involvement in multiple processes during the neutrophil swarming response (Lämmermann, 2016). Cathelicidin (LL-37) is a host defence peptide released by neutrophils through degranulation, NETosis, and necrosis (Minns et al., 2021). LL-37 is able to induce the release of LTB4 by neutrophils, and LTB4 induces LL-37 production in these cells (Wan et al., 2011). Treatment of human neutrophils with lipoxin A4 (LXA4) did not affect LTB4 production, but when both LL-37 and LXA4 were used for treatment in tandem, there was a significant decrease in LTB4 released from neutrophils (Wan et al., 2011). LXA4 has also been found to be produced in large amounts by human neutrophils in response to zymosan particles (Reátegui et al., 2017). LXA4 production in swarming neutrophils remained low until at least 1 hour after swarm initiation, and was significantly increased at 3 hours after swarm initiation (Reátegui et al., 2017). Pre-treatment of neutrophils and subsequent continued treatment of swarming neutrophils with LXA4 significantly decreased swam size and growth rate compared to controls (Reátegui et al., 2017). This study also proposes the inflammation resolution mediator resolvin E3 (RvE3) as a swarming resolution mediator, which production remained constant at 30 minutes, 1 hour, and 3 hours after swarm initiation, but its effect on swarm formation was not reported (Reátegui et al., 2017). RvE3 has been shown to significantly inhibit LTB4-induced activity of LTB4R1 in mouse dendritic cells, an effect that was consistently stronger than inhibition with RvE1 and RvE2 (Sato et al., 2019). Reports of the interaction between RvE3 and LTB4 signalling in neutrophils were not found. However, another resolvin, RvE1, was found to abolish LTB4-induced calcium responses in human leukocytes, and significantly inhibited LTB4-induced neutrophil recruitment towards zymosan in mice (Arita et al., 2007). Finally, oxidation of LTB4 by omega-hydroxylase results in formation of ω-OH-LTB4, which binds to LTB4R1 in competition with LTB4 (Song et al., 2023). The neutrophil response elicited by ω-OH-LTB4 binding is markedly weaker than that of LTB4 binding, meaning ω-OH-LTB4 can be considered a natural inhibitor of LTB4 signalling (Golenkina et al., 2022). This natural inhibition effect may have been displayed in a *Salmonella enterica* Typhimurium infection model, in which neutrophil stimulation with fMLP after infection increased the ratio of LTB4 in expense of ω-OH-LTB4 when the ratio of bacterial burden to human neutrophils increased (Golenkina et al., 2022). In short, there are a number of mechanisms that could be employed to attenuate neutrophil swarming, be it through inhibition of LTB4 or through another pathway.

1.6. Resolution of neutrophilic inflammation

Some of the mediators involved in the resolution of neutrophil swarming are also part of inflammation resolution in general, including resolvins (Serhan et al., 2015). Resolution is an active process in which anti-inflammatory and pro-resolution lipid mediators are rapidly biosynthesised (Serhan et al., 2008). These mediators are resolvins, lipoxins, protectins, and maresins, which induce clearance of cell debris and apoptotic neutrophils and attenuate pro-inflammatory cytokine signalling (Panigrahy et al., 2021). Macrophages are mainly responsible for the clearance of these apoptotic neutrophils and do so by efferocytosis, which triggers macrophage to switch to an anti-inflammatory phenotype (Ortega-Gómez et al., 2013). The return to homeostasis can be described in 4 steps: 1) the attenuation of further neutrophil recruitment by shutting down chemokine signalling, 2) a switch from neutrophil survival signalling to neutrophil apoptosis, 3) clearance of apoptotic neutrophils, and 4) the switch from a pro-inflammatory- to a pro-resolving macrophage phenotype (Ortega-Gómez et al., 2013; Sugimoto et al., 2016). Disruption of chemokine signalling can occur through a variety of mechanisms, like the truncation of chemokines, called proteolysis (Ortega-Gómez et al., 2013). For example, macrophage-derived matrix metalloproteinase-12 (MMP-12) cleaves a specific motif called the ELR motive of CXC chemokines, which renders their receptor binding dysfunctional (Ortega-Gómez et al., 2013). Other methods of chemokine signalling disruption include chemokine sequestration by atypical receptors and removal of NETs, which components may act as pro-inflammatory mediators (Sugimoto et al., 2016). Next, signals inside the inflammatory environment switch from signals that enhance neutrophil survival to signals that induce neutrophil apoptosis (Ortega-Gómez et al., 2013). Neutrophils can contribute to this by secretion of inflammation-resolving signals, such as lipoxins and resolvins (Serhan et al., 2008). These signals include TNF and annexin A1 (AnxA1), the former of which promotes survival at lower concentrations, but induces apoptosis at higher concentrations (Ortega-Gómez et al., 2013). AnxA1 is secreted by dying neutrophils and promotes neutrophil apoptosis, inhibits neutrophil recruitment, and induces macrophages to clear dead neutrophils (Ortega-Gómez et al., 2013). Efferocytosis is further facilitated by the production of find-me and eat-me signals by apoptotic neutrophils (Loh & Vermeren, 2022). These find-me signals direct macrophages towards apoptotic neutrophils, and eat-me signals interact with receptors on macrophages to initiate efferocytosis (Loh & Vermeren, 2022). Additionally, find-me signals have also been shown to enhance macrophage phagocytic activity and to modulate cytokine production (Loh & Vermeren, 2022). Finally, efferocytosis of apoptotic neutrophils by macrophages causes a switch from a pro-inflammatory macrophage phenotype to an inflammation-resolving phenotype, which is reflected in the change in macrophage inflammation mediator production (Ortega-Gómez et al., 2013). For example, efferocytosis

results in the downregulation of pro-inflammatory TNF, IL-6, IL-12, and IL-1β (Jones et al., 2016), and increases production of IL-10 and TFG-β (Jones et al., 2016; Ortega-Gómez et al., 2013). Macrophages also secrete LXA4, which attenuates neutrophil recruitment and ROS formation, and promotes efferocytosis of apoptotic neutrophils (Ortega-Gómez et al., 2013). Proper functioning of these inflammation-resolving pathways is of utmost importance since uncontrolled inflammation can contribute to disease pathology in many diseases (Herrero-Cervera et al., 2022; Serhan et al., 2008).

1.7. Neutrophils in disease progression

1.7.1. Inflammatory disease

Failure to inhibit the signals that govern the pro-inflammatory environment, or the dysfunction of inflammation resolution, may result in chronic inflammation that leads to deadly diseases like diabetes mellitus and atherosclerosis (Herrero-Cervera et al., 2022). Rheumatoid arthritis (RA) is an autoimmune disease in which chronic inflammation causes erosion of bones and damage of cartilage (Riaz & Sohn, 2023). Neutrophil numbers are highly elevated in the synovial joint spaces of RA patients where they inflict damage through degranulation and release of ROS and NETs (H. L. Wright et al., 2014). Not only do they directly damage the tissue environment through these processes, they also produce a wide range of cytokines and chemokines that recruits additional immune cells towards the joints (H. L. Wright et al., 2014). Contributors to this disease feature many regulators of processes that have been discussed in this thesis so far, including LTB4, CXCL1, CXCL2, CXCL8, LL-37, MPO, and NE (Riaz & Sohn, 2023). Another very common inflammatory disease is diabetes mellitus, which can be categorised in type 1- and type 2 diabetes, the latter of which is the most prominent (Herrero-Cervera et al., 2022). Glucose concentrations in patients with these diseases is elevated, causing a shift in neutrophil metabolism to avoid toxicity from intolerable, intracellular glucose levels, and resulting in the upregulation of pro-inflammatory genes like NF-κB (Dowey et al., 2021). The subsequent upregulation of cytokine production further results in excessive recruitment of neutrophils (Dowey et al., 2021). Neutrophils contribute to disease progression in both types of the disease (Herrero-Cervera et al., 2022). In type 1 diabetes, neutrophils infiltrate into the pancreas where they contribute to destruction of insulin-producing β-cells by undergoing NETosis (Herrero-Cervera et al., 2022). In type 2 diabetes, neutrophils contribute to a state of chronic inflammation and contribute to insulin resistance in a NE-dependent manner (Keeter et al., 2021). Again, previously discussed neutrophil behavioural mediators like CXCL1, CXCL2, PAD4, MPO, and NE play a role in diabetes mellitus disease progression (Herrero-Cervera et al., 2022).

1.7.2. Infectious diseases

Neutrophils play a key role in the defence against pathogens, and while defence mechanisms like oxidative burst and NET formation are of great importance in sequestering bacterial or viral threats, these mechanisms may become detrimental to the host if unregulated (Ma et al., 2021). The recently discovered coronavirus disease 2019 (COVID-19) is one of these diseases where neutrophils have been designated as a major contributor to disease pathogenesis, even though their roles in fighting the virus are not clear (Rong et al., 2024). It has repeatedly been shown that neutrophilia indicates severe respiratory symptoms and poor disease prognosis (Cavalcante-Silva et al., 2021). Infection with this disease triggers high neutrophil activation, leading to tissue damage from uncontrolled ROS release and subsequent cytokine storms (Cavalcante-Silva et al., 2021), from degranulation (Rong et al., 2024), and from the presence of an excessive amount of NETs (Ackermann et al., 2021). For example, sera of hospitalised COVID-19 patients contained elevated levels of NET-markers MPO-DNA and citrullinated histone H3 (Cit-H3), and treatment of control neutrophils with these sera triggered NET release (Zuo et al., 2020). While the exact mechanisms in which COVID-19 induces tissue damage have not yet been elucidated, there is clear evidence that neutrophils play a major part in disease progression that is perhaps most clearly indicated by the poor prognosis of high neutrophil counts paired with a low number of circulating lymphocytes (deKay et al., 2021; Rong et al., 2024; Shafqat et al., 2023).

The dual role of neutrophils in fighting disease as well as progressing disease is well-exemplified by the human immunodeficiency virus (HIV) (Hensley-McBain & Klatt, 2018). Neutrophils fight HIV infection in a variety of ways, most notably using defensins in granules and release of ROS. For instance, neutrophils have been shown to be viricidal towards HIV in a H_2O_2-dependent manner, H_2O_2 being a product of respiratory burst by neutrophils (Klebanoff & Coombs, 1992). Neutrophils of both healthy individuals and individuals with acquired immune deficiency syndrome (AIDS) were cytotoxic to HIV after stimulation with G-CSF and GM-CSF in cell cultures (Baldwin et al., 1989). Furthermore, neutropenia and decreased neutrophil function are associated with advanced disease progression and risk to secondary infections (Hensley-McBain & Klatt, 2018). However, neutrophils can also play a role in the pathogenesis of the disease by, for example, modulating T cell function (Bowers et al., 2014). T cells are important in the defence against many pathogens, but loss of their function is a key event in HIV disease progression (Bowers et al., 2014). Neutrophils are partially responsible, as they have an immunosuppressive effect on T cells by binding program death ligand 1 (PD-L1) on T cells, which negatively impacts T cell function (Bowers et al., 2014). In infection with HIV/SIV, a subtype of HIV infection, neutrophils contribute to tissue damage and destruction of immune cells by release of NETs (Moreno de Lara et al., 2023).

A final example of neutrophil duality is their involvement in the bacterial infection tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) (Kroon et al., 2018). The role of these cells in TB infection is controversial, as their recruitment can be both beneficial and detrimental to the host, depending on whether they are capable of handling the infection or not (Kroon et al., 2018). Neutrophils are capable of killing *Mtb* in a number of ways, including phagocytosis, release of ROS, and release of nitric oxide (NO) synthase (NOS) (L. Zhuang et al., 2024). However, effectiveness of these killing mechanisms are controversial as they are not always effective against *Mtb*, and they may contribute to significant damage caused by neutrophils during late stage TB (Kroon et al., 2018). This makes TB a particularly interesting model to study neutrophil behaviour, and the knowledge obtained could potentially be of use in research of neutrophil behaviour in the context of different pathogens.

1.7.3. Neutrophil swarming in disease

It is difficult to observe neutrophil swarming in human diseases, but a variety of diseases show pathological evidence that is compatible with neutrophil swarming (Brown & Yipp, 2023). One of the main swarming mediators, LTB4, has been shown to contribute to disease progression in rheumatoid arthritis, atherosclerosis, and both types of diabetes mellitus (Brandt & Serezani, 2017). For example, neutrophils of diabetic mice, which have poor antimicrobial functions, significantly increased non-healing skin lesions and bacterial burden following *Staphylococcus aureus* skin infections due to increased neutrophil migration dependent on LTB4 signalling (Brandt et al., 2018). Inhibition of LTB4 signalling reversed these effects (Brandt et al., 2018). *In vivo* studies have also found neutrophil swarming is involved in a number of other infectious diseases, like *Candida albicans* (Hopke et al., 2020; E. K. S. Lee et al., 2018) and *Cryptococcus neoformans* (D. Sun & Shi, 2016). Some studies report beneficial effects of neutrophil swarming, like a mouse model in which *Pseudomonas aeruginosa* bacterial clearance was shown to be aided by neutrophil swarming and neutrophil arrest within swarms (Kienle et al., 2021). This was also found in zebrafish model, where clearance of *P. aeruginosa* was rapidly initiated upon formation of neutrophil clusters around the bacteria (Poplimont et al., 2020). Neutrophil swarming has not been extensively reported in TB, but reports of important neutrophil-mediated killing of *Mtb* (Kisich et al., 2002; C.-T. Yang et al., 2012) indicate the potential of investigating neutrophil swarming in response to these bacteria.

1.8. Tuberculosis

TB is an infectious disease caused by airborne transmission of *Mtb* and is the leading cause of death in HIV-infected individuals (WHO, 2023). According to the Global Tuberculosis Report

of 2023 released by the World Health Organisation (WHO), 10.6 million people were estimated to be infected globally and 1.3 million succumbed to TB infection in 2022 (WHO, 2023). Infection with *Mtb* may not immediately result in active disease, and individuals may instead carry *Mtb* asymptomatically for years (Pai et al., 2016). Such asymptomatic *Mtb* infections are classified as latent TB infection (LTBI) and are stipulated to affect around 2 billion people globally (Alsayed & Gunosewoyo, 2023). Reactivation of LTBI occurs in about 5% to 15% of infected individuals, but the underlying mechanisms that trigger active disease are unclear (Kiazyk & Ball, 2017). There are, however, certain conditions and risk factors that put the patient at risk of developing active TB (Kiazyk & Ball, 2017). The most dangerous comorbidity is HIV infection, which increases the risk of developing active disease by over 100-fold (Kiazyk & Ball, 2017). Symptoms of active TB include a cough, fever, weight loss (Pai et al., 2016), fatigue, night sweats, and loss of appetite (Alsayed & Gunosewoyo, 2023). Dissemination of the disease from the lungs to other parts of the body can also lead to development of symptoms such as chronic back pain (Smith, 2003).

The TB infection cycle (Figure 1.2) starts with the transmission of *Mtb* within aerosol particles after, for example, coughing by an infected host (C. J. Cambier et al., 2014). These particles reach the lung of an uninfected individual where the particles are initially picked up by alveolar macrophages (Chandra et al., 2022a). Infected macrophages recruit more immune cells towards the infection, including tissue-resident macrophages, neutrophils, dendritic cells (DCs) (Chandra et al., 2022a), monocytes (Ernst, 2012), and natural killer cells (Ehlers & Schaible, 2013). This eventually results in the formation of a granuloma, one of the hallmarks of TB, which is a collection of macrophages and other immune cells that is meant to restrict spreading of the bacteria (Alsayed & Gunosewoyo, 2023). However, the bacteria can instead abuse the granuloma during its early stages and replicate within newly recruited macrophages (C. J. Cambier et al., 2014). In fact, the early innate immunity response to *Mtb* is ineffective in curtailing infection (Ehlers & Schaible, 2013), and an adequate response by T cells and B cells in cooperation with activated macrophages is required for bacterial control instead (Chandra et al., 2022a). For example, antigen priming of T cells by DCs in the lymph nodes results in T cell differentiation into T-helper 1 (TH1) cells, TH17 cells, and cytotoxic T effector cells, which are important for macrophage activation (Ehlers & Schaible, 2013). Recruitment of all these immune cells eventually results in a granuloma with a core of infected macrophages surrounded by lymphocytes, in which the bacterium is contained (Ehlers & Schaible, 2013). These macrophages may become necrotic, resulting in a caseating granuloma (Assari et al., 2014), which allows the bacteria to further replicate (C. J. Cambier et al., 2014). Untenable replication of the bacteria causes the granuloma to rupture, thereby releasing the bacteria into the airways, rendering the host infectious to others (Alsayed & Gunosewoyo, 2023).

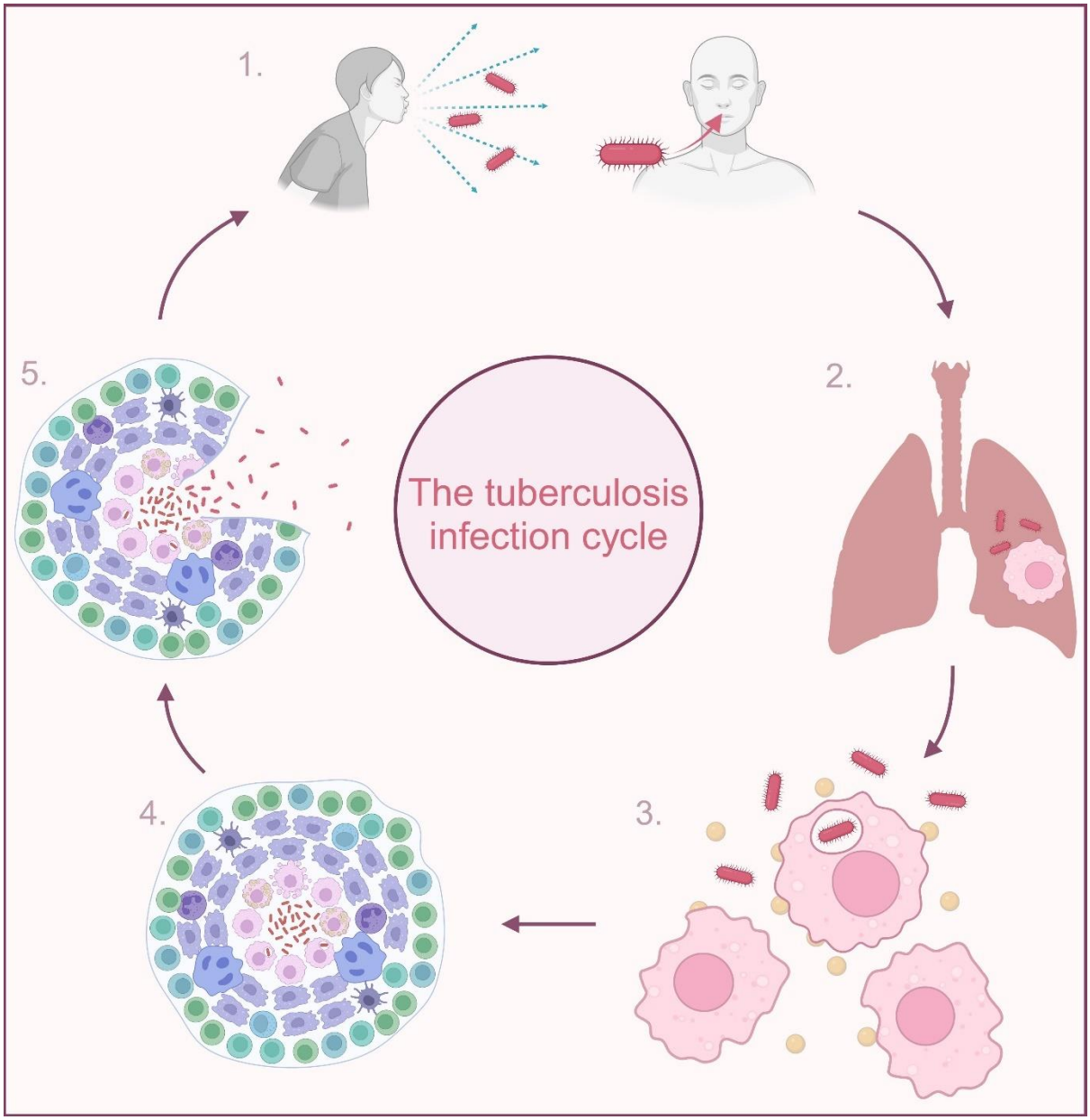


Figure 1.2 | The tuberculosis infection cycle. 1. Transmission of *Mtb* (red rods) in aerosol particles occurs after, for example, sneezing or coughing of an infected host. A new host inhales these particles containing the bacteria and relocates towards the lungs. 2. Alveolar macrophages (pink cell) are the first to encounter *Mtb* and subsequently phagocytose the bacteria. 3. Through cytokine release (orange orbs), infected macrophages recruit additional alveolar macrophages and, later, additional types of immune cells, including neutrophils, dendritic cells, monocytes, and more. 4. The continuous inability of immune cells to kill the bacteria, paired with continuous recruitment of immune cells, leads to formation of a collection of immune cells in which the bacteria are contained, termed a granuloma. 5. Uncontrolled replication within the granuloma leads to a necrotic core called the caseum and may eventually result in rupturing of the granuloma. This causes active disease in the patient and renders

them infectious to others. Created using Biorender.com. Adapted from (C. J. Cambier et al., 2014).

Treatment of active disease includes administration of multiple antibiotics and is challenging due to varying disease profiles and drug resistances (Dartois & Rubin, 2022). Diagnosis of LTBI includes measuring interferon(IFN)-γ release which is induced in T lymphocytes by *Mtb*, and active disease can be diagnosed through a variety of methods, including microscopy of sputum or bronchial secretion, sputum culture, and sputum PCR (Heyckendorf et al., 2022; Suárez et al., 2019). Individuals with LTBI may be treated preventatively, but this only occurs in those with significant predisposition to developing active TB, like people infected with HIV (Dartois & Rubin, 2022). Preventative therapy could reduce reactivation rates by up to 90% (Kiazyk & Ball, 2017). The standard treatment regimen employed here is a range of antibiotic treatments: three regimens based on rifampicin and two alternative monotherapy regimens supplied with daily isoniazid, provided that the *Mtb* strain carried by the patient is not resistant to these drugs (Dartois & Rubin, 2022). For active disease, treatment can generally be divided into two steps: killing of the population of bacteria with a high replication rate in the initial phase, and killing of semi-dormant bacteria in the second phase (Sotgiu et al., 2015). Treatment duration may vary significantly depending on the type of infection and its susceptibility to treatment (Sotgiu et al., 2015). For example, 12% to 14% of TB patients in Germany between 2013 and 2017 showed resistance to at least one of the treatments (Suárez et al., 2019). Of these patients, those resistant to rifampicin had significantly higher increases in treatment duration compared to those with isoniazid (Suárez et al., 2019). The rise in drug resistance poses a significant issue by increasing treatment failure rates and increasing treatment costs of alternative, and potentially more toxic, alternatives (Dartois & Rubin, 2022). An alternative to targeting *Mtb* directly, thereby potentially circumventing the problem of drug resistance, is by targeting the host instead. This requires understanding of the immune response to *Mtb* and how *Mtb* affects the host cells in this response.

1.8.1. Macrophages in tuberculosis

A wide variety of immune cells are involved in the response to *Mtb* infection, as is evident from the range of cell types that respond to infection in the early stages and the cell types that end up as part of the granuloma (Chandra et al., 2022a; Ehlers & Schaible, 2013; Ernst, 2012). Alveolar macrophages are the first cells to encounter *Mtb* and are considered to be particularly permissible to infection establishment by the bacteria (Chandra et al., 2022a). These cells are able to recognize PAMPs of *Mtb*, partially through TLRs, which activates them without activating phagocytosis (Queval et al., 2017). Binding of PAMPs with TLR2 triggers NF-κB signalling and thereby release of pro-inflammatory cytokines (Chandra et al., 2022a).

However, *Mtb* has also been reported to mask PAMPs with a surface lipid called phthiocerol dimycocerosate (PDIM), which prevents the bacteria from being detected (C. J. Cambier et al., 2014). Instead, this induces macrophage production of the macrophage chemokine CCL2 that recruits other macrophages that may be more permissible for *Mtb* growth (C. J. Cambier et al., 2014). Uptake of *Mtb* is likely regulated by the recognition of *Mtb* by the macrophage-inducible C-type lectin (Mincle), and subsequently results in the activation of the NF-κB pathway by recognition of bacterial components by TLRs (Goldberg et al., 2014). This is another step where *Mtb* may affect the effectiveness of the immune system, as the structural characteristics of the bacteria inhibit acidification of the phagolysosome by decreasing vacuolar ATP and GTP accumulation (Zhai et al., 2019). *Mtb* may even replicate within macrophages by perforating the phagosomal membrane and moving the cytosol (Ahmad et al., 2022; Goldberg et al., 2014). This is done through the use of a component of its type VII secretion system (T7SS), with the name of early secretory antigenic target-6 (ESAT-6) secretion system-1 (ESX-1) (Goldberg et al., 2014). Other components of the T7SS are also capable of modulating macrophage apoptotic pathways through, for example, induction of ROS production to induce apoptosis, and inhibition of the ROS/JNK signalling pathway to curtail apoptosis (Ahmad et al., 2022). These examples show how *Mtb* abuses host macrophages to prevent its destruction and instead proliferate in a hostile environment.

This does not mean macrophages are completely defenceless against *Mtb* infection. IL-1β is capable of killing *Mtb* within human and murine macrophages directly, but also indirectly by inducing TNF secretion (Xu et al., 2014). IL-1β is therefore a target of *Mtb* to facilitate its survival, which it may do by reducing production of IL-1β by triggering host signalling of type 1 IFNs (Xu et al., 2014). IL-36γ, which is closely related to IL-1β, has been shown to suppress *Mtb* growth in macrophages by facilitating the formation of the autolysosome through WNT5A, which part of the WNT family of signal transducers (Gao et al., 2019). Furthermore, LPS-stimulation of human macrophages was shown that TLR4-induced production of ROS was effective in killing *Mtb* (Lv et al., 2017). This bactericidal activity of ROS was also shown in a study investigating the role of peroxisomes in *Mtb* infection (Pellegrino et al., 2023). Peroxisomes are organelles responsible for regulation of important metabolic processes, including metabolism of ROS (Pellegrino et al., 2023). ROS derived from these peroxisomes was shown to be important for restriction of *Mtb* in macrophages (Pellegrino et al., 2023). Finally, apoptosis may serve as a mechanism to kill *Mtb* and deprive them from their growth environment, as TNF-α-induced apoptosis of infected macrophages reduced bacterial viability (Oddo et al., 1998). In conclusion, there is a clear dichotomy in the role that macrophages play in *Mtb* infection, and targeting specific processes in the macrophage response may be a viable way to target *Mtb* pathogenicity.

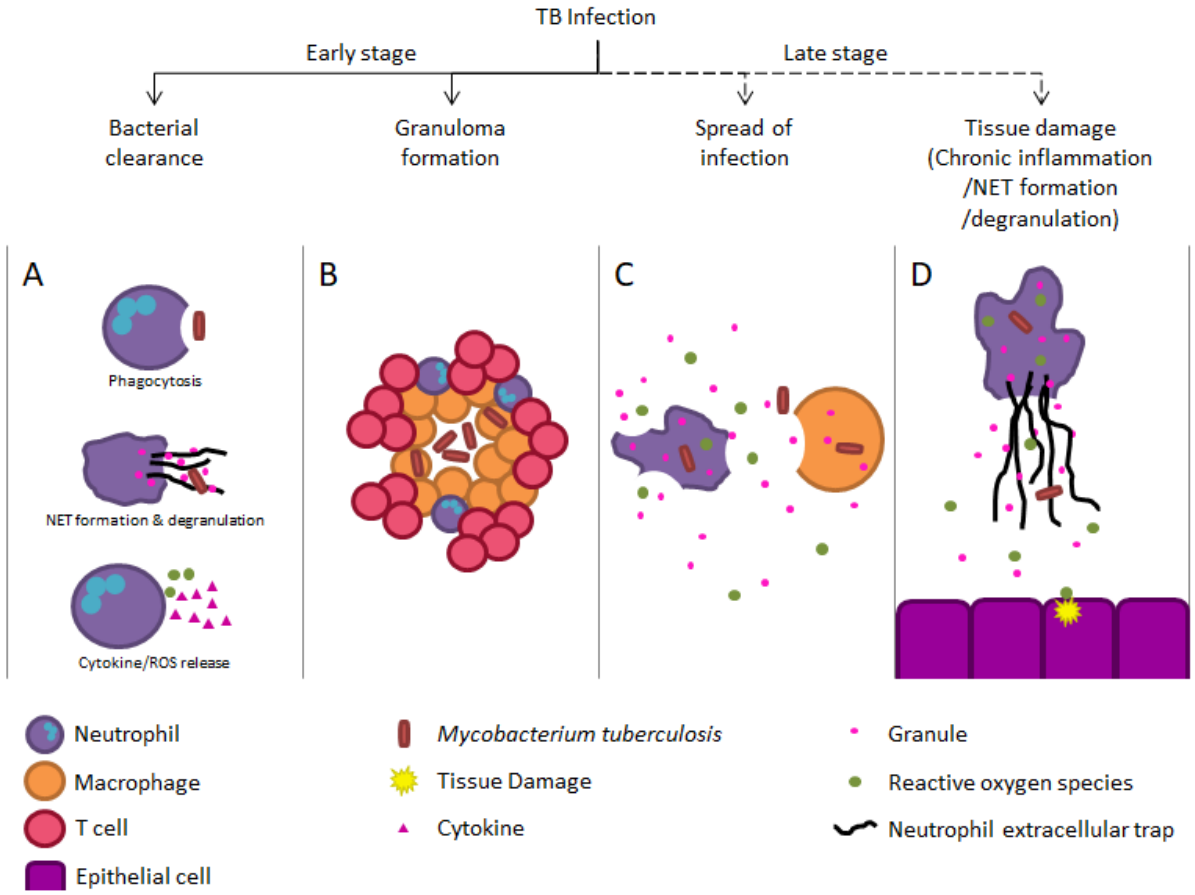


Figure 1.3 | Neutrophils in host-defence and disease pathology during early and late TB infection. Following infection with *Mtb*, immune cells are recruited to the lungs to kill the bacterium. Among these immune cells are neutrophils, which contribute to the killing of *Mtb* during early infection, but become pathogenic during late-stage TB through chronic neutrophilic inflammation and subsequent tissue damage. A) Neutrophils employ several defence mechanisms to kill *Mtb*, including phagocytosis, NET formation, degranulation, and release of ROS. Neutrophils produce several proinflammatory cytokines, such as IL-1β and IL-6, to initiate a stronger immune response and recruit more immune cells to fight infection. B) Initially, infected cells, usually macrophages, recruit more immune cells to the site of infection after internalisation of the bacteria. Inability to kill *Mtb* and prolonged immune cell recruitment ultimately results in formation of granulomas which may or may not contain *Mtb*. Depicted is a simplified schematic of a granuloma, which normally contains other cell types in addition to those shown, like dendritic cells and B cells. In immunocompetent individuals the bacterium is alive but contained within the granuloma and prevented from spreading further, termed latent TB. If the host becomes immunocompromised, the granuloma may rupture and release the bacteria into the surrounding tissue, leading to active, or “late stage” disease. C) Spread of the infection occurs when the immune cells lose control over the infection and succumb after internalisation of *Mtb*. This *Mtb*-induced necrosis leads to further spread of the

infection, and efferocytosis of necrotic neutrophils by macrophages results in necrosis of the macrophage as well. D) *Mtb* may also induce NET formation by upregulating ROS production, but NETs are only capable of capturing, not killing, *Mtb*. Release of granules and ROS after necrosis or NET formation may lead to damage of surrounding tissue.

1.8.2. Neutrophils in tuberculosis

Like with macrophages, neutrophil involvement in TB is controversial, as their uncontrolled deployment is a significant problem during late-stage TB (Gaffney et al., 2022). Recruitment of neutrophils towards *Mtb* infection occurs after macrophages release pro-inflammatory cytokines, such as TNF, IL-6, IL-8, and IL-1β, following their encounter with the bacteria (Kroon et al., 2018). Macrophages may also release IL-1, which promotes neutrophil production through the IL-17-G-CSF axis (Alcantara et al., 2023). Once arrived at the site of infection, neutrophils employ their entire bactericidal arsenal against *Mtb*, including degranulation, ROS release, and NET release (Kroon et al., 2018). Whether these neutrophil advances are successful appears to partially depend on the stage of the disease (Figure 1.3) (Alcantara et al., 2023). For example, IL-6- and IL-23-mediated production of IL-17 by neutrophils in response to *Mtb* infection was found to contribute to bacterial killing during early infection by increasing neutrophil recruitment towards the infection and by inducing ROS production (Hu et al., 2017). However, this production of ROS eventually also contributed to the development of collagen-induced arthritis in mice (Hu et al., 2017). Such a problematic effect of neutrophils during late *Mtb* infection is evident from research into the role of alarmins S100A8/A9 in the pathology of *Mtb* infection (Gopal et al., 2013; Scott et al., 2020). These proteins were found to be associated with lung inflammation in patients with active TB, and were responsible for excessive neutrophil recruitment towards TB infection in a mouse model (Gopal et al., 2013). This is potentially due to an increase in S100A8/A9 signalling, which increased as TB disease progressed in an *Mtb* mouse model (Scott et al., 2020). Furthermore, deficiency of S100A9 significantly limited neutrophil accumulation during chronic infection and thereby improved *Mtb* control (Scott et al., 2020). In contrast, a rat model of *Mtb* showed neutrophilia induced by LPS in early stages of the disease significantly reduced infection levels, but this protective effect was lost when neutrophilia was induced 10 days after infection (Sugawara et al., 2004). There is also evidence for neutrophil protectiveness in later stages, one example of which showed that neutrophils significantly decreased bacterial load in infected mice with deficiency for natural IFN-γ production 28 days after infection (Feng et al., 2006). In short, there is clear evidence for both a protective and damaging effect of neutrophils in *Mtb* infection, but the underlying mechanisms remain unclear.

The ability of neutrophils to kill *Mtb* also remains disputed (Gaffney et al., 2022). Like with macrophages, *Mtb* has developed several mechanisms that allow it to evade death through neutrophil phagocytosis (Borkute et al., 2021). One such mechanisms is preventing neutrophil granules from fusing with the phagosome, potentially through Rab5-mediated arrest of phagosome maturation (Borkute et al., 2021). *Mtb* has also been shown to induce neutrophil necrosis both actively and passively, as phagocytosis of both heat-killed *Mtb* and live *Mtb* induced necrosis in human cells, with increased levels of necrosis found after phagocytosis of live *Mtb* (Corleis et al., 2012). Neutrophils from patients with a disease called granulomatous disease that causes dysfunctional NADPH-oxidase, and therefore the inability to produce ROS, did not succumb to necrotic cell death after infection with *Mtb*, indicating that *Mtb* induces ROS production in neutrophils to cause necrotic cell death (Corleis et al., 2012). Uptake of such necrotic infected neutrophils by macrophages has been shown to exacerbate *Mtb* infection, and inhibition of ROS and necrosis restored the ability of macrophages to control growth of *Mtb* (Dallenga et al., 2017). However, there are reports of successful killing of *Mtb* by neutrophil phagocytosis (Kisich et al., 2002). Neutrophils of healthy human donors were able to phagocytose and subsequently kill *Mtb* in cell cultures, which was potentiated by treatment with TNF-α (Kisich et al., 2002). This appeared to be partially due to exposure of intracellular *Mtb* to neutrophil defensins, and did not appear to be affected by reactive nitrogen intermediates, reactive oxygen intermediates, or neutrophil apoptosis (Kisich et al., 2002). Neutrophils may even phagocytose dying, infected macrophages within the granuloma and kill their internalised *Mtb* through NADPH-oxidase-dependent mechanisms (C.-T. Yang et al., 2012).

Release of NETs during *Mtb* infection is another way for the innate immune system attempts to eradicate the bacterium (Cavalcante-Silva et al., 2023). *Mtb* can directly induce NET formation, which may directly damage host tissue or cause recruitment of additional neutrophils through stimulation of macrophage cytokine production (Cavalcante-Silva et al., 2023). For example, ESAT-6 secreted by the ESX-1 T7SS expressed by *Mtb* was involved in NET formation following necrosis of neutrophils in a calcium-dependent manner (Francis et al., 2014). Another route of NETosis activation is through specific proteins produced by ESX5, a gene cluster that is only present in slow-growing mycobacteria species (García-Bengoa et al., 2023). Stimulation of human neutrophils with these proteins induced NET formation in a response that was potentially ROS-dependent (García-Bengoa et al., 2023). Such NET-inducing effects of ROS were previously seen in cell cultures of neutrophils obtained from TB patients, where *Mtb* infection significantly increased neutrophil ROS levels and initiated NET release (Su et al., 2019). Blocking of ROS production abrogated the release of NETs (Su et al., 2019). Finally, treatment of neutrophils with conditioned medium derived from

macrophages stimulated with *Mtb* induced ROS production and NET formation, indicating *Mtb* may also indirectly induce NET formation by activating non-neutrophil immune cells (Murphy et al., 2024).

These various ways in which *Mtb* induces NET may at first glance suggest that this may be a successful way in which neutrophils combat the bacterium, but the effectiveness of NETs in the killing of *Mtb* remains disputed (Cavalcante-Silva et al., 2023). NETs have been described to be microbicidal against a range of pathogens, but numerous studies suggest NETs are unable to eradicate *Mtb* and are only capable of capturing the bacteria (Cavalcante-Silva et al., 2023). One such study used human neutrophil cell cultures to describe the effect of NETs on two different strains of *Mtb* with different levels of virulence: a highly virulent *Mtb* strain and the low virulence *Mycobacterium canettii* strain (Ramos-Kichik et al., 2009). Exposure of neutrophils to these bacteria initiated NET release that was preceded by production of ROS, but these NETs were unable to kill bacteria or attenuate their multiplication in either of the strains of bacteria (Ramos-Kichik et al., 2009). Similarly, *Mtb* infection in the skin of guinea pigs induced NET formation as early as 30 minutes post inoculation, but these were unsuccessful in killing the bacteria (Filio-Rodríguez et al., 2017).

Neutrophils may also affect the response of other, non-neutrophil immune cells through, for example, efferocytosis of apoptotic neutrophils or release of NETs (Cavalcante-Silva et al., 2023). Macrophages have been shown to benefit by such an interaction in a model of HIV and *Mtb* co-infection (Andersson et al., 2020). Apoptotic neutrophils were added to human macrophages infected with HIV and *Mtb*, and efferocytosis of these neutrophils by the macrophages aided in *Mtb* bacterial killing in an MPO-dependent manner (Andersson et al., 2020). Similarly, efferocytosis of apoptotic neutrophils by *Mtb*-infected macrophages decreased intracellular *Mtb* burden in human cell cultures (Tan et al., 2006). Granules of these apoptotic neutrophils entered macrophage endosomes after efferocytosis and exerted antimicrobial activity, as growth of *Mtb* was inhibited both extracellularly and intracellularly by these granules (Tan et al., 2006). It is important to note that these studies used induction of neutrophil apoptosis in a manner independent of *Mtb*, and may therefore not accurately reflect macrophage-neutrophil interactions in *Mtb* infection. This is a particularly important distinction due to the findings that *Mtb* may actively drive neutrophils away from apoptosis by inducing necrosis, which contributes to *Mtb* growth (Dallenga et al., 2017). NETs may also affect the response of dendritic cells, which were activated into producing type I IFNs when co-cultured with NET-producing, *Mtb*-infected neutrophils (A. M. Lee et al., 2023). Production of these IFNs is associated with *Mtb* disease progression (A. M. Lee et al., 2023). Finally, *Mtb*-induced NET formation was found to induce macrophage IL-6, TNF-α, IL-1β, and IL-10 cytokine

release after they bound to and phagocytosed NETs (Braian et al., 2013). Macrophages also internalised LL-37 complexes found on NETs, which localised towards the macrophage phagolysosome and aided in growth inhibition of intracellular *Mycobacterium bovis* (Stephan et al., 2016). This suggests that such an interaction may be present in *Mtb* infection, but this has not been shown in published literature.

It is clear that neutrophils play a dual role in TB disease progression, and the general consensus appears to be that neutrophils are host-protective during the early granuloma stages, but develop a pathological role by damaging tissue during late granuloma stages (Alcantara et al., 2023). Neutrophil abundance was found to relatively highly correlate with severe tuberculosis, including pulmonary destruction and bacteria excretion (Panteleev et al., 2017). Neutrophil mediators such as MMP-8, S100A8, IL-8, and TNF were significantly increased in TB patients with severe lung damage, and a majority of these mediators was decreased after TB therapy (Muefong et al., 2021). NETs are likely implicated in disease pathology, as plasma markers of NETs, such as MPO-DNA and MPO, were strongly associated with disease severity and decreased after antibiotic therapy (Schechter et al., 2017). Furthermore, sera of TB patients contained significantly increased levels of NET parameters, including NE-DNA and MPO-DNA, and patients with extensive tissue damage had increased NET formation and NE activity compared to those with minor damage (Zlatar et al., 2024). Targeting of neutrophils instead of *Mtb* itself may therefore be a fruitful strategy in combating TB and reducing neutrophil-mediated tissue damage during active TB.

1.9. Hypoxia and HIF

Hypoxia is a state of low oxygen and is a hallmark of tissue inflammation, infection, or damage (Krzywinska & Stockmann, 2018). It is major contributor to the pathogenesis of wide variety of diseases, including cancer, chronic heart disease, inflammatory bowel disease, lung disease, and more (P.-S. Chen et al., 2020; Della Rocca et al., 2022). Hypoxia occurs when the requirement for oxygen exceeds the supply, and ultimately results in the death of cells if the hypoxic state is maintained for too long (Taylor & Colgan, 2017). For example, an immune challenge can lead to immune cell influx into the affected tissue, resulting in an increased demand for oxygen that may not be met by supply (Watts & Walmsley, 2018). Oxygen is required for oxidative phosphorylation (OXPHOS), a process that generates ATP in aerobic conditions, and is vital to cell survival (Taylor & Colgan, 2017). This does not necessarily imply that hypoxia harmful, as it is seen in normal processes unrelated to disease or harm, such as stem cell differentiation (Abdollahi et al., 2011). Additionally, it plays a key role in immune cell function, as these cells often encounter hypoxic environments in areas where they express

their functions (Krzywinska & Stockmann, 2018). Such effects of hypoxia on immune cells includes increasing neutrophil survival, enhancing macrophage phagocytosis, and increased B lymphocyte glycolytic metabolism (Krzywinska & Stockmann, 2018).

One of the master regulators of hypoxia is hypoxia-inducible factor (HIF) (Figure 1.4), which targets many genes involved in a variety of processes, including angiogenesis, cell proliferation, and metabolism (Tirpe et al., 2019). There are three types of HIFs: HIF-1, HIF-2, and HIF-3 (Tirpe et al., 2019). HIF-1 is the most ubiquitously expressed and most well-described HIF isoform (Krzywinska & Stockmann, 2018). It can affect target genes by binding to them either directly, which is associated with gene upregulation, or indirectly, which is associated with gene downregulation (Semenza, 2012). HIF-2 is less widely expressed, is not as well-described as HIF-1, and may affect a number of the same genes as well as different genes than HIF-1 (Krzywinska & Stockmann, 2018). During inflammation, HIF-1 is expressed at the early stages, while HIF-2 is seen at later stages (Watts & Walmsley, 2018). HIF-3 is poorly understood and has not been as extensively researched as the other HIF, and is suggested to play a role in mediating HIF-1- and HIF-2-mediated gene expression (S.-L. Yang et al., 2015). This thesis will focus on HIF-1 due to its well-described effects on neutrophil function (Krzywinska & Stockmann, 2018).

The HIF transcription factor is heterodimeric and has two subunits called HIF-1α and HIF-1β (Masoud & Li, 2015). HIF-β is constitutively expressed in excess and translocates into the nucleus after its production (Masoud & Li, 2015). This means the protein levels of HIF-1α determine the transcriptional activity of HIF-1 (Semenza, 2012). HIF-1α is also constantly produced but is targeted for proteasomal degradation in normoxic conditions (Semenza, 2012). This is a result of the hydroxylation of HIF-1α proline residues by prolyl hydroxylase domain (PHD) enzymes, which allows the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to bind to HIF-1α (Krzywinska & Stockmann, 2018; Semenza, 2012). VHL then ubiquitinates HIF-1α which targets it for proteasomal degradation (Krzywinska & Stockmann, 2018). This reaction only occurs during normoxic conditions, as the hydroxylation of the prolyl residues requires oxygen (Masoud & Li, 2015). Hypoxia therefore results in the constitutive expression of HIF-1α, which allows it to translocate into the nucleus (Krzywinska & Stockmann, 2018). Here, HIF-1α forms a transcription complex with HIF-1β and subsequently recruits co-activator p300/CBP (Krzywinska & Stockmann, 2018). This complex binds to hormone response elements (HREs) within target genes, resulting in the transcription of target genes and subsequently affects target processes (Krzywinska & Stockmann, 2018).

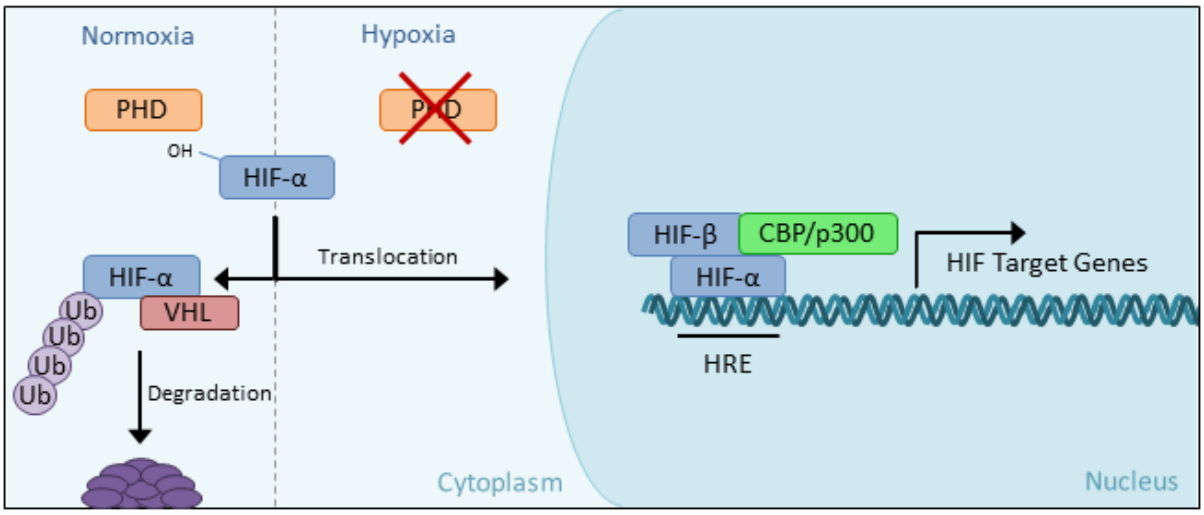


Figure 1.4 | Overview of the HIF signalling pathway during normoxia and hypoxia. The master regulator of the hypoxia pathway is Hypoxia Inducible Factor (HIF), which consists of 2 subunits called HIF-α and HIF-β. During normoxic conditions, prolyl hydroxylases (PHDs) continuously hydrolyse prolyl residues on HIF-α in an oxygen-dependent manner. This results in the binding of the von Hippel-Lindau (VHL) protein to HIF-1α, which recruits a ubiquitin ligase and targets HIF-α for proteasomal degradation. During hypoxia, PHD enzymes lose their oxygen-dependent function and are unable to hydroxylate HIF-α. HIF-α is therefore not targeted for proteasomal degradation, becomes constitutively expressed, and translates to the nucleus. Here, HIF-α can bind to its HIF-β subunit, which present in abundance within the nucleus. This transcription complex can then bind to co-activators like CBP/p300 and subsequently to hypoxia responsive elements (HREs), which results to transcription of HIF target genes.

1.9.1. HIF and neutrophil function

HIFs play a crucial role in immune cell adaptation to hypoxic conditions and therefore affects a wide range of immune cell effector functions, including those of neutrophils (Krzywinska & Stockmann, 2018). Survival of mouse-derived HIF-1α knockout neutrophils was significantly reduced in hypoxic conditions compared to normoxic conditions in cell cultures (Walmsley et al., 2005). This was dependent on NF-κB signalling, as inhibition of the pathway resulted in a loss of the hypoxic survival effect, and NF- κB transcript abundance during hypoxia was significantly reduced in HIF-1α knockout mice (Walmsley et al., 2005). This effect on neutrophil survival was also seen in human neutrophils from patients with hypoxia-inducing myocardial infarction, where neutrophil survival was accompanied by increased intracellular levels of HIF-1α (Dölling et al., 2022). HIF also modulates the energy generation by facilitating the switch to anaerobic ATP generation through glycolysis (Cramer et al., 2003). Deletion of HIF-1α in mouse neutrophils significantly reduced their ATP generation in hypoxic conditions (Cramer

et al., 2003). This may have severe consequences for essential neutrophil processes, including migration, ROS production, and degranulation (Jeon et al., 2020). Indeed, production of NE and cathepsin G, two important neutrophil granule proteases, was significantly decreased in HIF-1α-null neutrophils, and significantly increased in VHL-null mutants in mouse cell cultures, indicating HIF-1α is important for granule protease production (Peyssonnaux et al., 2005). These reports show the wide range of effects of HIF on neutrophil function, which implicates it as a key regulator during immune challenges.

1.9.2. HIF in TB

Stabilisation of HIF-1α during TB infection has been studied intensively and affects many processes involved in response to the disease, including inflammation and angiogenesis (C. Li et al., 2024). HIF-1α stabilisation has been described to enhance the bactericidal effect of macrophages against *Mtb* infection (Q. Li et al., 2021; Osada-Oka et al., 2019; Zenk et al., 2021). Pharmacological stabilisation of *Mtb*-infected human macrophages significantly increased inhibition of *Mtb* in a growth-dependent manner (Zenk et al., 2021). Human tissues of pulmonary tuberculosis contained significantly higher expression of Hif-1α compared to healthy lungs, and a marker for autophagy, LC3B, was also increased in infected lungs (Q. Li et al., 2021). Autophagy is a process in which intracellular components, or pathogens outside of phagosomes, are encapsulated in a structure called the autophagosome, that fuses with a lysosome (Golovkine et al., 2023). This process has been shown to be host-protective in *Mtb* infection, partially by protecting macrophages from *Mtb*-induced apoptosis (Golovkine et al., 2023). It was found that Hif-1α was responsible for enhanced macrophage autophagy, and induction of pro-inflammatory cytokines IL-6 and TNF-α (Q. Li et al., 2021). Stabilisation of Hif-1α significantly increased macrophage killing of *Mtb*, and strengthened the bactericidal effect of rifampin treatment (Q. Li et al., 2021). In mice, HIF-1α was also shown to be host-protective by curtailing the intracellular replication of *Mtb* in macrophages by inducing glycolytic energy generation (Osada-Oka et al., 2019). Glycolysis-metabolite pyruvate is used by *Mtb* to facilitate its replication, but is reduced during glycolysis by conversion into lactate (Osada-Oka et al., 2019). Finally, HIF-1α stabilisation in mouse macrophages initiated a shift to aerobic glycolysis, which was imperative for IFN-γ-mediated control of *Mtb* infection in macrophages (Braverman et al., 2016). Deletion of *Hif-1α* resulted in a significant inability to control *Mtb* infection, and these knockout mice succumbed to infection months before wildtype mice (Braverman et al., 2016).

Naturally, these pro-inflammatory functions of HIF-1α may be detrimental to the host during TB infection by facilitating *Mtb* growth and inducing neutrophil-mediated tissue damage (C. Li et al., 2024). For example, loss of VHL in T cells significantly increased susceptibility to *Mtb*

infection due to the loss of VHL-mediated accumulation of *Mtb*-specific T cells in mouse lungs (R. Liu et al., 2022). HIF-1α stabilisation in human cell cultures increased neutrophil production of MMP-8 and MMP-9, which contributed to collagen destruction during *Mtb* infection (Ong et al., 2018). In addition to these collagenases, production of MMP-1 by human macrophages and epithelial cells has also been shown to be increased in response to *Mtb*-driven stabilisation of HIF-1α (Belton et al., 2016). Finally, neutralisation of IL-17, which stabilises HIF-1α, in mice severely increased *Mtb* bacterial burden and pulmonary inflammation (Domingo-Gonzalez et al., 2017). Inhibition of HIF-1α reversed this increased susceptibility to *Mtb* in these mice (Domingo-Gonzalez et al., 2017). Evidently, HIF-1α stabilisation may induce both positive and negative effects in *Mtb* infection, and is therefore a promising target for further investigation.

The effect of HIF-1α stabilisation on neutrophils in the context of *Mtb* infection is not as well-studied as with macrophages, but shows clear potential for therapeutic interventions (Remot et al., 2019). HIF-1α and NO were shown to positively regulate each other in mice, but had opposing effects of pro-inflammatory cytokine production (Braverman et al., 2016). Production of IL-1α, IL-1β, which play a role in neutrophil recruitment, was significantly reduced in *Hif-1α* knockout macrophages, but was significantly increased in iNOS-deficient (*Nos2* knockout) macrophages stimulated by IFN-γ (Braverman et al., 2016). NO production may therefore prevent host tissue damage afflicted by neutrophils, but iNOS-deficient mice did not have increased life spans and had slightly exacerbated *Mtb* infection (Braverman et al., 2016). This interplay between Hif-1α and NO signalling has been more extensively described in zebrafish, which are natural hosts to a close relative of *Mtb* called *Mycobacterium marinum* (*Mm*) (Elks et al., 2013). Stabilisation of Hif-1α both pharmacologically and genetically resulted in the significant reduction of *Mm* bacterial burden in systemic infections (Elks et al., 2013). NO production was increased in neutrophils after stabilisation of Hif-1α, indicating a potential role of iNOS in this process (Elks et al., 2013). This was confirmed by addition of a range of iNOS blockers to genetic stabilisation of Hif-1α, which abrogated the host-protective effect of Hif-1α stabilisation (Elks et al., 2013). Furthermore, Hif-1α stabilisation was not host-protective in infected zebrafish depleted of neutrophils, while it remained host-protective when only macrophages were depleted, indicating that neutrophils are the driving factor behind the host-protective effect of Hif-1α stabilisation (Szkuta, 2020). Despite this, macrophages may still be important for the Hif-1α-driven neutrophil response to *Mm*, as they were shown to increase the neutrophil NO-driven destruction of *Mm* infection by producing IL-1β after stabilisation of Hif-1α (Ogryzko et al., 2019). These examples show the potential of modulating the neutrophil response in response to TB infection, and how the zebrafish is a suitable model to further explore such endeavours. However, much about the neutrophil response to TB and how

specific neutrophil behaviours like neutrophil swarming affect disease progression is still unclear and will require further research.

1.10. The zebrafish model organism

Zebrafish are a useful *in vivo* model that allows for studying of the combination of neutrophil swarming, tuberculosis, and Hif modulation. The zebrafish model has a variety of advantages over alternatives, including ease of maintenance, high fecundity, ease of genetic modification, and rapid development (Goldsmith & Jobin, 2012; Mastrogiovanni et al., 2024). They only become protected under the Animals (Scientific Procedures) Act 1986 at 5 days post fertilisation (dpf), when they become capable of independent feeding (Home Office, 2022), meaning any zebrafish-related work performed before this point effectively reduces the use of protected animals in research. Compared to other model organisms such as the mouse, zebrafish experimentation is relatively high throughput, less labour intensive, and less expensive (Goldsmith & Jobin, 2012). Drug treatment is an example of relatively easy labour, as it is applied by submersion through simply adding the drug to the fish media (Goldsmith & Jobin, 2012). Finally, the entire zebrafish genome has been sequenced and has high homology with the human genome, as around 70% of human genes have an orthologue in the zebrafish (Howe et al., 2013).

Genome sequencing has also drastically increased the available toolbox for gene manipulation, including CRISPR-Cas9 gene editing (M. Li et al., 2016; K. Liu et al., 2019). CRISPR-Cas9 can be used to target a gene and knock it out by introducing a deletion into the DNA, which can result in stable lines in which all fish carry this homozygous deletion, provided it does not induce lethality (M. Li et al., 2016). Constructs carrying a fluorescent element can be inserted into the DNA, allowing for the generation of transgenic reporter zebrafish lines, which indicate the activation of a protein of interest (Sassen & Köster, 2015). This can be specifically applied to, for example, a single cell type, meaning migration of these cells can be followed using fluorescence microscopy (Sassen & Köster, 2015). This is a clear benefit of zebrafish over other models such as mice, which require injection of fluorescently labelled donor neutrophils for migration assays (Stackowicz et al., 2020). Migration assays are particularly easy during the early development, as they remain transparent for the first week after fertilisation (Goldsmith & Jobin, 2012). The transparent benefit has been further enhanced by the generation of the *nacre* and *casper* lines, which are distinguished by lack of pigmentation (Lister et al., 1999) and almost fully transparent adult zebrafish (White et al., 2008) respectively. Transparency also lends well to infection experiments with fluorescent

bacteria, such as *Mm*, in which one can follow disease progression by quantifying bacterial burden using fluorescence microscopy (Davis et al., 2002).

Zebrafish are a natural host to *Mm*, which is the closest genetic relative to *Mtb* (Tobin & Ramakrishnan, 2008). TB research is most often performed in mice, but this model lacks the formation of caseating granulomas seen in TB in humans (Tobin & Ramakrishnan, 2008). Aside from zebrafish, guinea pigs and rabbits form granulomas reminiscent of human TB granulomas, but these models show other disease dissimilarities and suffer from maintenance difficulties and restricted modulatory tools (Fonseca et al., 2017). Infection of *Mtb* in macaques most closely resembles the disease progression in humans, but this model comes with significant ethical considerations and monetary costs (Tobin & Ramakrishnan, 2008). A rightful criticism of the zebrafish model is the significant morphological difference with humans, particularly the lack of lungs where *Mtb* initially causes infection (Tobin & Ramakrishnan, 2008). However, outside of the method of infection, *Mm* disease progression follows a very similar path to *Mtb*, with a significant macrophage response, macrophage-mediated *Mm* dissemination, and formation of granulomas (Speirs et al., 2024). In short, the zebrafish *Mm* model offers an alternative to available TB models with a genetically relevant alternative to *Mtb*, the ability to study neutrophil behaviours during *Mm* infection using imaging-based approaches, and the ability to examine the involvement of neutrophil swarming in the published host-protective effect of Hif-1α stabilisation during *Mm* infection (Elks et al., 2013).

The zebrafish immune system, like that of humans, consists of both innate and adaptive immunity that develop at different points in time after birth (Speirs et al., 2024). Innate immunity develops rapidly, and phagocytic activity can be witnessed as early as a day after fertilisation (van der Vaart et al., 2012). Adaptive immunity develops around 3 dpf, which allows for studying of innate immunity in isolation while adaptive immunity remains undeveloped (van der Vaart et al., 2012). Vertebrate innate immunity in zebrafish in highly conserved, and consists of neutrophils, macrophages, TLRs, and other similarities (Harvie & Huttenlocher, 2015). Both of these immune cells have similar morphology and functionality compared to mammalian neutrophils and macrophages (Varela et al., 2017). Many immune cell receptors in humans are also found on their zebrafish counterparts, but there are differences (van der Vaart et al., 2012). For example, the specificity of TLR2, TLR3, and TLR5 is conserved, but TLR4 is not activated by LPS like it is in humans (van der Vaart et al., 2012).

Development of neutrophils is initiated at around 24 hours post fertilisation (hpf) in the definitive wave of haematopoiesis (Speirs et al., 2024). Haematopoietic stem cells migrate to an area called the caudal haematopoietic tissue (CHT) where they develop into mature

neutrophils that contain granule contents (Speirs et al., 2024). These granules can be divided into categories similar to those in humans, including primary, secondary, and tertiary granules (Speirs et al., 2024). One of the key antimicrobial agents in primary granules, MPO, is present in zebrafish as the homolog MPX and is expressed during embryonic development (van der Vaart et al., 2012). Zebrafish neutrophils have similar effector functions to human neutrophils, including phagocytosis, ROS production, degranulation, NET formation, and cytokine production (Speirs et al., 2024; van der Vaart et al., 2012). Many cytokines and their receptors are conserved in zebrafish, including IL-1β, IL-6, IL-8, and receptors CXCR1 and CXCR2 (van der Vaart et al., 2012). Neutrophil swarming has also been investigated in zebrafish (Coombs et al., 2019; Isles et al., 2021; Poplimont et al., 2020), and has been shown to be conserved (Isles et al., 2021). In conclusion, the zebrafish is a versatile model that can combine methodological procedures that will adequately serve the purpose of studying neutrophil swarming and its modulation in a *Mm* infection background.

1.11. Hypothesis and aims

Neutrophil swarming is a behaviour that is not well-understood in the context of infection. In addition, neutrophil contribution to TB disease progression is poorly understood and controversial (Gaffney et al., 2022). The behaviour of neutrophil swarming in response to *Mm* infection has not seen extensive investigation and its role in fighting the bacteria remains unclear. Considering that swarming is conserved in zebrafish (Isles et al., 2021), and that neutrophils can play a significant role in early clearance of *Mm* infection (Elks et al., 2013), there is a substantial possibility that neutrophil swarming is involved in the neutrophil response to *Mm* infection. Investigation of this phenomenon could further elucidate the inner workings of the neutrophil swarming response, and might present new avenues for host-targeted therapies in combating TB.

I hypothesise that neutrophils swarm to mycobacterial infection in zebrafish, and that this behaviour can be manipulated to the benefit of the host.

The following aims were addressed to test this hypothesis:

1) Develop and characterise a zebrafish model that can be used to investigate neutrophil swarming in response to infection
    - Determine the optimal methodological procedures
    - Characterise the neutrophil swarming response to *Mm*

2) Investigate the dependency of *Mm*-initiated neutrophil swarming on LTB4 signalling and other swarming mediators
   - Achieve reliable and tractable knockdown of *lta4h* with CRISPR-Cas9
   - Determine the effect of *lta4h* knockdown on neutrophil swarming in response to *Mm*
   - Examine the effect of inhibition of potential swarming mediators on neutrophil swarming in response to *Mm*

3) Examine if neutrophil swarming is involved in the host-protective effect of Hif-1α stabilisation during *Mm* infection
   - Determine if the host-protective effect of Hif-1α stabilisation is maintained in the somite infection model
   - Investigate the effect of Hif-1α stabilisation on neutrophil swarming
   - Evaluate the effect of Hif-1α stabilisation on infection outcome

2. Materials and Methods

2.1. General Procedures

2.1.1. Zebrafish husbandry and ethics

All zebrafish lines used were maintained in the Bateson Centre at the University of Sheffield in facilities approved by the UK Home Office. Bacterial burden experiments were performed on *nacre* larvae and all clustering and swarming experiments used *Tg(mpx:GFP)i114* (Renshaw et al., 2006) larvae. Stabilisation of Hif-1α was confirmed using *Tg(phd3:GFP)i144* (Santhakumar et al., 2012) larvae. Adult fish were kept on a light/dark cycle of 14/10 hours at 28°C and marbling of tanks was done up to a few hours before initiation of the dark cycle on the day before the scheduled lay. Eggs were collected and maintained in Petri dishes with E3 medium (Cold Spring Harbor) containing a single drop of methylene blue. This E3 medium was replaced with clear E3 medium at 1 dpf. All larvae were kept up to a maximum of 124 hours and were culled by immersion in bleach before this time point or conclusion of the experiment. All procedures were in accordance with the Animals (Scientific Procedures) Act 1986 under project license PP7684817.

2.1.2. Dechorionation and anaesthesia

All embryos that were used in experiments were dechorionated at 1 or 2 dpf using tweezers (Sigma-Aldrich). The tweezer tips were pushed together, pressed against the chorion of the egg, and subsequently opened to rupture the chorion and remove the unharmed embryo from the chorion. Any procedures involving injections (excluding yolk and single cell injections), injury, and imaging involved anaesthesia of embryos with 0.168 mg/ml 3-amino benzoic acid ethyl ester (Tricaine, Sigma-Aldrich). This was achieved by immersion in E3 containing 5% Tricaine.

2.2. Injections and *Mm*

2.2.1. Preparation for injections

Kwik-Fill Borosilicate Glass Capillary needles (World Precision Instruments) were pulled using a micropipette puller device to create two individual needles per capillary that were used for all injections. Injection mixtures were pipetted into the needles using extended length gel loading tips (Eppendorf), and the loaded needles were put into a micromanipulator (Manual Micromanipulator MM33, Wärzhäuser Wetzlar) for injections. Needles were broken using tweezers to allow for efflux of injection mixes. Broken needles were kept as slim as possible to avoid a potentially larger wound induced by a wider needle. A PV820 Pneumatic PicoPump (H. Saur Laborbedarf) and an SMZ 745 microscope (Nikon) were used to adjust the droplet

size. A graticule (Pyser-Sgi) was used to measure a droplet of 1 nl, which is the desired volume for injections.

2.2.2. Yolk- and single cell injections

Zebrafish eggs were lined against a microscope slide within a petri dish lid with a Pasteur pipette. Excess water from transferring the eggs to the petri dish lid was removed before injections. For yolk injections, all eggs were injected into the middle of the yolk. Only eggs with up to 4 cells were used for injection. For single cell injections, eggs were rotated with the tip of the needle until the single cell of the egg was visible. The single cell was then injected. Only eggs with a single cell were used for injection. Any eggs with an undesired number of cells were discarded or injected with an excess amount of inoculum to cause developmental issues.

2.2.3. CRISPR-Cas9 injections

CRISPR experiments were performed with single cell injections. Only experiments that combined CRISPR-mediated knockdown with genetic Hif-1α stabilisation were performed with yolk injections. When using a single CRISPR guide, injection mixes contained 1 µl Cas9 protein (New England Biolabs), 1 µl TRACR RNA (Merck), and 1 µl 20mM sgRNA. A Cas9 control contained 1 µl DEPC instead of Cas9 protein and was used as a mock injection. When using 3 sgRNAs at the same time, mixes contained 0.5 µl of each sgRNA instead of 1 µl of a single sgRNA.

2.2.4. Preparation of *Mm* culture plates and culture media

Growth agar plates were made using 3.8g of 7H10 Middlebrook agar powder (BD) in 180ml distilled water in a 200ml screw top bottle, followed by addition of 1ml glycerol (Sigma-Aldrich). The bottle was subsequently autoclaved to sterilise the contents. Before pouring plates, the bottle was microwaved until the contents were dissolved. The bottle was cooled to 50-55°C and 50 ml OADC (Scientific Laboratory Supplies) and 200µl hygromycin (Sigma-Aldrich) were added. The mixture was poured into petri dishes and was allowed to solidify before storage at 4°C. 7H9 growth medium was prepared with 2.35 g 7H9 Middlebrook broth powder (BD Biosciences) added to 450ml distilled water in a 500 ml screw top bottle, followed by 1 ml glycerol (Sigma-Aldrich). The bottle was autoclaved before further use. Subsequently, 45ml of the media was transferred to a 50 ml falcon tube, and 5 ml ΔADC (Scientific Laboratory Supplies) and 50 µl hygromycin (Sigma-Aldrich) were added.

2.2.5. *Mm* routine culture

Glycerol stocks of *Mm* mCherry were used for routine *Mm* cultures. A single glycerol stock was pipetted onto a 7H10 culture plate and was streaked prior to incubation. Plates were kept

at 28°C and, after sufficient growth, the plate was re-streaked onto another 7H10 plate. Re-streaking of the same glycerol stock was performed every 10 days up to 6 times after the initial re-streak. When this limit was hit, or when bacteria were visually unhealthy (discolouration, crustiness), a new glycerol stock was plated on a 7H10 plate.

2.2.6. *Mm* mCherry culture preparation

7H9 (BD Biosciences) bacterial growth media was used for culturing of *Mm* mCherry. 10 ml 7H9 media containing Middlebrook ADC (Scientific Laboratory Supplies) and hygromycin (Sigma-Aldrich) was inoculated with *Mm* mCherry from streaked 7H10 plates and was cultured for 24 hours at 37°C in T25 culture flasks (Greiner Bio-One). The OD600 was measured using a Jenway 6300 spectrophotometer (Jenway) at both the time of inoculation and at the end of the culture. The OD600 at inoculation was aimed to be within range (±0.02) of 0.2 to ensure optimal conditions for the bacterium to grow and to prevent overgrowth at the end of the culture. The OD600 measured at the end of the culture was used to calculate the required volume for resuspension of the *Mm* mCherry pellet to obtain the desired CFU after various washing steps. *Mm* mCherry was resuspended in 2% PVP in PBS directly before injection to help prevent blocking of injection needles.

2.2.7. Preparation of injection plates

To make injection plates, agarose (Bioline) was added to E3 + methylene blue for a final concentration of 1% agarose. The bottle was microwaved until all agarose was dissolved. The mixture was allowed to slightly cool and was decanted into petri dishes until the bottom of each dish was covered with liquid. The mixture was allowed to set at room temperature and, after solidification, the dishes were maintained at 4°C until further use.

2.2.8. Somite injections

Mm mCherry was injected into the 23rd somite on the ventral side of the body at 2 dpf. The prepared *Mm* culture was pipetted into an injection needle which was loaded into a micromanipulator (Manual Micromanipulator MM33, Wärzhäuser Wetzlar) and was broken with tweezers to form a sharp edge and allow flow-through of the injection fluid. The target volume of 1 nl was obtained by calibrating the droplet size on a graticule (Pyser-Sgi). A PV820 Pneumatic PicoPump (H. Saur Laborbedarf) and an SMZ 745 microscope (Nikon) were used to adjust the droplet size and inject into the somite. Experiments generally used a final CFU of 500, which was achieved by rapidly injecting 2 droplets of 1 nl containing 250 CFU. Any deviations from the standard injection volume of 500 CFU will be specified in the relevant results sections. All larvae were anaesthetised prior to injection and were loaded onto an injection plate. Larvae were oriented identically on the plate to ensure injection of the somite

on the same lateral plane for each fish. More specifically, fish were all injected on the left lateral side, meaning this side was facing up during injections. This side was facing down towards the bottom of the glass slide during microscopy.

2.2.9. Caudal vein injections

Systemic infections were achieved by injecting 200 CFU *Mm* into the caudal vein of zebrafish of at least 30 hours old. Fish were anaesthetised and were transported to an injection plate. Zebrafish were injected at the blood island of the caudal vein as described in published literature (Benard et al., 2012).

2.3. CRISPR-Cas9

2.3.1. Guide and primer design

The guide RNA for *lta4h* CRISPR-mediated knockdown was available from a previous study (Isles et al., 2021). A forward primer was also available, but a reverse primer had to be designed. Ensembl was used to identify the gene code for *lta4h* in zebrafish: ENSDARG00000006029. This code was used in CHOPCHOP (https://chopchop.cbu.uib.no/) and a reverse primer was designed using this web tool. The guide RNA (IDT) and primers (IDT) used for knockdown of *lta4h* are described in Table 1. Injection of CRISPR mixes has been described in the "Injections and *Mm*" section above.

Table 1 | *lta4h* CRISPR guide RNA and primer sequences.

Gene	Guide RNA (5'-3')	Forward primer (5'-3')	Reverse primer (5'-3')	Enzyme
lta4h	AGGGTCTGAAACTG GAGTCATGG	CGTGTAGGTTAAAAT CCATTCGCA	TGACAGCTTTTGATAA ATCCGA	HinfI

2.3.2. Extraction of gDNA

CRISPR-injected zebrafish and control-injected zebrafish were dechorionated at 1 dpi. Up to 8 anaesthetised fish were collected per group for generation of gDNA, which was later used for genotyping. These fish were individually subjected to 20 minutes of 95°C in 100 µl NaOH. Afterwards, 10 µl 1M Tris-HCl (pH 8.0) was added to each fish and the mixture was stored at -20°C until further use.

2.3.3. PCR

The gDNA of *lta4h* knockdown fish and controls was used for PCR. The optimal annealing temperature used during the PCR protocol was first determined by running a temperature gradient for the annealing step of the PCR protocol, followed by gel electrophoresis to

determine the strongest band. Each sample used in PCR contained 5 µl DEPC-treated water, 1 µl forward primer, 1 µl reverse primer, 2 µl Firepol (Solis Biodyne), and 1 µl extracted gDNA. The PCR protocol can be found in Table 2 below.

Table 2 | Standard PCR protocol.

Step	Temperature (°C)	Time
Initiation	95	2 min
Cycle	95	30 sec
Annealing	57 (*lta4h* only)	30 sec
Extension	72	45 sec
Final extension	72	10 min
Hold	4	∞

2.3.4. Digestion of CRISPR PCR products

The PCR products obtained from the CRISPR PCR protocol were digested with the corresponding restriction enzyme. The *lta4h* CRISPR was digested with HinfI (New England Biolabs) at 37°C for a minimum of 3 hours. Up to 3 fish per group were left undigested to serve as digestion controls. Samples were stored at -20°C after digestion until further use.

2.3.5. Gel electrophoresis and guide validation

Digested PCR products and controls were used for gel electrophoresis to validate the efficacy of the *lta4h* CRISPR in knocking down of the gene. SYBR safe dye (Invitrogen) was added to 2% agarose (Sigma-Aldrich) gels (w/v in 1x TAE buffer, Bioline), with 4 µl per 50 ml agarose solution. The solution was microwaved and poured into a mould until solidified. The gel was then put in a gel tray (Geneflow) and was submerged with 1x TAE buffer. The gel was loaded with 5 µl of each sample per lane. The gel was also loaded with 1x DNA ladders (Bioline) to allow for identification of sample band sizes. Gels were run at 100V until the bands were close to the lower edge of the gel. Gels were photographed using a Syngene U:Genius gel doc (Avantor).

2.4. Imaging

2.4.1. Mounting of fish for microscopy

Fish were mounted on microscopy chambers (Thermo Fisher Scientific) using 1% low melting point agarose (LMP) (Sigma-Aldrich) for all experiments, excluding the systemic bacterial burden experiments. 0.1 g LMP agarose was dissolved in 10 ml clear E3 through vortexing after heating in a microwave. 420 µl LMP agarose was subtracted from the falcon tube and

was replaced with 420 µl Tricaine. A drop of E3 with up to 20 larvae for the clustering experiments or up to 6 for swarming experiments was transferred to a microscopy chamber. Roughly 500 µl LMP agarose + 4% Tricaine mixture was pipetted into the chamber and larvae were aligned using a pipette tip. All fish were oriented with their injected somite facing towards the bottom of the microscopy chamber to reduce the distance between the glass and the injected somite to as little as possible. For time-lapse experiments, all microscopy chambers were topped off with E3 containing Tricaine once the LMP agarose had solidified to ensure the fish would not dry out during imaging. For systemic bacterial burden experiments, fish were transferred onto a 50 mm glass bottom FluoroDish™ (Ibidi) and were maintained in E3 containing Tricaine.

2.4.2. Fluorescence screening

Larvae of the *Tg(mpx:GFP)i114* line were screened for fluorescent neutrophils at 1 or 2 dpf, prior to somite infections, tailfin injury, or drug treatments. Zebrafish were anaesthetised and a microscope with filter was used to visually inspect individual larvae. Larvae without apparent neutrophil fluorescence were discarded.

2.4.3. Fluorescence microscopy

A DMi8 microscope (Leica Microsystems) with an ORCA-flash4.0 digital camera (Hamamatsu) was used to image larvae at the specified time points. Leica Application Suite X (LAS X) (Leica Microsystems) was used for both widefield and confocal microscopy. Images of somites were acquired using either a 10x objective or a 20x objective (initial microscopy experiments only). Systemic bacterial burden was captured using a 2.5x objective. *Mm* mCherry was detected using a TRITC filter set and *mpx:GFP* was detected using a FITC filter set. All experiments used 2x2 binning, while clustering and bacterial burden experiments used 100% fluorescence intensity manager (FIM) compared to 30% for time-lapse experiments. Non-time-lapse experiments used 7.5 µm distance per z-stack, while 10 µm was used for time-lapses to reduce imaging time.

2.4.4. Time-lapse microscopy

Mark-and-find in LAS X software was used to designate locations to image during time-lapses. The sizes of the z-stacks were minimised per larva individually to reduce imaging time compared to a fixed range for the z-stacks for all larvae. After initiation of the time-lapse, the first cycle was monitored to ensure no mistakes were made during the set-up, and monitor if the cycle time did not exceed the expected standard 10 minutes. The latter was necessary

due to a lack of accuracy of the software in predicting the expected cycle time before initiation of the time-lapse.

2.5. Analysis

2.5.1. Processing of images and time-lapses

Max projections of images and time-lapses, where necessary, were created in LAS X software. Images of bacterial burden were exported as Tiff files for further quantification using ZF4, as described in (Stoop et al., 2011) or FIJI (Schindelin et al., 2012).

2.5.2. Imaging read-out: neutrophil counts

Neutrophil counting was done in LAS X. The annotation tools were used to outline the somite for each larva using the Brightfield channel images. The lateral somite lines were drawn on the z-stacks where *Mm* mCherry was in focus, while the upper and lower somite lines were drawn on the max projection. Neutrophil numbers were counted within the designated somite area, with all neutrophils residing on these lines being included in the counts. Neutrophils seen within the somite area that resided on the other side of the fish could be excluded by their z-stack displacement compared to somite-localised neutrophils and the somite infection.

2.5.3. Imaging read-out: neutrophil clusters and swarms

Neutrophil clusters were defined as: three or more neutrophils in close contact with each other at a single point in time. Neutrophil swarms were defined as: three or more neutrophils in close contact with each other for at least 30 minutes. Max projections were used to identify fish with potential clusters or swarms. Z-stack images of these fish were used to determine whether these neutrophils were indeed in close contact with each other, or if they were spatially separated in the z-plane.

2.5.4. Imaging read-out: bacterial burden

The *Mm* bacterial pixel count in the systemic bacterial burden experiments was calculated by loading the *Mm* mCherry max projections of each larva into ZF4 bacterial pixel counting software. Bacterial burden in somite experiments was done using the Color Pixel Counter plugin for FIJI. With this plugin, the somite area was outlined on the *Mm* mCherry max projection images using the ROIs from the neutrophil counts to only count pixels within the somite. The threshold (“Minimum intensity value (1-255)”) was set to 65 and the colour (“Color”) setting to “Red” to determine the number of pixels. This threshold was determined by comparing read-outs of a number of max projections to read-outs of the same pictures in ZF4.

The threshold that showed the closest results to the ZF4 read-outs was used in all clustering and time-lapse experiments.

2.5.5. Statistical analysis

Graphpad Prism 10 (GraphPad Software) was used for all statistical analyses. Unpaired, two-tailed t-tests were performed on bacterial burden experiments, which are presumed to be normally distributed. Experiments with non-normally distributed data used the Mann-Whitney test. A Spearman correlation test was done to assess the correlation between two groups. A Fisher's exact test was done on clustering/swarming prevalence between two groups. One-way ANOVA and two-way ANOVA was used on experiments with more than two groups. The statistical analysis used for a specific dataset is mentioned in the corresponding Figure legend. P values shown are: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Characterising neutrophil swarming to *Mm* infection

3.1. Introduction

3.1.1. *In vitro* approaches to investigating neutrophil swarming

The neutrophil swarming process in disease has been studied in a variety of model systems since a revolutionary publication in 2013 (Lämmermann et al., 2013), both *in vitro* and *in vivo*. *In vitro* studies often make use of neutrophils isolated from humans or mice. Over multiple publications, Reátegui and colleagues have used bioparticle microarrays to investigate neutrophil swarming (Glaser et al., 2024; Reátegui et al., 2017; Walters et al., 2021; Walters & Reátegui, 2020). These arrays function by applying cationic polyelectrolyte spots on glass slides and then adhering anionic bioparticles to these spots through electrostatic interaction, forming bioparticle clusters. Only the anionic bioparticles are designed to attract neutrophils and elicit a swarming response. Adding wells around the clusters then allows for the addition of solutions containing neutrophils and subsequently to monitor their behaviour in response to the anionic bioparticles (Walters & Reátegui, 2020). *Staphylococcus aureus* was typically used as the neutrophil attractant in these studies, but alternatives included *Saccharomyces cerevisiae* and *Escherichia coli* (Glaser et al., 2024; Reátegui et al., 2017; Walters et al., 2019, 2021). Microarray can also be utilised with fungi to study neutrophil swarming. *Candida albicans*, *Candida auris*, *Candida glabrata*, and *Aspergillus fumigatus* were successfully grown on microarrays and mimicked the growth of conventional fungus cultures (Hopke et al., 2020, 2022; Hopke & Irimia, 2020). Finally, neutrophil swarming was investigated in *Cryptococcus neoformans* with cell cultures and live imaging (D. Sun & Shi, 2016).

3.1.2. Examining neutrophil swarming *in vivo* in mice

A majority of neutrophil swarming experimentation is done *in vivo* in mice or zebrafish. A popular technique to study neutrophil behaviours in mice is two-photon- or multi-photon microscopy (Jeong et al., 2019; Kreisel et al., 2010; Lämmermann et al., 2013; Lekkala et al., 2024; W. Li et al., 2012; Liese et al., 2012; Uderhardt et al., 2019; Walters et al., 2021; Zhao et al., 2023). Two-photon microscopy uses a laser beam that generates a light spot of a micrometer in size by focusing the beam through a microscope objective (Helmchen, 2009). Compared to confocal microscopy, 2-photon microscopy is less susceptible to light scattering (Helmchen, 2009). A reduction in light scattering in biological tissue is a property of higher-wavelength light used in this type of imaging and allows it to reach higher levels of tissue penetration (Helmchen, 2009; Ishii & Ishii, 2011). Intravital 2-photon imaging includes imaging of a tissue of a live specimen. Generally, this involves exposing the tissue surgically under anaesthesia and maintaining the body temperature of the mouse and preventing drying-out of

the tissue in a chamber (Park, Choe, Lee, et al., 2018). These techniques have been applied to investigate neutrophil swarming in response to primarily inflammation (Kreisel et al., 2010; Lämmermann et al., 2013; W. Li et al., 2012; Uderhardt et al., 2019; Walters et al., 2021), but also infectious agent such as *Staphylococcus aureus* (Kamenyeva et al., 2015; Lekkala et al., 2024; F. Yang et al., 2023), *Salmonella enterica* Typhimirium*, and Pseudomonas aeruginosa* (Kienle et al., 2021).

3.1.3. Examining neutrophil swarming *in vivo* in zebrafish

The zebrafish is a common model organism for studying neutrophil behaviour (Bader et al., 2021; Elks et al., 2013; Harvie & Huttenlocher, 2015; Henry et al., 2013; Isles et al., 2021), yet the area of neutrophil swarming is relatively unexplored in zebrafish. In recent work it was demonstrated that neutrophil swarming is conserved in zebrafish, with a similar LTB4-dependent, bi-phasic response as seen in mice (Isles et al., 2021). This study used live imaging paired with transgenic neutrophil zebrafish lines and CRISPR-Cas9 knockouts to demonstrate dependency of injury-induced neutrophil swarming on *lta4h* (Isles et al., 2021). Similarly, a different wounding model, where a sterile wound was introduced near the CHT, was used to investigate the interplay between LTB4 and calcium signalling using live imaging with transgenic lines for neutrophil calcium, connexin43, *lta4h*, and 5-LO expression (Poplimont et al., 2020). This showed neutrophils further promote swarming through calcium signalling, in part because of the positive effect of calcium alarm signals on LTB4 biosynthesis (Poplimont et al., 2020). These studies demonstrated that neutrophil swarming is conserved in the zebrafish, and exemplify ways to apply the toolbox of this model to further examine neutrophil behaviours.

3.1.4. Neutrophil behaviour in response to injury and infection

Despite a lack of studies that describe neutrophil swarming in zebrafish, many studies have been done on neutrophil responses that make up part of the swarming response, such as neutrophil recruitment and clustering. While LTB4 is a key player in the swarming response (Isles et al., 2021), other factors may also be at play. In a burn-induced tailfin injury model, inhibition of adenosine triphosphate and hydrogen peroxide significantly reduced neutrophil recruitment towards the wound and knockout of the IL-6 receptor in a mutant line similarly showed reduced recruitment towards the wound (Barros-Becker et al., 2020). Similarly, other wounding assays found evidence for involvement of Cxcr1 and Cxcr2 (Coombs et al., 2019), pan-histone deacetylase (Fan et al., 2023), and the circadian regulator Clock1a (A. Chen et al., 2023) in neutrophil recruitment towards injury. These examples show that neutrophil recruitment and, by extension, swarming is a dynamic response that is affected by many different factors.

In addition to signalling of relevant genes, the location of neutrophil swarming may also determine the time point at which swarming can be observed. A laser wound introduced in the ventral fin near the CHT initiated neutrophil recruitment within 5 minutes post wounding and showed swarming by 20 minutes (Poplimont et al., 2020). A wound introduced relatively caudal to this previous study, and therefore further away from the CHT, introduced a peak in neutrophil numbers recruited towards the wound as early as 1 to 2 hours post wounding (Coombs et al., 2019). Finally, neutrophil counts in the well-described zebrafish tail transection model generally assess neutrophils around 6 hours (Bader et al., 2021; Ellett et al., 2015; Kaveh et al., 2020; Petrie et al., 2014; Ren et al., 2017), which has been described as the peak of inflammation (Coombs et al., 2019; Ellett et al., 2015), which implies that swarming is likely to occur around this time as well. These findings indicate the possible effect of the location of inflammation on the time it takes for neutrophils start swarming, but it might not be fully translatable to neutrophil responses to infection.

In zebrafish, swarming in response to infection is not well-understood, particularly in the case of *Mm*. There were no studies found that specifically investigate the swarming response to mycobacteria in zebrafish, but one study has utilised the pathogen *Staphylococcus aureus* to investigate swarming. The otic vesicle, where the main sensory components of the inner ear develop (Haddon & Lewis, 1996) was injected with *Staphylococcus aureus* and assessed for neutrophil swarming at 6 hours post infection (Isles et al., 2021). The clusters that formed were reminiscent of those found in mammalian swarming responses to infection (Isles et al., 2021), indicating that neutrophil swarming may be conserved in both injury and infection models. Similarly, the otic vesicle was infected with *Mm* and elicited a neutrophil response at 3 hours post infection (Benard et al., 2012). Wright and colleagues investigated the microRNA miR-206 in its effect on neutrophil recruitment and retention after *Mm* infection into the trunk. Knockdown of miR-206 significantly increased recruitment to the site of infection, with recruitment peaking between 6 and 12 hours for the control, and retained neutrophils there for longer (K. Wright et al., 2021). A comorbid wound and infection model showed that stabilisation of Hif-1α increased neutrophil retention at the site of a localised infection near the tailfin would at 6 hours post wounding (Schild et al., 2020). These studies again illustrate the dynamic and variable nature of neutrophil behaviour in response to infection, and much is still unknown in terms of neutrophil swarming in response to *Mm* infection.

3.1.5. Hypothesis and aims

The zebrafish model has been used extensively in examination of the neutrophil swarming in both inflammation and a variety of infections, as outlined above. However, there is a general

lack of studies into neutrophil swarming and TB, and there are no studies on neutrophil swarming to *Mm* infections in zebrafish in particular. One of the main swarming mediators, LTB4, has been shown to be both host-protective (Peres et al., 2007; Tobin et al., 2010) and host-destructive (Bafica et al., 2005; Sorgi et al., 2020; Tobin et al., 2013) during *Mtb* or *Mm* infection. Considering neutrophils are involved in the immune response towards these infections (Alcantara et al., 2023; Elks et al., 2013), these findings highlight the potential of neutrophil swarming to occur in response to *Mm* infection.

I hypothesise that neutrophils swarm to *Mm* infection in zebrafish.

To investigate this hypothesis, localised *Mm* infection in transgenic zebrafish lines was paired with fluorescent imaging to address the following aims:

- Determine a tractable zebrafish model for investigation of swarming
- Characterise the neutrophil swarming response to *Mm*

3.2. Materials and Methods

3.2.1. Heat-killing of *Mm*

In experiments involving heat-killing (HK) of *Mm*, *Mm* in the HK-*Mm* group was subjected to 30 minutes at 80°C in a heat block (brand). After injections, the injection mixes of the PVP control group, *Mm* group, and HK-*Mm* group were injected once (250 CFU) onto 7H10 injection plates to confirm successful heat-killing of *Mm*. These plates were grown at 28°C for three days and were checked for bacterial growth under a fluorescence microscope. The Leica MZ10F stereo 14 microscope was fitted with a GXCAM-U3 series 5MP camera (GT Vision) and photos of the 7H10 plates were taken with GXCapture-T (GT Vision) software.

3.4. Results

3.4.1. Visualisation and determination of neutrophil swarming to a localised *Mycobacterium marinum* infection site

Neutrophil swarming in zebrafish has been investigated in injury models (Isles et al., 2021; Poplimont et al., 2020). but not in a *Mm* infection model specifically. Such an infection model would require a number of practical adjustments to ensure swarming events can be captured with live imaging without compromising the quality of imaging or the number of fish that can be imaged, and to allow for analysis of multiple parameters from the resulting time lapse movies. Therefore, the optimal site of infection and the most efficient microscopy had to be determined before doing any experiments.

Firstly, the site of infection needs to be appropriate to visualise neutrophil swarming. Infection with *Mm* is most commonly performed systemically into the caudal vein (Benard et al 2012), however where infection foci and granuloma form in this model is not predictable. The somite, a muscle block of the zebrafish, is a suitable local infection site alternative as it generates a local infection at the injection site. The distance from the caudal haematopoietic tissue, where neutrophils develop, to infection would also remain relatively constant if the same somite is consistently injected in every fish. Imaging setup is also positively affected, as this consistent location is quick and easy to find in each larva. For these reasons, the 23rd-24th somite of the zebrafish was chosen as the preferred site of infection (Figure 3.1 A).

Secondly, the day at which the fish are infected could impact results. At 2 days post fertilisation (dpf) the larvae are robust enough to withstand somite injections. Neutrophils are already present at this day, as they develop around 1 dpf (Speirs et al., 2024). At 3 dpf zebrafish generally have developed more neutrophils than at 2 dpf, but somite injections are harder to perform due to an increased thickness of the skin, which may induce excess wounding from injections. While a high number of neutrophils may be desirable, it could also hinder the ability to distinguish individual neutrophils from each other. As such, day 2 was chosen as a starting point for infection experiments that would not require any changing if recruitment and swarming levels were deemed sufficient.

Thirdly, the optimal way to image the injected fish had to be established. Time lapse imaging requires a balance between image quality and speed of each imaging cycle, so movies are clear enough to be analysed for multiple parameters without compromising the number of organisms that can be imaged per experiment. Additionally, cycles should remain close

enough together to reduce the possibility of a swarm having disappeared and reformed between each subsequent image.

Finally, our lab initially had two options to image the fish: line-scanning confocal microscopy and widefield microscopy. Line-scanning confocal microscopy reduces out of focus light and individual neutrophils are therefore more easily distinguishable. This imaging technique also offers high resolution but suffers from relatively slow imaging times per fish. For instance, imaging of a single fish using confocal imaging may take 3 minutes (Figure 3.1 B), while this same fish may only require 30 seconds of imaging time with widefield imaging (Figure 3.1 C). The absence of a brightfield image with our confocal imaging set-up also makes it difficult to determine the edges of the somite, which is problematic for analysis as discussed in the next section. While confocal microscopy would have provided an overall preferable image quality, the time restraints that come with this type of imaging are not suitable for time-lapsing experiments that require a statistically relevant number of fish to be imaged within short time windows and are the main reason that widefield imaging was henceforth used as the default image acquisition method. Finally, a 10x lens provided lower resolution of individual neutrophils (Figure 3.1 D) than a 20x lens (Figure 3.1 E), the latter of which was deemed the most appropriate for visualisation of both bacterial burden and neutrophils.

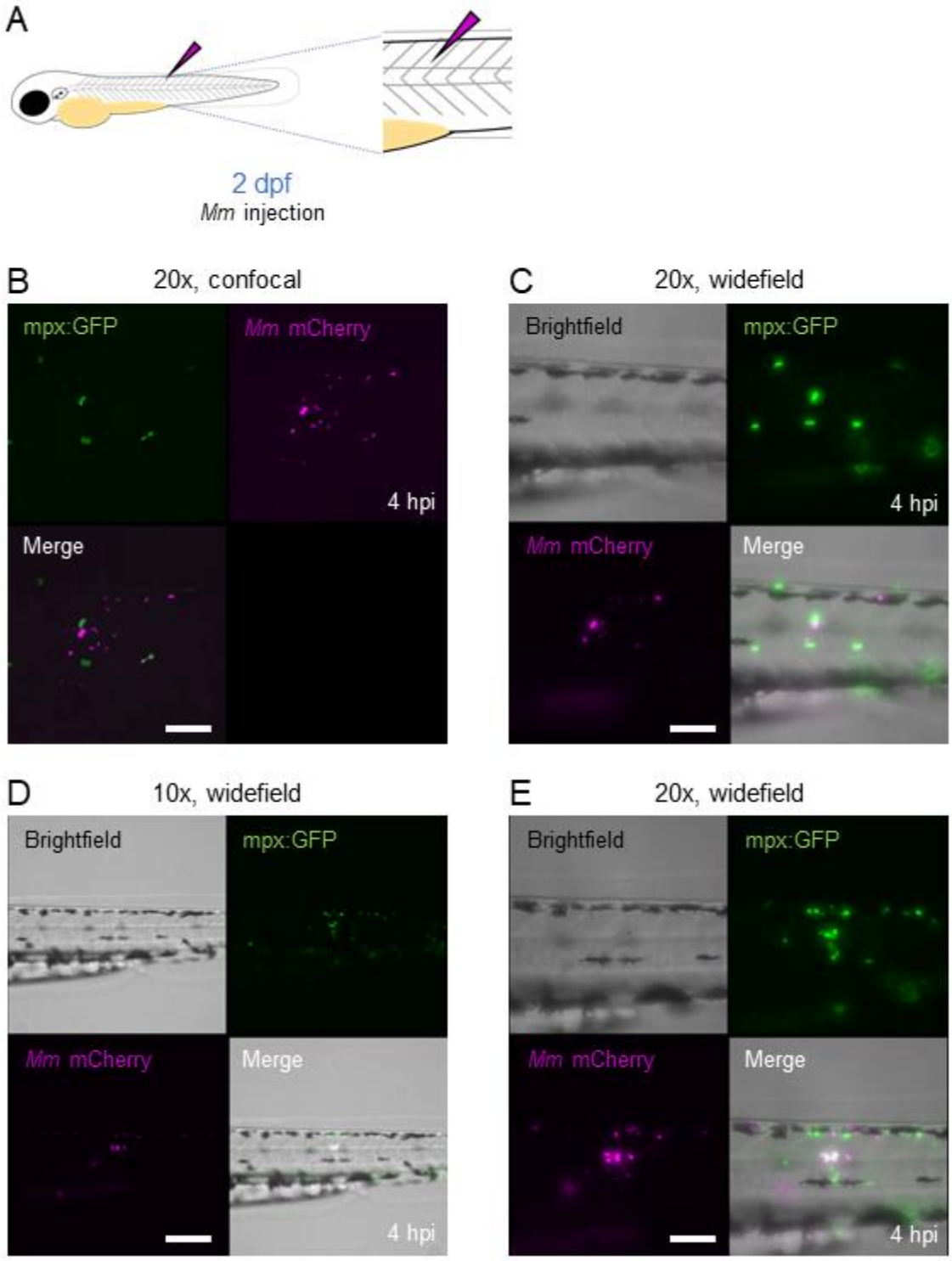


Figure 3.1 | Determination of the optimal neutrophil swarming imaging strategy. A) Zebrafish larvae were infected with *Mm* into the somite at the end of the yolk extension, the place where neutrophils develop. B) Confocal imaging was compared to C) widefield imaging at 20x magnification, and the latter technique was chosen as the more suitable method due to its inclusion of brightfield images and faster imaging speed. Widefield imaging with a E) 10x objective and F) 20x objective were compared to determine the preferential magnification of

the images. 20X imaging was chosen for its relatively high detail in infection levels and distinction of individual neutrophils. Scale bars in B, C, E are 200 μm, and 400 μm in D.

3.4.2. Analysis: Neutrophil numbers and clustering, and bacterial burden

Parameters, including the number of neutrophils or bacterial burden, were assessed specifically within the somite area. The edges of the somite were approximated by using the brightfield images (Figure 3.2). The number of neutrophils was then determined by manual counting by scrolling through the z-stacks. Somite bacterial burden was quantified using max projections of the somite area and using the “color pixel counting” FIJI plugin to count bacterial pixels.

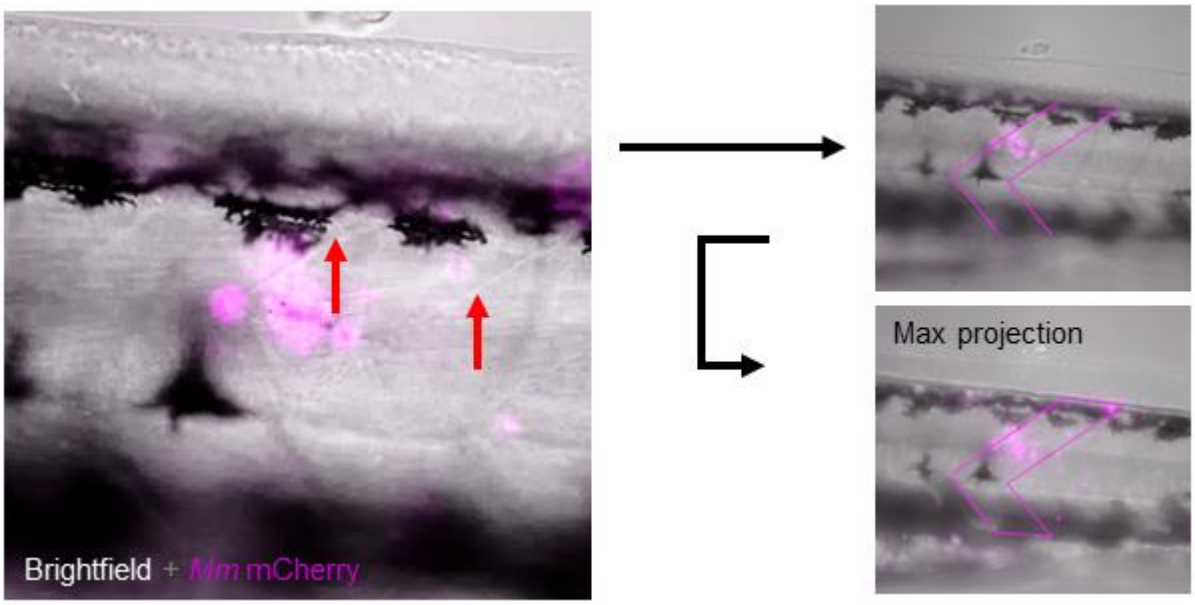
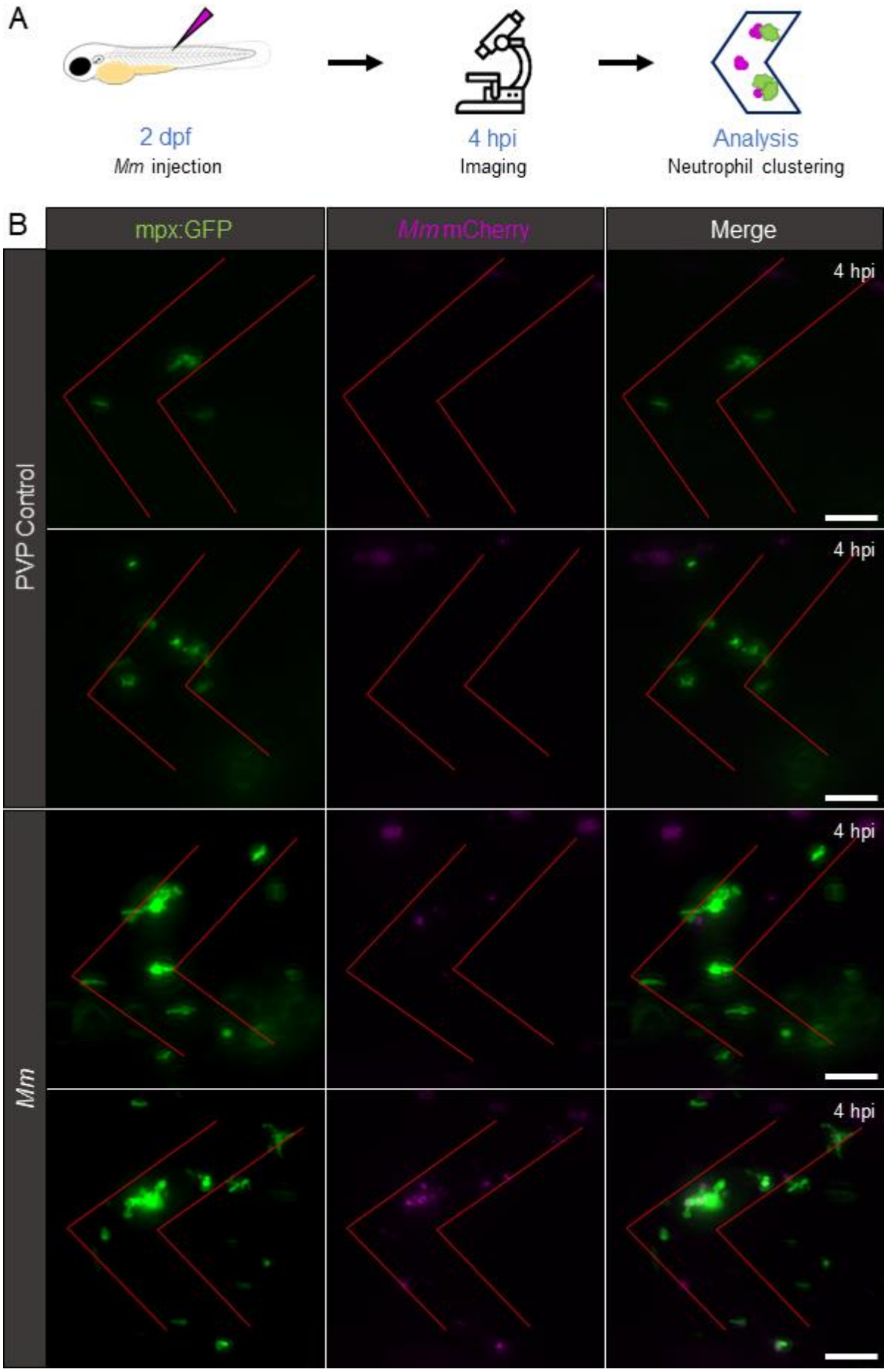


Figure 3.2 | The analysis process of clustering and swarming imaging. A) To determine certain parameters such as the number of neutrophils within the somite, first the somite edges had to be outlined. Image brightness and contrast were adjusted for visual clarity. Scrolling through z-stacks reveals faint lines (red arrows) that indicate the approximate sides of the injected somite. The max projection can then be used to find the upper and lower bounds of the somite.

3.4.3. *Mycobacterium marinum* is primarily responsible for neutrophil accumulation within the somite

Neutrophil swarming is a process that may occur in response to both inflammation and infection (Kienle & Lämmermann, 2016). Therefore, it stands to reason that the needle injury introduced while injecting pathogens may also cause neutrophils to be recruited towards the somite. To make sure that *Mm*, and not the needle injury, is responsible for neutrophil recruitment towards the somite, larvae were injected with 500 CFU *Mm* mCherry at 2 dpf. They were then imaged at 4 hpi, which time point is based on data from Isles and colleagues in which the neutrophil swarm area peaked at 4 hours post injury in a tailfin injury model (Isles et al., 2021) (Figure 3.3 A). Analysis showed that neutrophils are present after both PVP injections and *Mm* injections (Figure 3.3 B). However, injection with *Mm* had significantly increased neutrophil accumulation within the somite compared to the PVP injection ($p < 0.0001$) (Figure 3.3 C). These results indicate that neutrophil accumulation in the somite is primarily orchestrated by the addition of the bacteria, and not because of the needle injury introduced while injecting.


A
2 dpf
Mm injection
4 hpi
Imaging
Analysis
Neutrophil clustering
B
mpx:GFP
Mm mCherry
Merge
PVP Control
Mm
4 hpi
4 hpi
4 hpi
4 hpi

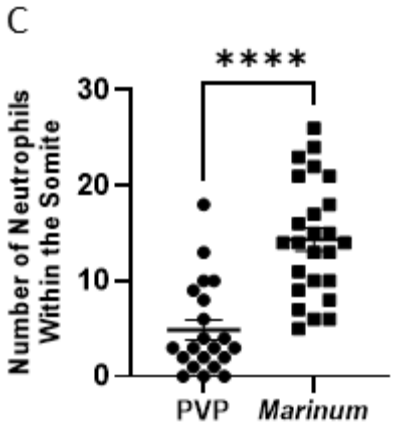


Figure 3.3 | Neutrophils accumulate at localised *Mm* infections. A) Timeline of the experimental procedures. Zebrafish larvae were injected with *Mm* or PVP control at 2 dpf. The PVP control indicates whether or not the injury introduced from injections is enough to trigger neutrophil recruitment. The injected larvae were imaged at 4 hpi and were analysed for neutrophil counts within the somite. B) Neutrophil recruitment towards the injected somite. The lines indicate the edges of the somite. C) The number of neutrophils within the somite. *Mm* injection recruited significantly more neutrophils towards the somite than injection with PVP ($p < 0.0001$, Mann-Whitney test). Error bars depicted in B are mean + SEM, N = 21 and 25 for PVP and *Mm* respectively, taken from 3 experimental repeats. Scale bars are 100 µm.

3.4.4. Neutrophils cluster at sites of *Mycobacterium marinum* infection within the zebrafish somite

Recruitment and clustering of neutrophils at the site of infection are indicators of potential neutrophil swarm formation (Isles et al., 2021). Neutrophil clustering was defined as the close contact of 3 or more neutrophils at the single point in time and was determined visually. Examining clustering allows for the use of larger sample sizes, as each fish only needs to be imaged once instead of multiple times in a pre-determined time frame for neutrophil swarming, and is less methodologically demanding compared to swarming. The presence of clustering serves as a means of identifying whether swarming has the potential to occur after *Mm* infection within the somite. To this end, the somite was injected with 500 CFU *Mm* at 2 dpf and imaging was performed 4 hours after injections (Figure 3.4 A). As mentioned above, this time point was based on data from a previous study that saw a peak in neutrophil swarm size at 4 hours post wounding (Isles et al., 2021). Neutrophil clusters were observed around sites of *Mm* infection within the zebrafish somite (Figure 3.4 B). In total, 22 out of 158 larvae showed neutrophil clusters within the somite, which equals to 13.9% of larvae (Figure 3.4 C). In one instance, two neutrophil clusters could be observed within the same somite. A PVP injected negative control was not included in these experiments to maximise the number of fish that could be imaged at the 4 hpi time point. These results indicate that neutrophils cluster around *Mm* infection.

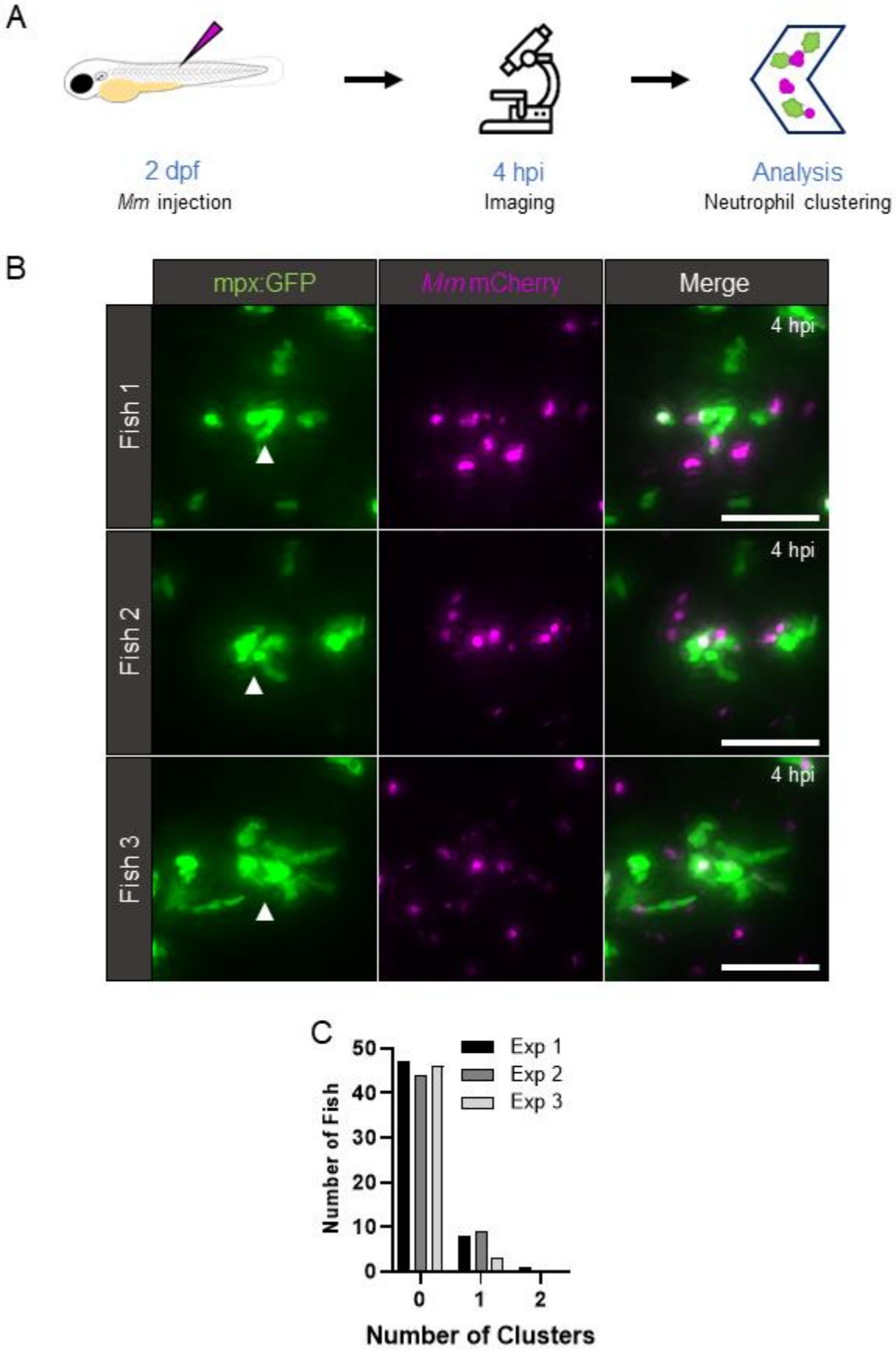


Figure 3.4 | Neutrophils cluster around *Mycobacterium marinum*. A) Experimental overview. Zebrafish larvae were injected with *Mm* into the top half of the somite at 2 dpf, and

injected larvae were then imaged at 4 hpi. Images were then analysed, and clusters were defined as 3 or more neutrophils in close contact with each other at a single time point. B) Examples of clusters found in 3 different larvae. C) The number of clusters observed per larva. Accumulated from 3 repeats, 13.9% of larvae had at least 1 neutrophil cluster at the time of imaging. N = 49-53 larvae from 3 experimental repeats, and scale bars are 100 μm.

3.4.5. Infection levels and neutrophil numbers are consistent over multiple experiments

For the experimental somite swarming model to work, it is important to remain as consistent with the experimental procedures as possible to prevent unnecessary variation in the data. Similarly, this should result in a consistent trend in the data over multiple experiments. Larvae were infected with *Mm* in the somite at 2 dpf and were imaged at 4 hp. Larvae showed similar levels of infection (Figure 3.5 A) over the 3 experiments, indicating the practical aspect of infecting fish was performed consistently. The number of neutrophils within the somite was also averagely equal between the experiments (Figure 3.5 B). Taken together, these results show that infection levels were consistent between experiments and that they lead to consistent neutrophil numbers.

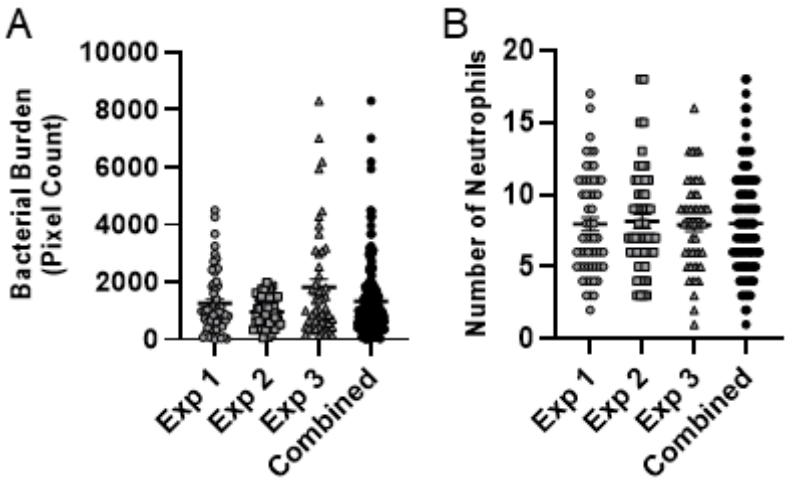


Figure 3.5 | *Mm* bacterial burden and somite neutrophil numbers remain consistent over multiple experiments. Zebrafish larvae were infected with *Mm* at 2 dpf and were imaged at 4 hpi. A) Bacterial burden within the somite for each experiment. B) Neutrophil numbers within the somite for each experiment. Both burden and neutrophil numbers remained consistent between experiments.

3.4.6. Neutrophil abundance correlates with *Mm* bacterial burden within the somite

The previous set of data was combined to determine whether the bacterial load affects the number of neutrophils that are recruited to the somite. A Spearman correlation test showed the number of recruited neutrophils positively correlated significantly with the amount of bacterial burden within the somite (*p = 0.009*) (Figure 3.6 A). However, a spearman r of 0.2071 indicates this correlation is weak. When comparing bacterial burden within the somite of fish without clusters and fish with clusters (Figure 3.6 B), no difference was found (*p = 0.6619*). In summary, this means an increase in bacterial burden is likely to be accompanied by an increase in neutrophil number and vice versa, but this increase will be minimal, and that the amount of bacterial burden does not affect clustering prevalence.

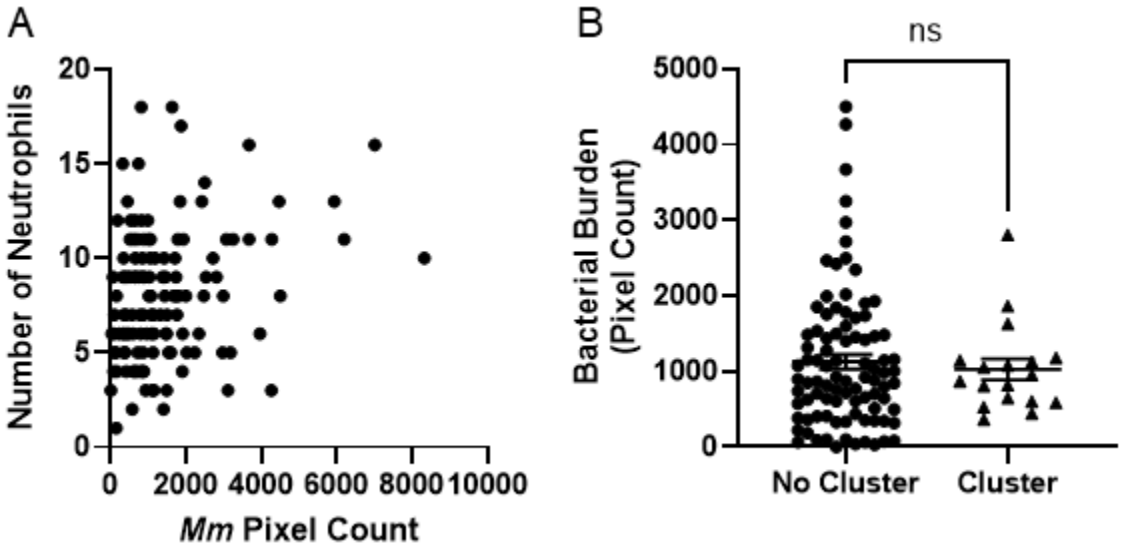


Figure 3.6 | Neutrophil numbers within the somite weakly, positively correlate with *Mm* bacterial burden. A) A Spearman correlation test showed there is a positive correlation (*p = 0.009*) between neutrophil numbers and bacterial burden within the somite. However, a spearman r of 0.2071 indicates this correlation is weak. N = 158 over 3 experimental repeats. B) There was no difference in bacterial burden pixel counts within the somite in fish with neutrophil clusters compared to fish without clusters (*p = 0.6619*, unpaired t-test). N = 91, 18, for the no cluster group and cluster group respectively, taken from 2 experimental repeats.

3.4.7. Localised infection with *Mycobacterium marinum* induces neutrophil swarming

As mentioned above, injection with *Mm* into the somite induced neutrophil clustering in 15.33% of fish (Figure 3.4 C). Additionally, neutrophil numbers within the somite averaged 7 (Figure 3.5 B), meaning that with the current set-up it would require nearly half the number of neutrophils found within the somite to be considered as clustering. In a zebrafish tailfin injury model, neutrophil swarming occurred in around 50% of injured fish (Isles et al., 2021). Together these results and the findings in other swarming models raise the question whether the time point chosen for clustering examination is viable. The fish were therefore imaged by time-lapse microscopy every 10 minutes for 10 hours to increase the likelihood of capturing both the formation, peak, and resolution of potential neutrophil swarming. First, neutrophil recruitment into the somite after mock injection and *Mm* injection was re-examined at the earliest time point, 1 hpi, to confirm the previous findings that needle injury is not responsible for a majority of the neutrophil recruitment (Figure 3.7 A). Consistent with the previous results, a mock injection with PVP recruited significantly fewer neutrophils into the somite compared to the *Mm* injection ($p < 0.0001$) (Figure 3.7 B, C, D). This confirms that *Mm* is primarily responsible for neutrophil recruitment towards the somite during the first hour of infection.

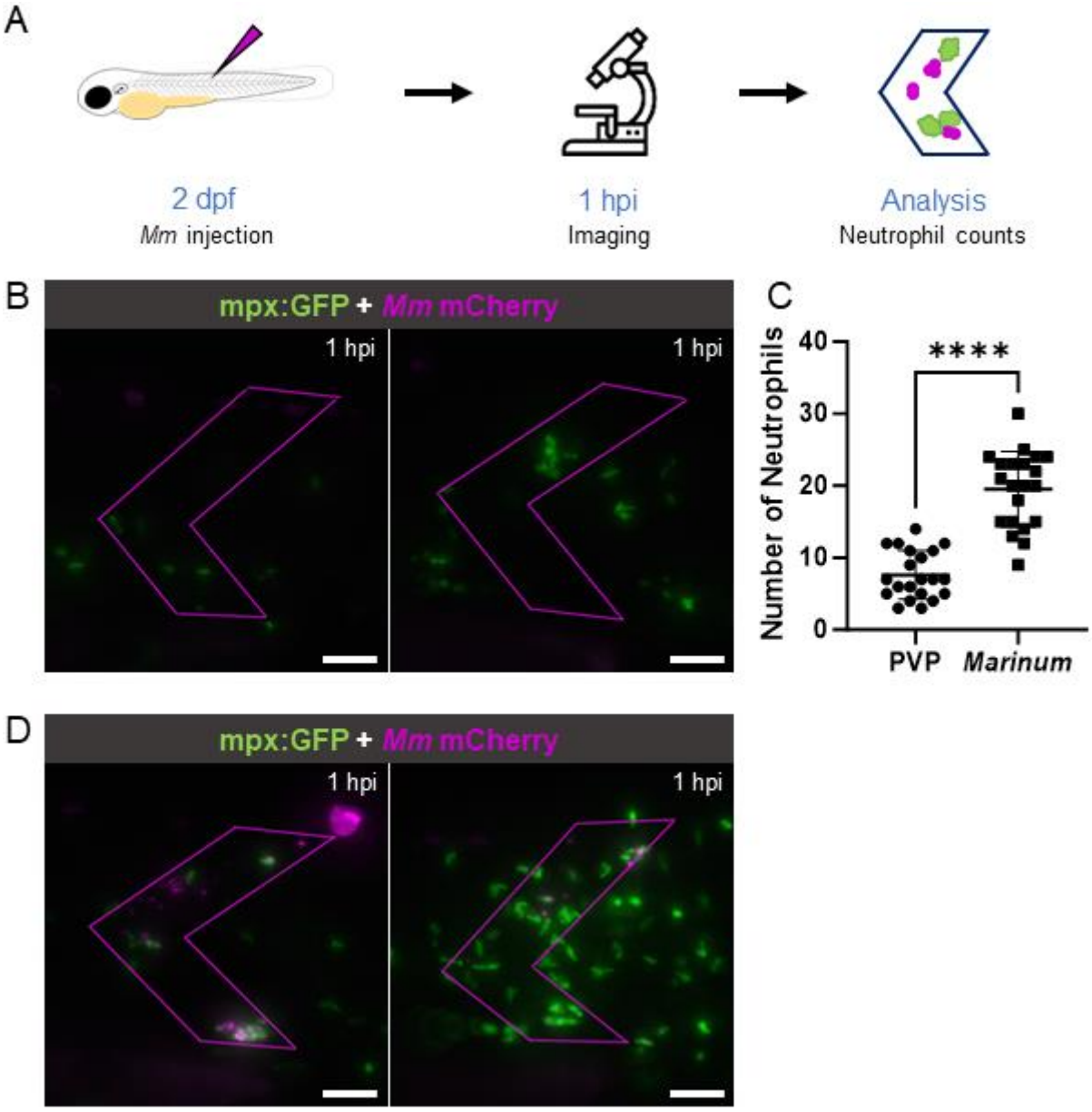


Figure 3.7 | *Mm* infection, not needle injury, is primarily responsible for neutrophil recruitment towards the somite during the first hour of infection. A) Experimental overview. Zebrafish were infected with *Mm* at 2 dpf, were imaged at 1 hpi, and were examined for neutrophil counts within the infected somite**.** B) Neutrophil recruitment towards the somite in PVP-injected larvae at 1 hpi, 2 dpf. Left: example of a larva with relatively minimal neutrophil recruitment in the PVP group. Right: example of a larva with relatively high neutrophil recruitment in the PVP group. C) *Mm* recruited significantly ($p < 0.0001$) more neutrophils towards to somite compared to the PVP control according to a Mann-Whitney test. N = 21 for both groups, taken from 3 experimental repeats. Scale bars are 100 µm. D) Neutrophil recruitment towards the somite in *Mm*-injected larvae. Left: example of a larva with relatively minimal neutrophil recruitment in the *Mm* group in C. Right: example of a larva with relatively high neutrophil recruitment in the *Mm* group in C.

After it had been determined that PVP injection recruits significantly fewer neutrophils towards the somite than *Mm* within the first hour of infection, two separate experiments were performed without PVP controls to maximise the number of fish that could be imaged. Neutrophil swarms were defined as three or more neutrophils in close contact with each other for at least 30 minutes, determined visually. Fish were infected with *Mm* at 2 dpf and were imaged for 10 hours afterwards (Figure 3.8 A). Time-lapse videos were assessed for neutrophil swarming. It is important to note that experiment 1 commenced imaging at 2 hpi, and experiment 2 commenced at 1 hpi due to an improvement in experimental skill by the second attempt. Time lapse data of as early as 1 hpi and up to 12 hpi, up to a total of 10 hour per fish per experiment, showed neutrophil swarming in 18 out of 39 larvae (=46.15% of larvae) (Figure 3.8 B, C). All of the swarms were present at the start of imaging which suggests they start forming before 1 hpi. These results confirm that the time point of 4 hpi used in the clustering experiments is substantially later than the onset of neutrophil swarms in this model.

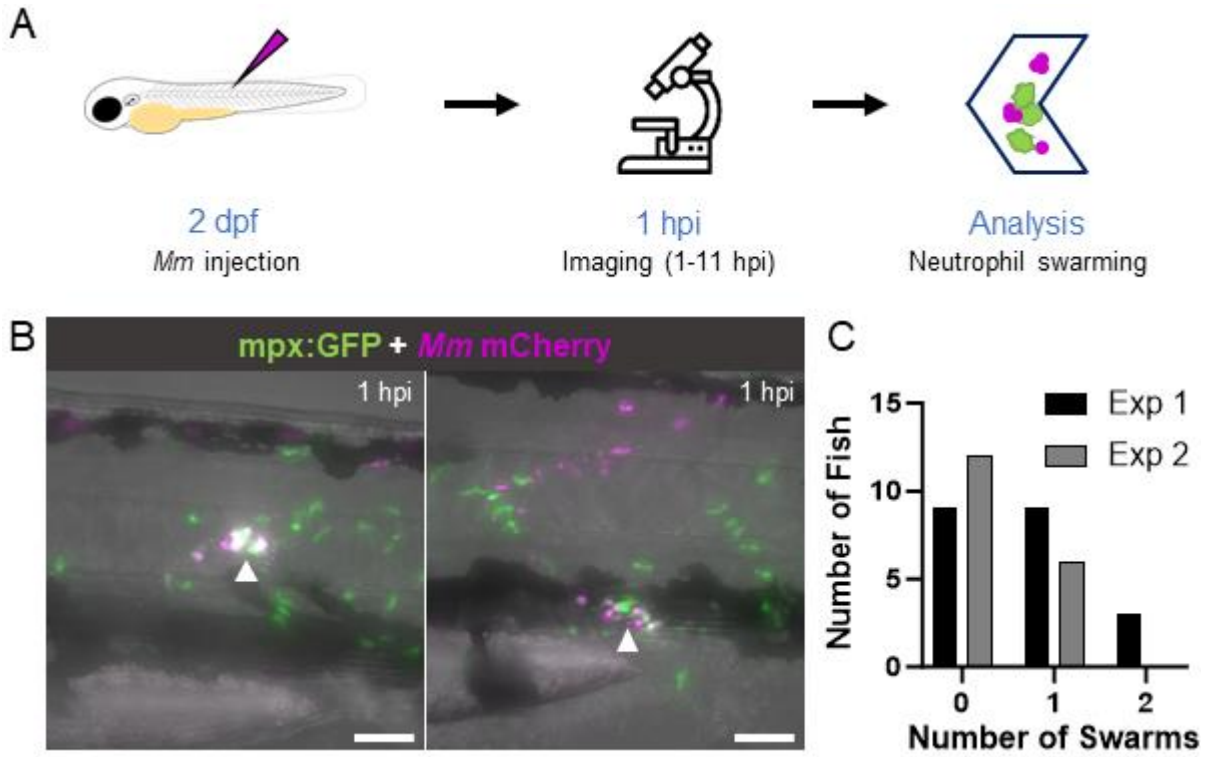


Figure 3.8 | Neutrophil swarming was found in ~46% of infected larvae. A) Zebrafish larvae were infected with *Mm* into the somite at 2 dpf, after which they were imaged from 2 hpi to 12 hpi for experiment 1 and 1 hpi to 11 hpi for experiment 2, for a total of 10 hours per fish. Neutrophil swarms were defined as 3 or more neutrophils in close contact with each other for at least 30 minutes. B) Examples of neutrophil swarms (arrows). C) Prevalence of

neutrophil swarming in zebrafish larvae during 2 separate experiments. A total of 21 neutrophil swarms were found in 18 different fish out of 39 infected fish, which is equivalent to 46.15% swarming prevalence in infected fish. Scale bars are 100 µm.

3.4.8. Different somite injection sites may not be suitable for the swarming model

As shown above, capturing the onset of swarming becomes difficult because it frequently occurs before the fish are being imaged. Injection sites away from the CHT may elicit a later neutrophil response (Coombs et al., 2019) than the standard injection site used so far in this thesis. Different sites of injection may therefore be more suitable if neutrophil recruitment and swarming is still plentiful despite moving away from the CHT, and if swarming onset occurs later than in the standard injection site. This would provide more time to inject and prepare the fish for imaging, making it easier to catch swarm formation in the larvae. Larvae were injected at 2 dpf in 3 different injection sites (Figure 3.9 A). In the first experiment, larvae were infected in the upper body and standard injection sites, and in the second experiment larvae were infected in the mid-body and standard injection site. It was hypothesised that choosing locations further away from the CHT would delay the onset of swarming while not impacting the frequency of swarming or the recruitment of neutrophils. Imaging was performed at 40 mpi for 6 hours and time-lapse videos were analysed for neutrophil numbers and the onset of swarming. Compared to the standard injection site, both alternative sites appeared to recruit fewer neutrophils towards the somite (Figure 3.9 B, C). In addition to the standard injection site, neutrophil swarming only occurred in the upper body injection site (Figure 3.9 B). Taken together, both experiments show a reduction in the amount of swarming and the number of neutrophils within the somite, and it was determined that the standard injection site would remain optimal for further experimentation.

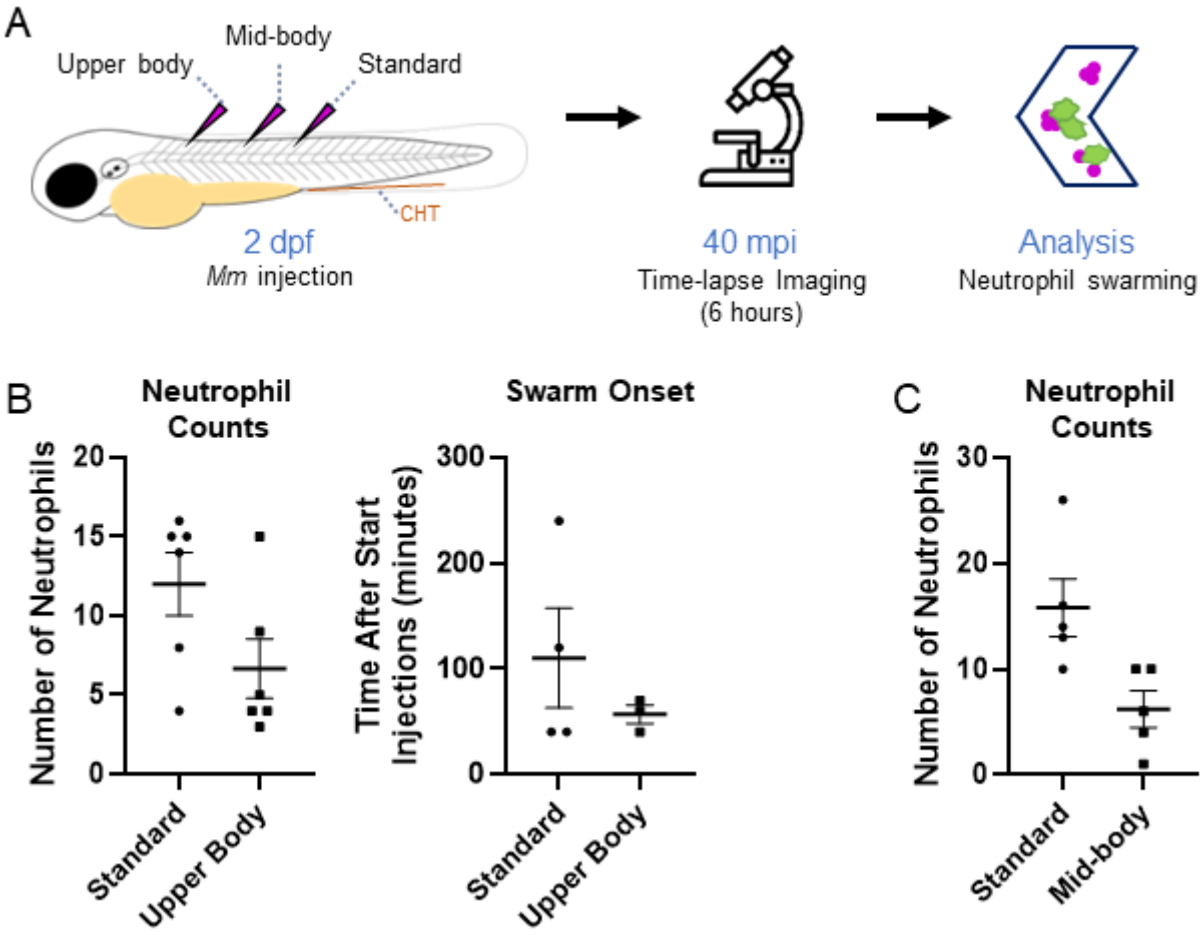


Figure 3.9 | Testing different somite injection sites. A) Zebrafish larvae were injected with *Mm* at 2 dpf to examine differences in neutrophil recruitment and swarming onset. Larvae were injected in 2 separate experiments in 1: the upper body and the standard injection site, and in 2: the mid-body and the standard injection site. They were imaged 40 minutes after starting injections for 6 hours and were then analysed for neutrophil counts and swarming onset. B) Neutrophil counts within the infected somite and onset of neutrophil swarms in experiment 1. N = 6 fish per group. C) Neutrophil counts within the infected somite in experiment 2. No swarms were found in the mid-body group (data not shown). N = 5 fish per group. Error bars are mean ± SEM.

3.4.9. Neutrophil swarming occurs as early as 24 minutes post *Mm* infection

The onset of neutrophil swarming had not been seen in any of the previous swarming experiments, which started as early as 1 hpi. For reference, the experiments outlined in the previous paragraphs started at 1 hpi, which does not take into account the 20 to 30 minutes that was spent injecting the larvae. To capture and analyse swarming onset, the number of fish per experiment was significantly decreased to a maximum of 8 to enable quicker experimental procedures and thereby lowering the amount of time between infections and the start of imaging. This resulted in time windows of a minimum of 24 minutes to a maximum of 36 minutes in which all experimental procedures, from infections to preparing the time lapse, were carried out (Figure 3.10 A). The cycle time, the time between subsequent images of each fish, of the time lapses were also shortened to 6 minutes to more accurately pinpoint exact starting points of swarms without decreasing the sample size too significantly. Neutrophil swarming commenced at ~70 minutes after starting infections on average (Figure 3.10 B). Notably, 3 larvae contained swarms that were present at the start of imaging for their respective experiment: 2 swarms at 24 mpi and 1 at 30 mpi, indicated by green data points. These relatively early swarmers were all located closely towards the CHT where neutrophils develop (Figure 3.10 C). Figure 3.10 D and Figure 3.10 E show examples of swarms over time. Panel 1 shows the first frame of the time lapse, panel 2 shows the commencing of swarming, panel 3 shows the peak of swarming, and panel 4 shows the resolution of swarming if the swarm dispersed before the end of the time lapse.

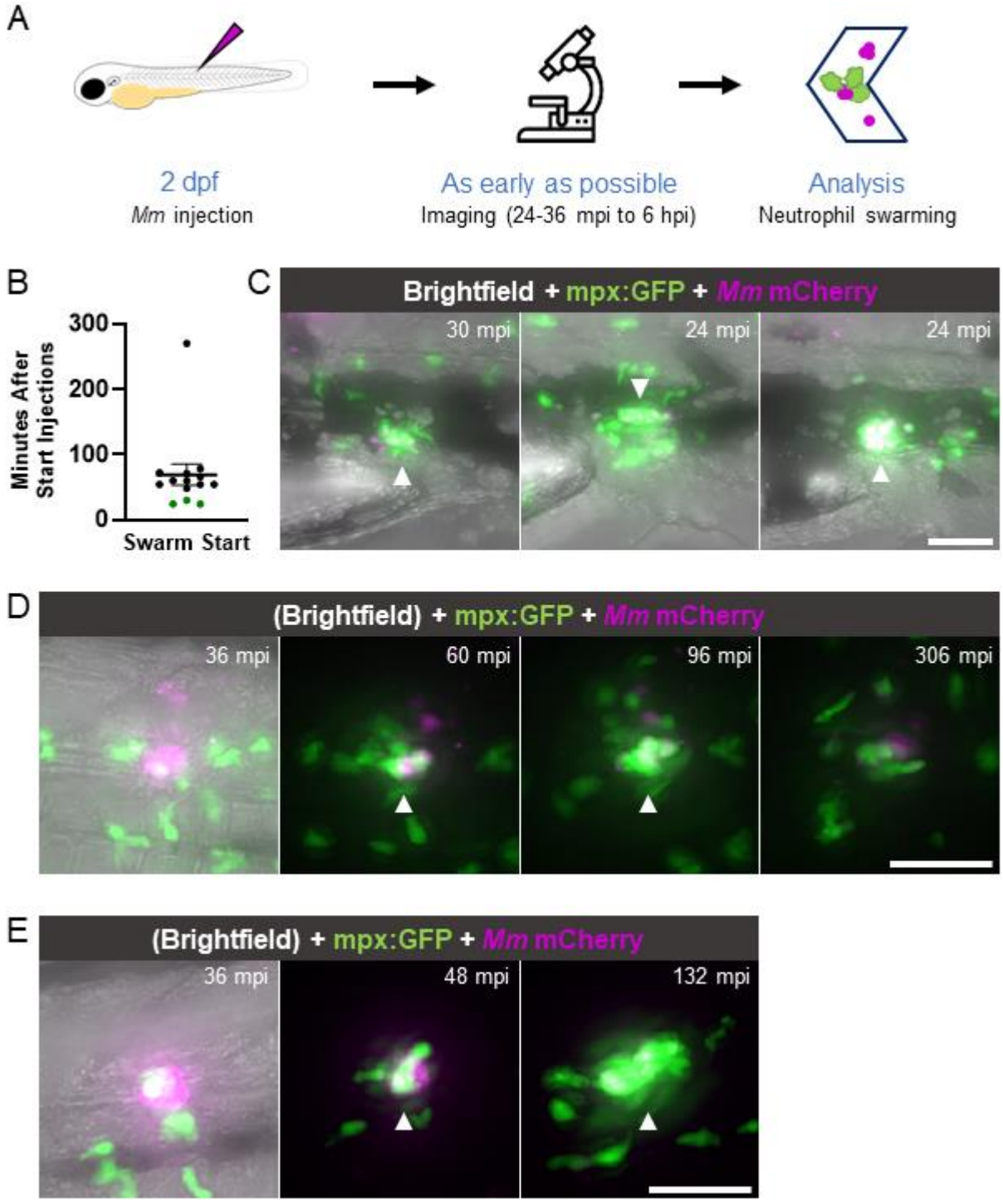


Figure 3.10 | Neutrophil swarming can be observed as early as 24 minutes post infection. A) Zebrafish were infected with *Mm* at 2 dpf and were imaged as soon as possible to be able to capture the onset of neutrophil swarming. B) The time points at which neutrophil swarms started after infection with *Mm*. In total, 14 swarms were captured in 32 infected fish from 3 independent experiments. Of these swarms, 3 were already present at the start imaging, indicated by green data points and visualised in B. D, E) Visualisation of neutrophil swarms over time. Panel 1: First frame of the time lapse. Panel 2: Start of the neutrophil

swarm. Panel 3: Peak of the swarm. Panel 4: The end of the swarm, if this occurred within the time lapse. Scale bars are 100 µm.

3.4.10. Neutrophil swarms resolve after 4 hours and 17 minutes after infection on average

Considering all swarms were already present at the start of imaging, the total duration that swarms were present could not be confirmed. However, all swarming resolution events, with the exception of 1, were captured within the 10-hour time lapses. Neutrophils were considered to have ended swarming once there were no longer 3 neutrophils in close contact with each other in the swarm area. Following infections at 2 dpf, fish were imaged for 10 hours at 1 hpi, and time lapse movies were analysed for neutrophil swarm resolution (Figure 3.11 A). Neutrophil swarm resolution occurred at 257 minutes after injections on average (Figure 3.11 B, C). This offers an explanation for the low frequency of neutrophil clustering observed in the aforementioned clustering experiment (Clustering Figure), as 8 out of 18 swarms dissipated before 4 hours (240 minutes). Out of all observed swarms, a single neutrophil swarm lasted for the entire duration of the time lapse (Figure 3.11 D)

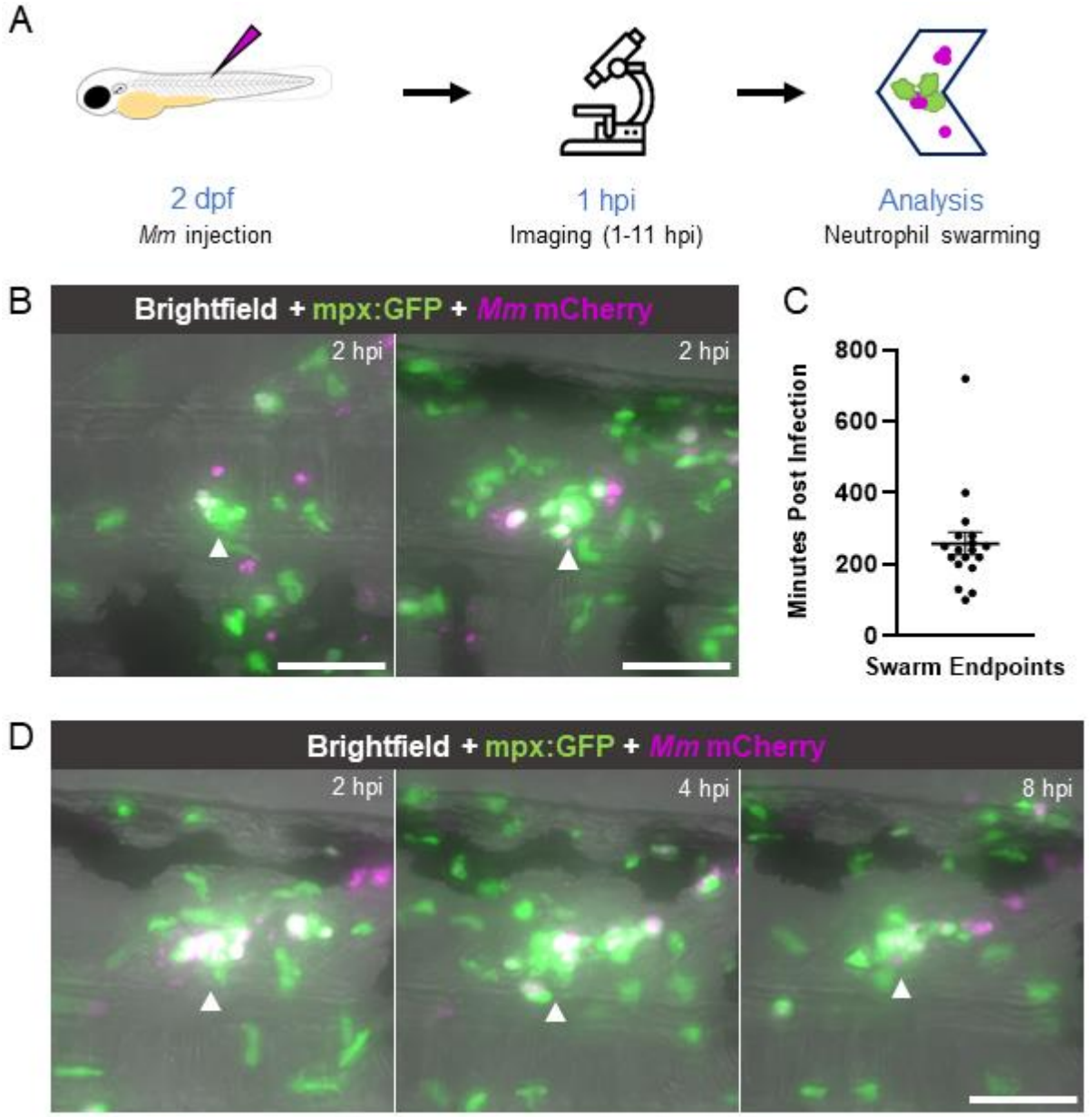


Figure 3.11 | Neutrophil swarms resolve at 257 minutes post infection on average. A) Zebrafish were infected with *Mm* into the somite at 2 dpf and were time-lapsed for 10 hours. Two experiments were performed, with the first commencing imaging at 2 hpi and the second starting imaging at 1 hpi. If a larva contained 2 swarms, the longest-lasting swarm was chosen for visualisation in these experiments. B) Examples of neutrophil swarms found during the time lapses. C) The time points at which neutrophil swarms dispersed, for a total of 18 swarms found in 39 fish. Neutrophil swarming events were considered to have ended once there were no longer 3 neutrophils in close contact with each other at the location of the swarm. On average, neutrophil swarming concluded at 257 minutes post infection. D) One infected larva showed a neutrophil swarm that was persistent throughout the time lapse from 2 hpi to 12 hpi. Scale bars are 100 µm.

3.4.11. Somites of infected zebrafish with swarms contain more neutrophils than those without

From the previous results it is evident that the bacteria initiate recruitment of neutrophils towards the somite, but it is unclear if neutrophil swarming plays a role in additional recruitment. Studies have shown that recruitment of more distant neutrophils takes part in the secondary response of swarming (Lämmermann *et* al, 2013). This is difficult to examine, as it would be unclear if swarming itself induces more recruitment of neutrophils, or if a higher number of neutrophils within the somite increases the likelihood of swarm formation. Despite this, comparing neutrophil numbers within the somite between fish with swarms and those without swarms could give an indication that this interaction is worth investigating further. Zebrafish were infected with *Mm* at 2 dpf, and time-lapses were generated for 10 hours as of 1 dpi (Figure 3.12 A). Time-lapse movies were analysed for neutrophil counts, and neutrophil numbers of fish with and without swarms were assessed at 190 minutes post infection, the point at which a majority of the neutrophil swarms started resolving (Figure 3.11 A). On average, 15 neutrophils were recruited towards the somite after infection with *Mm* (Figure 3.12 B). Fish with neutrophil swarms had significantly more neutrophils within their infected somite on average compared to in fish without swarms ($p = 0.0226$), at respectively 17 and 13 neutrophils on average (Figure 3.12 C). A different experiment will have to be performed to determine if neutrophil swarming initiates more recruitment towards the somite.

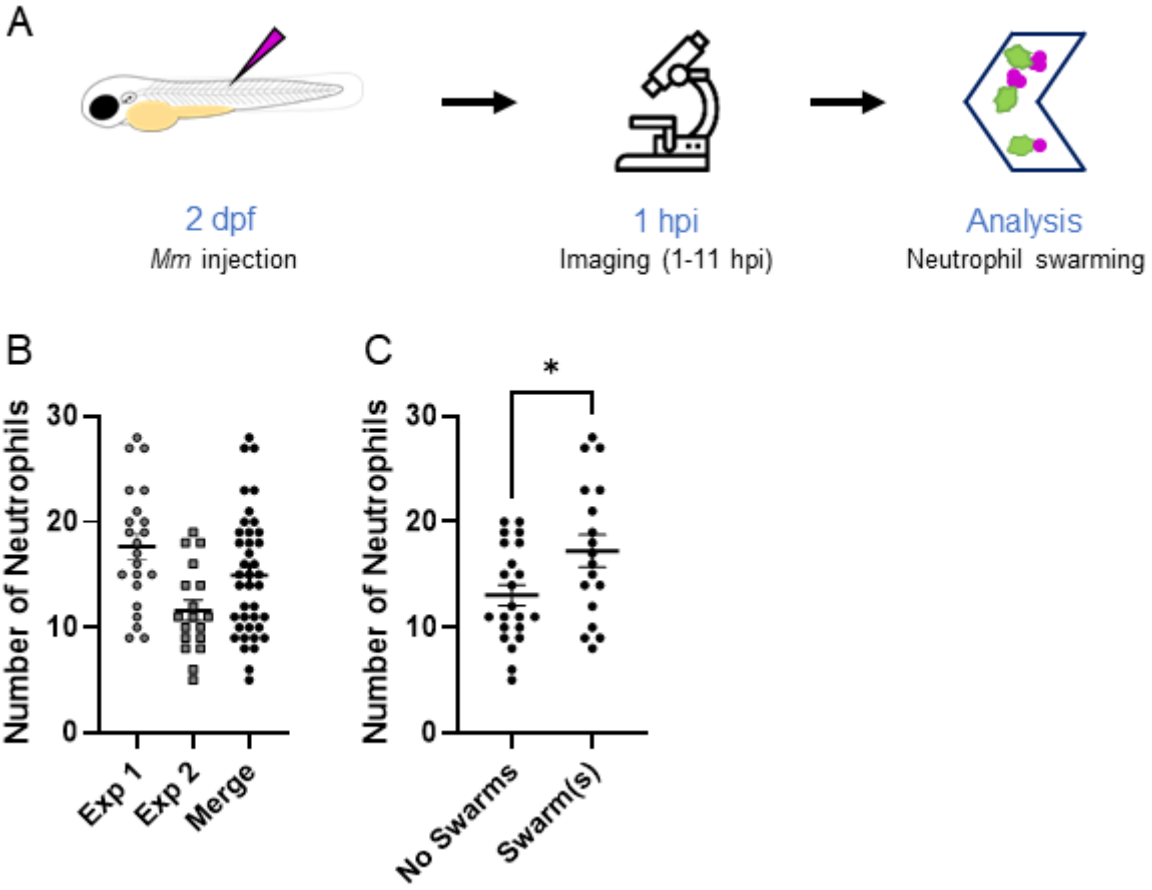


Figure 3.12 | Neutrophil numbers are increased in somites of fish with swarms. A) Zebrafish were infected with *Mm* at 2 dpf and neutrophil numbers within the somite were counted at 190 mpi. B) The number of neutrophils within the somite in both experiments. C) Neutrophil numbers in the somites of infected fish with swarms were significantly increased compared to neutrophil numbers in fish without swarms ($p = 0.0226$, unpaired t-test). Error bars are mean ± SEM. N = 22, 18 larvae, gathered over 2 experimental repeats.

3.4.12. Live *Mycobacterium marinum* is not required for neutrophil swarming to occur

The previous results have shown that *Mm* induces neutrophil swarming towards the site of infection, but it is unclear if the bacteria actively modulate neutrophil recruitment or if the immune system drives swarming. To examine the role of *Mm* in swarming, both *Mm* and heat-killed (HK) *Mm* were injected into the somite at 2 dpf and neutrophils were imaged over time as of 1 hpi. PVP injections were used as a negative control (Figure 3.13A). Both *Mm* and HK *Mm* induced a swarming response with stable swarms over time (Figure 3.13 B). There was no significant difference in swarming prevalence between *Mm* and HK *Mm* ($p = 0.9936$) (Figure 3.13 C). In comparison with PVP, there was a higher swarming prevalence in *Mm*-injected fish ($p = 0.0082$) and HK *Mm*-injected fish ($p = 0.0074$). There was no difference in the swarming endpoints between any of the groups (Figure 3.13 D). In contrast with previous experiments PVP injections also induced 3 swarms, lasting 30, 70, and 440 minutes within the timeframe of the time lapse. Considering such responses after PVP injections were not seen previously, this anomaly is likely a result disproportionately large injuries caused during infection. Occasionally, neutrophil clusters could be observed in the PVP group, but these often dispersed before they could be classified as swarms (Figure 3.13 E).

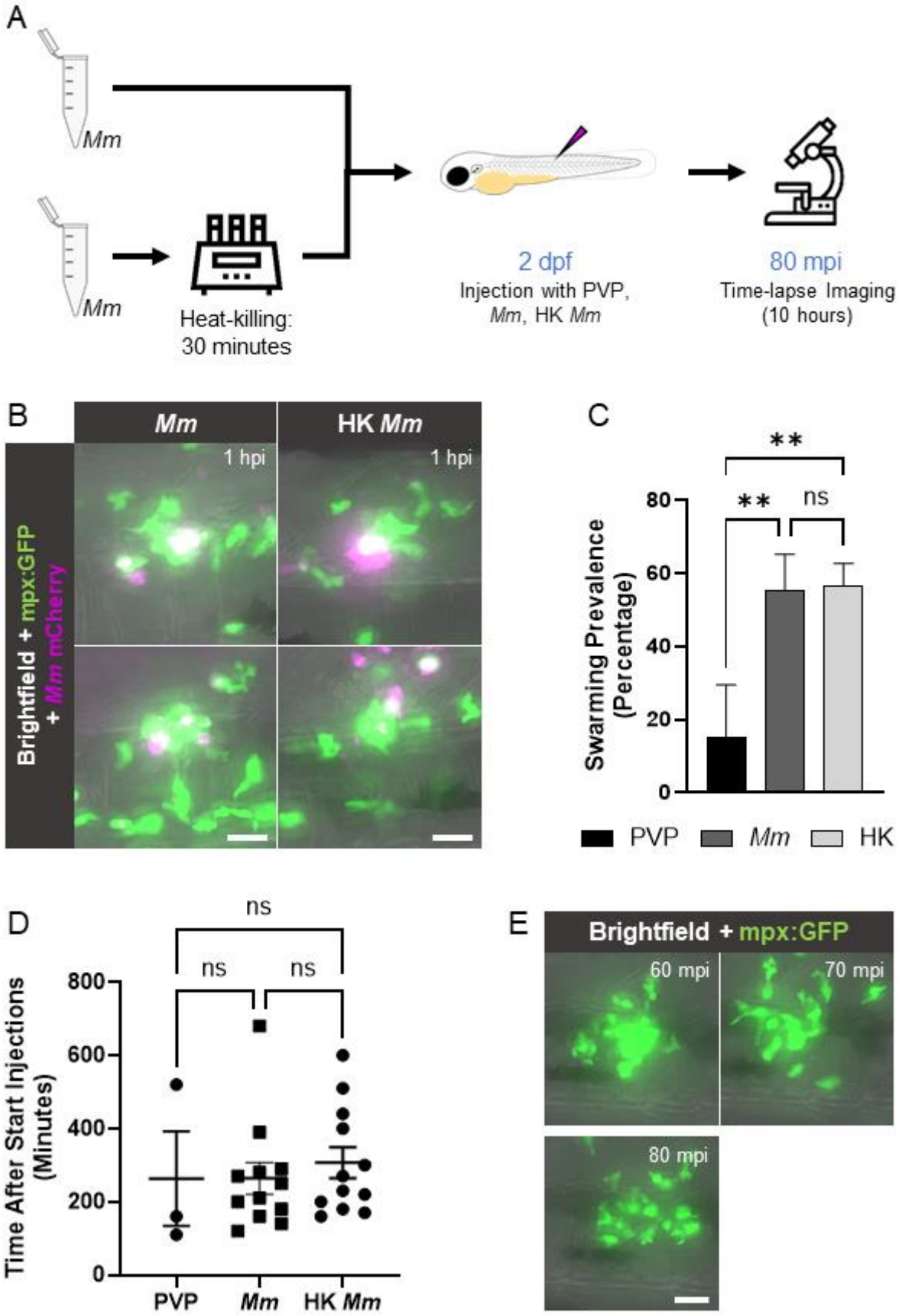


Figure 3.13 | Heat-killed Mycobacterium marinum initiates a swarming response similar to live bacteria. A) Experimental overview. Prior to infection, the *Mm* culture was split into 2

tubes. The bacteria in one of the tubes were heat-killed at 95 °C for 30 minutes. Afterwards, PVP, *Mm*, or HK *Mm* were injected into the somite at 2 dpf and time-lapse imaging was performed at 80 mpi for 4 hours. B) Examples of neutrophil swarms seen in the *Mm* and HK *Mm* groups. C) There was no difference in swarming prevalence between live *Mm* and HK *Mm* ($p = 0.9936$). PVP-injected larvae had significantly lower swarming prevalence compared to the *Mm* group ($p = 0.0082$) and to the HK *Mm* group ($p = 0.0074$). Statistics of swarming prevalence were performed on the percentage of fish with swarms using a multiple comparisons one-way ANOVA. D) Endpoints of neutrophil swarms in fish injected with PVP, *Mm*, or HK Mm. A Kruskal-Wallis test showed there was no significant difference between any of the groups. E) Example of a neutrophil cluster in the PVP group that dissipates quickly over time. Error bars represent mean ± SEM. N = 20-21 fish per group from 3 experimental repeats. Scale bars in all images are 50 µm.

3.4.13. Heat-killing *Mm* does not affect neutrophil recruitment towards the somite

To examine whether neutrophil recruitment is affected by heat-killing *Mm*, neutrophil numbers within the somite were evaluated over time (Figure 3.14 A). There was no significant difference between the 2 *Mm* groups over time ($p = 0.5481$) (Figure 3.14 B). In contrast, there was a significantly lower number of neutrophils present in the somite of PVP-injected fish compared to the *Mm* group ($p < 0.0001$) and the HK *Mm* group ($p = 0.0005$). Interestingly, the curve of the HK *Mm* group suggests the neutrophil response may have increased since the start of the time lapse until peaking at 200 minutes after starting injections, and then slowly decreasing. This pattern is not present in the *Mm* group.

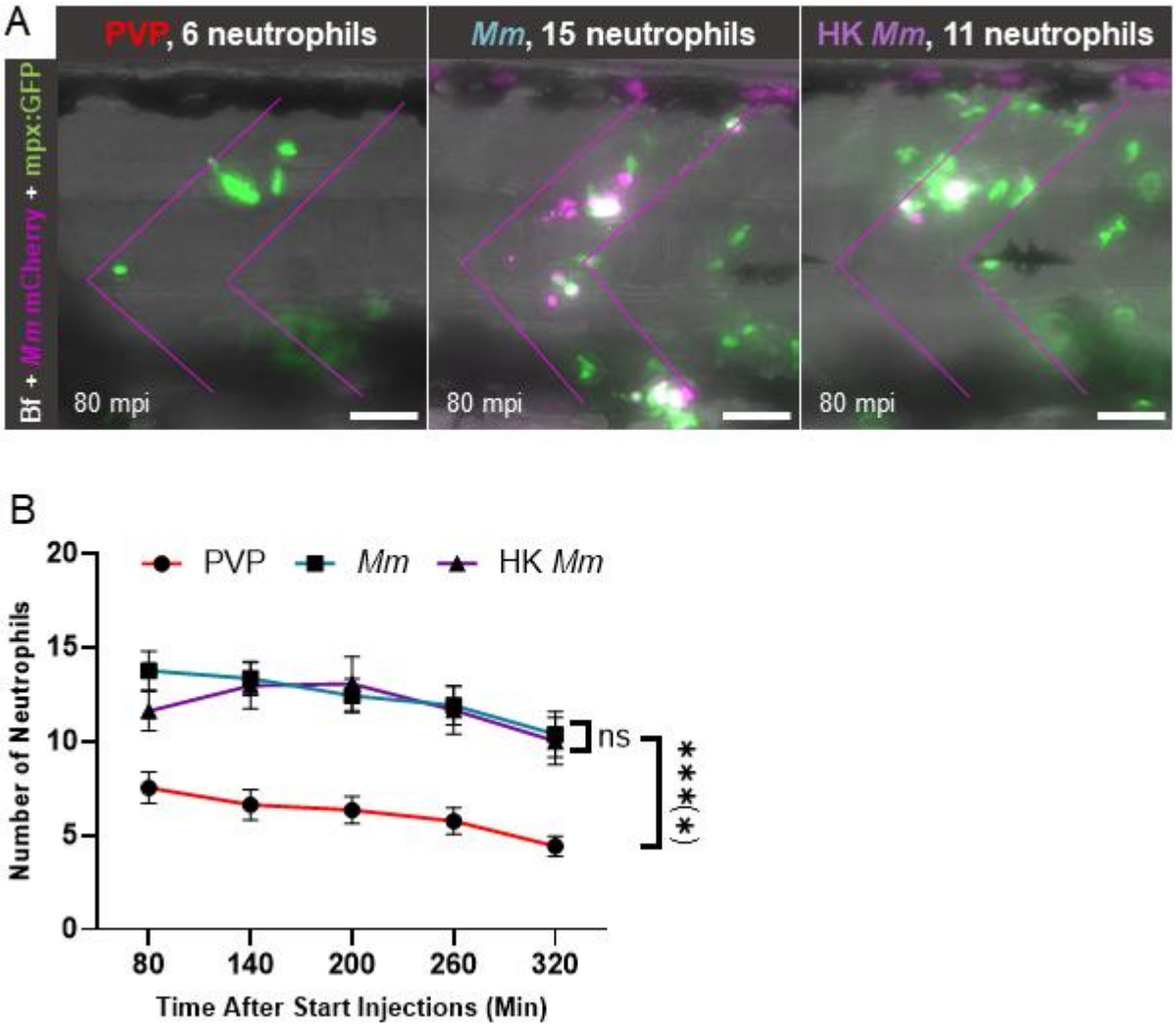


Figure 3.14 | Neutrophil numbers within the injected somite over time are not different between live or heat-killed *Mm*. Zebrafish were injected into the somite with PVP, *Mm*, and HK *Mm* at 2 dpf. Imaging started at 80 minutes post infection. A) Heat-killed bacteria still recruited neutrophils into the somite. All pictures were taken at the 80 mpi time point. In these images, PVP, *Mm*, and HK *Mm* injection recruited 6, 15, and 11 neutrophils towards the somite

respectively. B) Number of neutrophils in the injected somite per group over time. A repeated measures one-way ANOVA showed a significant difference in recruited neutrophils when comparing the PVP group to the *Mm* group ($p < 0.0001$) and the HK *Mm* group ($p = 0.0005$). There was no difference between the *Mm* group and the HK *Mm* group ($p = 0.5481$). Error bars represent mean ± SEM. N = 20-21 fish per group from 3 experimental repeats.

3.4.14. Neutrophils internalise *Mm* in both live *Mm* and heat-killed *Mm*

Phagocytosis of *Mm* is one of the actions neutrophils may employ against *Mm* infection, but zebrafish studies have shown varying phagocytic activity depending on method of infection (Belon et al 2014). To assess if phagocytosis possibly occurs in the somite infection models, time-lapses of fish infected with either live *Mm* or HK *Mm* were assessed on colocalisation of *Mm* mCherry signal with mpx:GFP signal (Figure 3.15). Simultaneous colocalised movement of both signals suggests internalisation of *Mm* by a neutrophil has taken place. Figure 3.15 A shows a neutrophil with internalised, live *Mm* (white arrowhead). Movement of the neutrophil over time is indicated by a green circle (original position) and a white arrow (direction). In this example, the neutrophil migrates through the somite over time, and eventually dies at 410 mpi. Figure 3.15 B shows a neutrophil with internalised HK *Mm* moving through the somite over time. At 400 mpi, the neutrophil appears to have died, leaving the bacteria behind (blue arrowhead). Interestingly, this colony of bacteria is later seen moving upwards, potentially due to being internalised by a macrophage. Bacteria were seen moving through the infected larvae without colocalisation of mpx:GFP in all analysed movies, 22 *Mm*-injected larvae and 21 HK *Mm*-injected larvae, again likely indicating uptake by macrophages. similarly, 22 *Mm*-injected larvae and 21 HK *Mm*-injected larvae, suspected internalisation of *Mm* by neutrophils could be observed at least once. In contrast with these observations, neutrophils with potentially internalised *Mm* and partaking in swarming near infections appear to meander near the site of infection (Figure 3.15 C). These results suggest that *Mm*, whether alive or heat-killed, is internalised by neutrophils and potentially macrophages, but further evidence is required to confirm the observed fluorescence colocalisation is indeed internalisation.

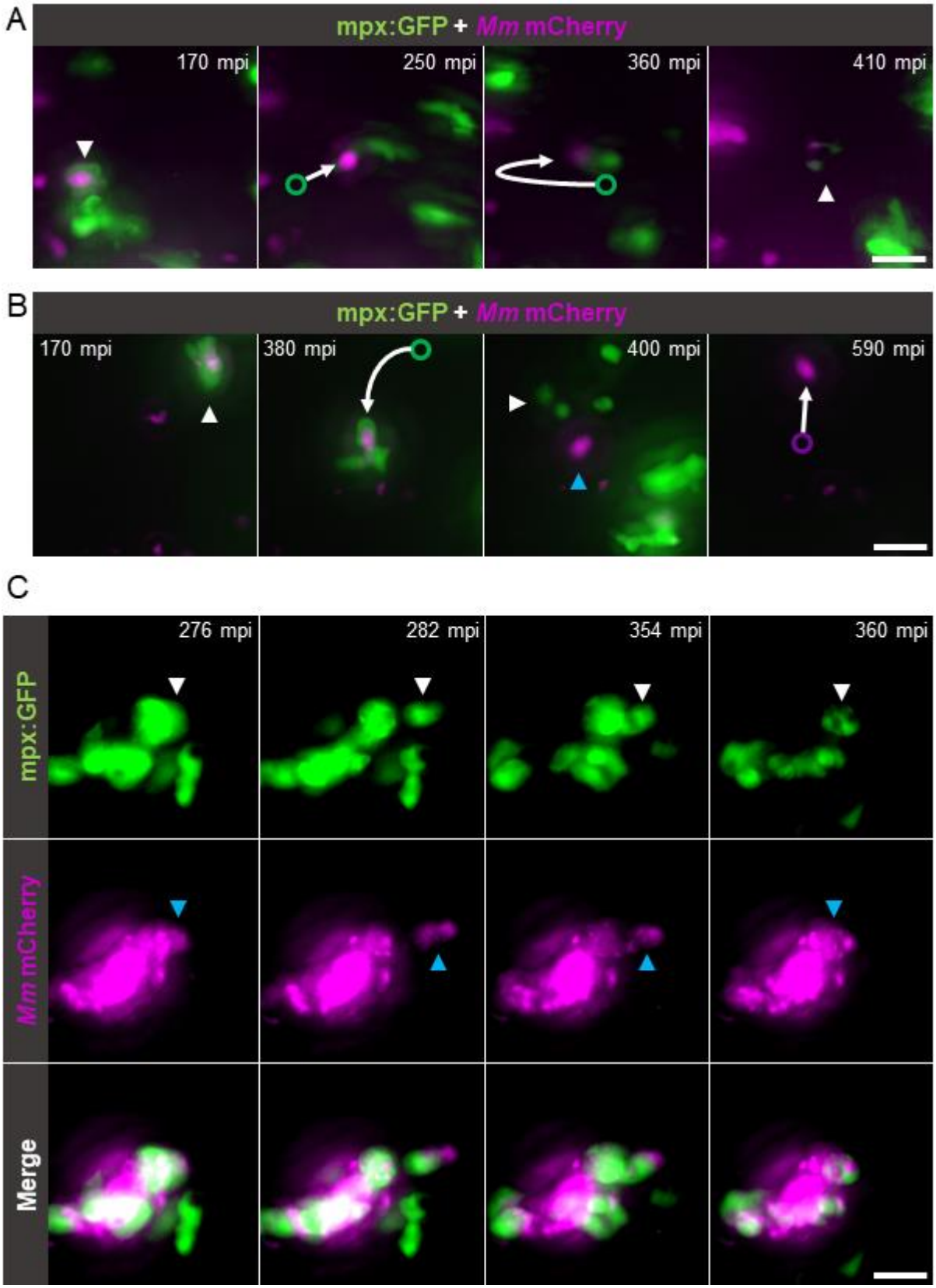


Figure 3.15 | Potential internalisation of (HK) *Mm* by neutrophils after somite infection. 22 *Mm*-injected and 21 HK *Mm*-injected time-lapse movies of zebrafish were analysed for simultaneous colocalised movement of bacteria and neutrophils to examine if these cells

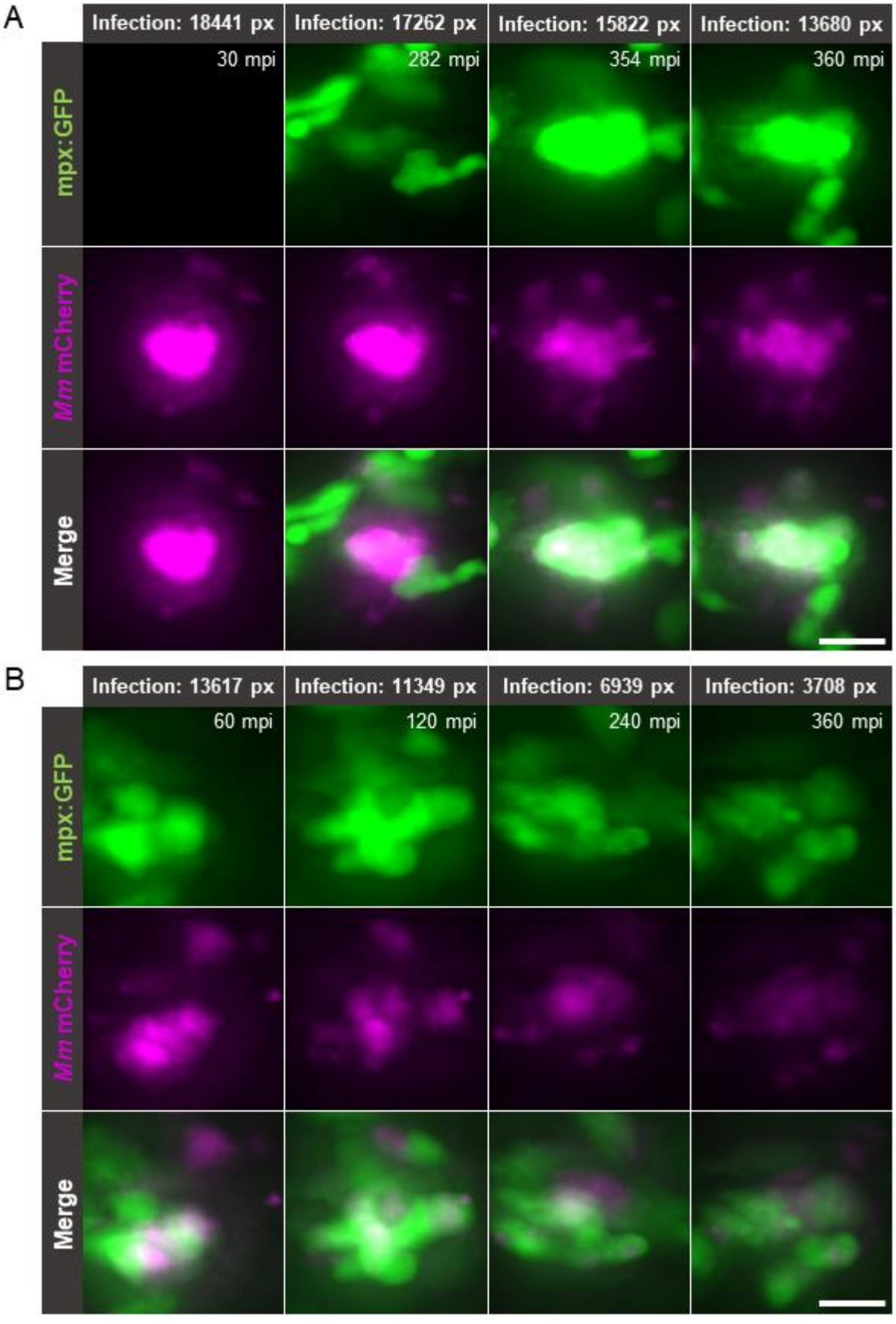
internalise *Mm*. A) A neutrophil (white arrowhead) is seen moving through the somite (green circle: starting position, white arrow: direction) over time, together with a live *Mm* colony. At 410 mpi the neutrophil appears to have died. B) The movement of a neutrophil with internalised HK *Mm* over time. At 400 mpi the cell appears to have died, leaving behind the HK *Mm* colony (blue arrowhead). This colony (purple circle: starting position) is then seen moving upward in the absence of a neutrophil fluorescent signal, potentially indicating internalisation by a macrophage. C) Neutrophils with internalised *Mm* in swarms appear to meander close to the neutrophil swarm and the accompanying site of infection. A neutrophil is seen partaking in the swarm and has exited the swarm by 282 mpi. This cell is then seen moving slightly left and right until it re-enters the area of infection at 360 mpi, where it was originally partaking in swarming. N = 22, 21 for the *Mm* group and *HK* group respectively, taken from 3 experimental repeats. Scale bars are 30 μm.

3.4.15. Neutrophil swarming does not appear to increase their effectiveness in clearing *Mm* infection.

Neutrophils have had a controversial role in fighting *Mm* infection, with reports of neutrophils both contributing to disease pathology (Lienard et al., 2023) and to clearance of the bacteria (Feng et al., 2006; Sugawara et al., 2004). Considering they can be seen internalising *Mm* both inside and outside of swarms, this suggests swarming neutrophils employ defensive mechanisms like phagocytosis during the swarming process. To assess whether neutrophil swarming affects bacterial burden, neutrophil swarms from experiments in Figure 3.10 were inspected for bacterial burden over time. In Figure 3.16 A, the bacteria at the depicted locus were in contact with up to 1 neutrophil for half the duration of 30 mpi until 282 mpi. At 282 mpi, 2 neutrophils entered the infection area and rapidly started swarming. This swarm remained stable over time, until the end of the time lapse. Between 30 mpi and 282 mpi (=252 minutes total), bacterial burden reduced by 6.4% (18441 px to 17262 px). Between 282 mpi and 360 mpi (=78 minutes total), bacterial burden reduced by 20.8% (17262 px to 13680 px), indicating the reduction in bacterial burden over time increased when neutrophils were actively swarming. In Figure 3.16 B, neutrophil swarming at the infection site started at 60 mpi and neutrophils were present there throughout the time lapse. The infection also appears to be cleared over time, indicating neutrophil swarming may play a host-protective role during early infection.

To further examine these observations, bacterial burden within the entire somite of fish without swarms and fish with swarms was quantified. For fish with swarms, bacterial burden was assessed at the first frame of neutrophil swarming and at the last frame of the time lapse, which was 6 hours long. For the group of non-swarmers, 54 mpi was chosen as the starting point from which to quantify bacterial burden. This is based on Figure 3.10 B, in which the average initiation of neutrophil swarming was 54 mpi after exclusion of the upper time point (270 mpi), which was considered to be an outlier. The final frame of the time lapse was also analysed for the non-swarmers. Bacterial burden at the initiation of swarming was not significantly different between fish without swarms and fish with swarms (1650 px vs 2459 px respectively, $p = 0.1017$) (Figure 3.16 C), indicating the amount of initial bacterial burden did not affect the likelihood of neutrophil swarming to occur. The reduction in burden was quantified from the initiation of swarming until the end of the time lapse (Figure 3.16 D). There was no significant difference in the percentile reduction of bacterial burden in fish without swarms compared to fish with swarms ($p = 0.4458$). Considering this analysis included the reduction in bacterial burden of the entire somite, an additional analysis was performed on a more specific group of swarms (Figure 3.16 E). Swarms included in this analysis were those that surrounded an isolated colony of bacteria, like in Figure 3.16 A. Neutrophils were present

at these infections until the end of the time lapses. On average, bacterial burden decreased on average ~60% in the presence of these swarms. This is similar to the levels of reduction seen in Figure 3.16 D, indicating that swarming may not potentiate neutrophil clearance of *Mm*. In short, bacterial burden reduces over time in the presence of neutrophils, but neutrophil swarming does not appear to increase this reduction.


A
Infection: 18441 px
Infection: 17262 px
Infection: 15822 px
Infection: 13680 px
30 mpi
282 mpi
354 mpi
360 mpi
mpx:GFP
Mm mCherry
Merge
B
Infection: 13617 px
Infection: 11349 px
Infection: 6939 px
Infection: 3708 px
60 mpi
120 mpi
240 mpi
360 mpi
mpx:GFP
Mm mCherry
Merge

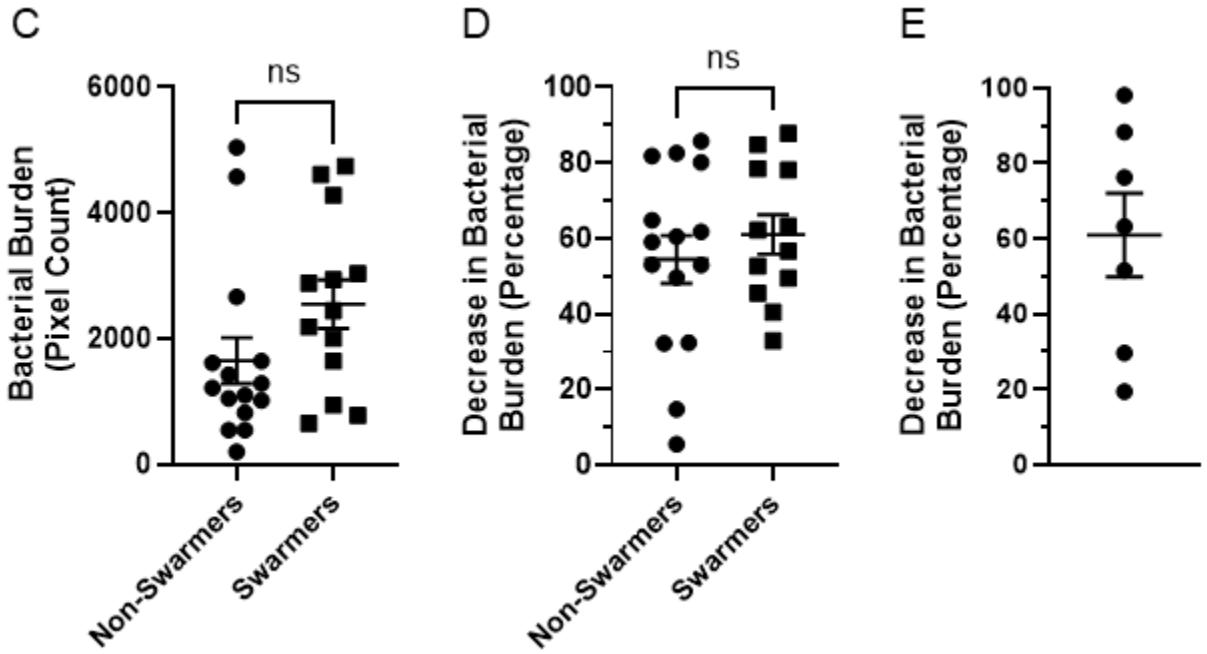


Figure 3.16 | Neutrophil swarming does not appear to potentiate bacterial clearance. The data used in this Figure originate from the experiment detailed in Figure 3.10. A) From 30 mpi until 282 mpi, neutrophils are rarely seen interacting with this bacterial infection. At 282 mpi, neutrophils started entering the infection area and eventually formed a swarm. Over the next 2 hours, the bacteria appear to be dispersing and are potentially being cleared by the neutrophils. Scale bars are 30 µm. B) A different infection where neutrophils start to swarm at 60 mpi. Neutrophils are present at this infection throughout the remainder of the time-lapse, and burden appears to be decreasing steadily over time. Scale bars are 30 µm. C) Bacterial burden of fish with and without swarms in Figure 3.10 B was analysed at the first frame of swarming. If a fish had no swarm, the beginning time point was set to 54 mpi, based on the average swarm starting time in Figure 3.10 B. The upper time point was excluded in this decision on the grounds of being an outlier. There was no significant difference in bacterial burden between these groups ($p = 0.1017$, unpaired t-test). N = 15 non-swarmers and 13 swarmers accumulated from 3 experimental repeats. D) Fish from Figure 3.10 were examined for bacterial burden reduction from the start of swarming until the end of the time lapse. There was no significant difference in the percentile decrease in bacterial burden within the entire somite between fish without swarms compared to fish with swarms ($p = 0.4458$, unpaired t-test). N = 15 non-swarmers and 13 swarmers over 3 experimental repeats. E) Stable neutrophil swarms that surrounded an isolated infection, like in A, were analysed for the reduction in bacterial burden over time. The decrease in burden was examined from the start of swarming until the end of the time lapse, at which point there were still neutrophils interacting with the infection. On average, the reduction in bacterial burden in these swarms amounted to 60%. N = 7 from experimental repeats. Error bars are mean ± SEM.

3.5. Discussion

Since an elaborate investigation in a mouse wounding model (Lämmermann et al., 2013), neutrophil swarming has emerged as an important mediator during infection with various diseases (Hopke et al., 2020; Kienle et al., 2021; Poplimont et al., 2020), yet much about this process in *Mm* infection remains unclear. Swarming in zebrafish has previously been shown to be conserved in zebrafish in an injury background (Isles et al., 2021; Poplimont et al., 2020), but neutrophils may respond differently to varying infections. This chapter explores the optimal methods and how to best visualise neutrophil swarming with the currently available tools in zebrafish, as well as characterising this behaviour in response to *Mm* with multiple parameters. This resulted in a localised infection model where *Mm* was injected into the somite close to the CHT. Injection with *Mm* was primarily responsible for neutrophil accumulation within the somite, and PVP mock injections rarely resulted in swarms. Neutrophil swarming was found to occur in around 50% of fish infected with *Mm*, and this response was similar to both live and HK *Mm*. This neutrophil behaviour was observed to occur as early as 24 minutes after starting infections, and swarms resolved close to 4 hpi on average. Additionally, a higher number of neutrophils was found in infected somites of fish with swarms compared to fish without swarms. Non-swarming and swarming neutrophils were seen internalising *Mm* and HK *Mm*, and neutrophil presence around bacteria reduced live *Mm* bacterial burden over time, but swarming did not appear affect the rate of this decrease. This marks the first in-depth investigation into neutrophil swarming in an *Mm* infection background, but the tools developed and provided may be applied to investigate other pathogens as well.

This thesis presents a localised infection model to study neutrophil behaviour in response to infection in zebrafish. Zebrafish are a popular model for studies that utilise microscopy as they possess multiple properties that make them suitable for imaging, like transparent bodies and the availability of transgenic lines (Stream & Madigan, 2022). The zebrafish has also been used to show neutrophils are host-protective during early, systemic *Mm* infection after stabilisation of one of the hypoxia pathway modulators (Elks et al., 2013). This brought forth a clear avenue for manipulation of neutrophil behaviour and, therefore, neutrophil swarming in response to *Mm* infection, which will be further discussed in Chapter 4. I used a transgenic line that labels myeloperoxidase in neutrophils with GFP (Renshaw et al., 2006), to adequately allow for visualisation of neutrophils with live imaging. Fluorescent *Mm*, labelled with mCherry in this thesis, has frequently been used in the Elks lab to visualise *Mm* infections and can be paired with the transgenic neutrophil line (Elks et al., 2013).

Using these tools, it was determined that a 20x objective with cycle times, the amount of time between each subsequent image per fish, of 10 minutes would be optimal in the somite infection model. This higher magnification, as opposed to a 2.5x or 10x objective, was needed to be able to distinguish individual neutrophils from each other. Objective magnifications of 10x and up to 40x have also been used in previous zebrafish swarming studies (Isles et al., 2021; Poplimont et al., 2020). In contrast, a ventral fin wound used in one of these studies introduces a much smaller area in which swarms may occur (Poplimont et al., 2020) compared to the somite infection model, meaning higher magnifications are easier to implement. Additionally, the fin tissues where swarming occurs in these models (Isles et al., 2021; Poplimont et al., 2020) is considerably thinner than the somite, meaning the cells have less space to move within the z-plane. The somite allows for more movement of neutrophils within the z-plane, meaning a higher magnification makes it easier to distinguish individual cells from each other based on morphological changes. However, a 20x objective magnification is the highest magnification at which the somite can be observed in full, which is a necessary requirement for many of the parameters in this thesis. In short, this explains the disparity in methodological approach for imaging of swarming in the somite infection model compared to existing swarming literature.

In order to first test the acquisition method and familiarise with the practical proceedings, neutrophil clustering was examined at a time point based on previous literature. In a collaboration between the Renshaw and Elks lab, tailfin amputation introduced neutrophil swarms with a peak in size at 4 hours post injury (Isles et al., 2021). This time point was applied to the somite infection model to investigate clustering as a starting point. This resulted in a neutrophil clustering prevalence of less than 15%. If, hypothetically, clustering prevalence is similar in both infection and injury, close to 50% of larvae should have shown clustering events like in the study of Isles and colleagues (Isles et al., 2021). Further experimentation showed that neutrophil swarms in the somite infection model form as early as 24 minutes after starting infections, and most swarms dissipated before 4 hpi. This discrepancy in the expected peak of swarming between the infection and injury models may stem from differences in neutrophil responses to infection and injury, and the distance in location of the site of challenge from the CHT (Coombs et al., 2019).

An example to illustrate possible effects of injury versus infection on neutrophil recruitment is a study that investigated recruitment to a cutaneous wound in mice, which showed that infection of the wound with *Staphylococcus aureus* increased recruitment by 100% while increasing the systemic neutrophil count by 250% (Kim et al., 2008). Another study in zebrafish found that addition of a *Pseudomonas aeruginosa* infection to a laser wound in the ventral fin

did not alter the neutrophil swarming response compared to the response in presence of the wound only (Poplimont et al., 2020). While both studies imply that there is no additional effect of the infection on neutrophil recruitment, the former did introduce a significant increase in the number of circulating neutrophils at 2 dpi. This is unlikely to be found in the zebrafish study or this thesis, as recruitment was assessed shortly after infection. Neutrophils also have distinct subsets that behave differently between infection or immunity (Liew & Kubes, 2019), and may therefore have influenced when the peak of swarming was observed. In contrast with the uncertainty surrounding neutrophils in infection versus wounding, previous work in zebrafish has clearly shown that the location of wounding plays a role in determining when neutrophils can be found at the site of injury (Bader et al., 2021; Coombs et al., 2019; Ellett et al., 2015; Kaveh et al., 2020; Petrie et al., 2014; Poplimont et al., 2020; Ren et al., 2017). Therefore, the location of the somite infection used, which is close to the CHT, is likely to be at least partially responsible for the deviation in the observed time of clustering compared to the expectation, whereas the influence of *Mm* infection versus injury on recruitment remains unclear.

Neutrophil accumulation in the somite at 4 hpi was found to have a statistically weak correlation with the amount of bacterial burden within the somite in a clustering experiment. Later, fish with swarms had close to 50% more bacterial burden around the time of swarm onset compared to fish without swarms, but this was not significant. This suggests that bigger infections may be more likely attract more neutrophils towards the somite and that they may be more likely to cause swarming events. An infection introduced by needle injury will likely feature danger signals that are both DAMPs and PAMPs, which contribute to the recruitment of neutrophils (de Oliveira et al., 2016). In inflammation, larger wounds correlate with an increase in swarm size and duration (Lämmermann, 2016). Additionally, knockout of the swarming mediator LTB4 significantly reduced neutrophil recruitment of distant neutrophils towards the site of injury, a phenotype that was exacerbated with smaller wounds (Lämmermann et al., 2013). It stands to reason that a bigger infection would elicit bigger signalling events and would therefore recruit more neutrophils. Moreover, zymosan particles only initiated neutrophil swarming on a microarray with human neutrophils after reaching a certain size (Reátegui et al., 2017), indicating a certain neutrophil number threshold needs to be reached in order for swarming to occur. Together, this implies that bigger infections can cause influx of additional neutrophils towards the site of infection compared to smaller infections, and that this, in turn, results in an increased likelihood of swarming to occur. However, further research will need to be conducted to examine if this is definitively the case in localised *Mm* infection.

Neutrophil swarming was defined as 3 or more neutrophils in close contact with each other for at least 30 minutes. While arbitrary, these cut-offs were chosen based on a number of reasons. Zebrafish larvae contain significantly fewer neutrophils throughout the body than mice, at around 115 cells per larva at 2 dpf (Isles et al., 2019). In contrast, neutrophil swarms in mice have been described to contain up to 300 neutrophils (Brown & Yipp, 2023). Visualisation of neutrophil behaviours in mice is often done by injection of reporter neutrophils into areas of interest such as the ear dermis, as described in a mouse wounding model used to examine swarming (Lämmermann et al., 2013). This study did not report the number of neutrophils found in swarms specifically, but these swarms were visually considerably larger than swarms reported in zebrafish (Isles et al., 2021; Lämmermann et al., 2013; Poplimont et al., 2020). Neutrophil swarms at mouse *Pseudomonas aeruginosa* footpad infections were reported to have a diameter of slightly below 60 µm on average (Lämmermann et al., 2013), which converts to slightly above 2800 μm^2. In zebrafish, cluster sizes at a ventral fin wound infected with *P. aeruginosa* did not reach 1200 μm^2 (Poplimont et al., 2020), and swarms areas towards a tailfin wound were not reported to be higher than 800 μm^2 (Isles et al., 2021). Both these reports in zebrafish show that swarms are considerably smaller than reported in the mouse study. Due to the discrepancy in neutrophil numbers between mice and zebrafish, the number of neutrophils required to be considered for swarming in the somite model was set at a balance of being low enough to not undervalue neutrophil swarms, and high enough to decrease the likelihood of cells being close to each other by chance. Together, a minimum of neutrophil counts and time spent swarming significantly decreases the likelihood of the observed behaviour being the result of chance, rather than a coordinated response by the neutrophils.

In the somite infection model, neutrophil swarming occurred in about 50% of fish, initiated as early as 24 minutes after the start of injections, and resolved at 257 mpi on average. Neutrophil swarm prevalence does not appear to be discussed often in literature. The experiments in these studies may be designed to avoid non-swarmers by, for example, injection of neutrophils near the site of challenge in mice (Lämmermann et al., 2013), the use of strong neutrophil recruiters like zymosan on microarrays (Reátegui et al., 2017), the addition of a large number of neutrophils to microarrays (500.000 in this example) (Hopke et al., 2020), or by introducing a laser wound directly next to the CHT in zebrafish (Poplimont et al., 2020). Nevertheless, a zebrafish tailfin injury model described swarming to be present in around 50% of injured fish. The initiation and duration of swarming is also highly dependent on the model and methodology. The mouse ear dermis injury model reported the initial neutrophil recruitment phase to take place between 1 and 15 minutes after laser wounding, but did not describe swarm resolution (Lämmermann et al., 2013). On microarrays, neutrophil swarming in response to zymosan initiated within a few minutes, and was stable for at least 2.5 hours

(Reátegui et al., 2017). Microarrays with Zymosan induced rapid neutrophil influx and swarming to *Candida albicans* within 30 minutes, and these swarms were stable for over 12 hours (Hopke et al., 2020). In zebrafish, a ventral fin laser wound induced stable neutrophil clusters by 20 mpw, but resolution was not assessed (Poplimont et al., 2020). Finally, a zebrafish tailfin injury model reported swarms to initiate from 40 mpw, which had to potential to remain stable until at least 4 hpw (Isles et al., 2021). In short, all these models show vast differences in swarming behaviour and therefore do not necessarily lend themselves well to comparison. However, characterisation of swarming in each of these models, including the somite infection model, lays the foundation for future examination into the neutrophil swarming response.

In the somite infection model, neutrophil accumulation in the infected somite was significantly increased at 190 mpi in larvae that showed swarming compared to those that did not. It is difficult to discern whether this is a result of increased signalling originating from the neutrophil swarms themselves, or if higher neutrophil counts coincide with an increased likelihood of swarm formation, or a combination of both. After an immune challenge, neutrophils close to the site of challenge may respond to signals released from the affected tissue (Lämmermann, 2016). A relatively high availability of neutrophils within this small, affected area may therefore increase the likelihood of swarm formation and subsequent neutrophil recruitment. For instance, in a mouse ear dermis injury model, neutrophil clusters at the wound site did not proceed to form stable swarms over time unless a certain threshold in neutrophil number within the cluster was reached (Park, Choe, Park, et al., 2018). Failure to reach this threshold resulted in migration away from the site of injury, while numbers above the threshold initiated recruitment of more neutrophils towards the swarm (Park, Choe, Park, et al., 2018). Conversely, swarming neutrophils produce LTB4 waves in order to recruit more neutrophils towards the swarm, but a self-extinguishing wave mediated by an NADPH-oxidase-based negative feedback mechanism may attenuate this process (Strickland et al., 2024). Similarly, knockout of GPCR kinase (GRK) 2 in neutrophils in of a skin injury mouse model resulted in improved aggregation and swarm-like behaviour compared to controls, indicating GRK2 plays a role in self-limiting of swarming (Kienle et al., 2021). These mechanisms of self-limitation may already have been initiated at the time of neutrophil counting, as this was done close to time window at which swarms generally started dissipating. Taken these mechanics together, it is likely that both neutrophil swarming and initial neutrophil numbers contribute to the observed higher neutrophil count in the somites of fish with swarms.

Presence of additional neutrophils in fish with swarms also introduces the question if swarming contributes to bacterial clearance. Neutrophil swarming has been described to contribute to

clearance of *Candida albicans* (Hopke et al., 2020), *Pseudomonas aeruginosa* (Kienle et al., 2021; Poplimont et al., 2020). In the somite infection model, the presence of neutrophils near infections resulted in a stable reduction of *Mm* bacterial burden over time, but swarming did not appear to increase this reduction. The initial neutrophils responding to- and swarming at a tailfin wound in zebrafish have been shown to release NET-like structures, and inhibition of NE and MPX, which are swarming components, reduced swarming prevalence (Isles et al., 2021). This suggests NET release during neutrophil swarming contributes to bacterial clearance directly, as well as through recruitment of Neutrophils to the site of challenge. Neutrophils release NETs in response to *Mtb*, but are unsuccessful in killing *Mtb*, which employs several pathways that aid in evading this killing mechanisms (Cavalcante-Silva et al., 2023; Chandra et al., 2022a). However, internalisation of these NETs by macrophages has been shown to aid these cells in internal killing of *Mycobacterium bovis* (Stephan et al., 2016), but it is unclear if this occurs in *Mm* infection. Further research will have to be conducted to examine the involvement of NETs in recruitment and swarming to *Mm* infection.

Phagocytosis is another defence mechanism of neutrophils against pathogens (Alcantara et al., 2023) that may be responsible for the observed reduction in bacterial burden over time. However, its effectiveness in response to *Mtb* infection is controversial (Gaffney et al., 2022). Following *Mm* infection in the somite model, neutrophils were seen to have internalised *Mm*, possibly indicating neutrophils employed phagocytosis. Similarly, neutrophils with internalised *Mm* were seen at 2 hpi after a tailfin injection (Szkuta, 2020), and were also seen within 24 hours in systemic infections (Elks et al., 2013). However, multiple studies have reported a distinct lack of *Mm* phagocytosis by neutrophils in early stages of systemic infections (Clay et al., 2007; Kenyon et al., 2017; C.-T. Yang et al., 2012). Only after phagocytosis of dying, infected macrophages by neutrophils were neutrophils seen internalizing *Mm* (C.-T. Yang et al., 2012). Another study showed both initial phagocytosis of *Mm* and efferocytosis of infected leukocytes by neutrophils after tailfin injection with *Mm* (Hosseini et al., 2016). Whether phagocytosis of *Mm* by neutrophils is host-beneficial remains disputed. However, neutrophils were able to rapidly kill internalised *Mm* after efferocytosis of infected macrophages through NADPH oxidase-dependent mechanisms (C.-T. Yang et al., 2012). Overall, it is unclear whether internalisation of *Mm* by neutrophils seen after somite infection is from direct phagocytosis of *Mm*, or if it is the result of efferocytosis of infected macrophages, and whether this contributes to the clearance of *Mm*.

A drawback of the somite infection model is the requirement for long imaging cycle times during time lapses due to the number of fish per experiment and the long exposure time of, particularly, *Mm* mCherry. This meant different types of swarms could not be adequately

distinguished. A relatively long cycle time introduces the possibility of neutrophil swarms forming and dissipating multiple times between each image. These transient swarms are smaller than persistent swarms, and likely disperse due to a lack of neutrophil-derived, pro-swarming signalling and recognition of other attractant signals (Lämmermann, 2016). Transient swarms were observed in response to tailfin transection in zebrafish (Isles et al., 2021), and it is possible they exist in the somite swarming model when, for instance, small swarms disband upon recognition of signals derived from other loci of *Mm* infection. However, due to the time required to image each fish, it was difficult to justify lowering the cycle time any further than the 10 minutes maintained throughout most of the experiments, as this would drastically reduce the number of fish that could be imaged and, thereby, would substantially decrease statistical power.

Other imaging techniques or improvements to available equipment may speed up the imaging process and could reduce cycle time or increase the sample size per experiment. The Wolfson Light Microscopy Facility at the University of Sheffield now possesses a spinning disk confocal microscope, which could be used instead of the laser scanning confocal microscope used in this thesis. This microscope could be used to decrease cycle times, as spinning disk microscopy has higher acquisition speed than laser scanning microscopy. It also brings other benefits, such as reduced photobleaching and increased resolution (Lam et al., 2014). Additionally, the spinning disk microscope can be used to generate three-dimensional images of the neutrophil swarms (Ahmadian et al., 2024), which would more accurately represent the morphology of these swarms than two-dimensional images could. However, the Elks Lab houses its own confocal microscope that is not limited in its availability and does not require additional payment for use, unlike the spinning disk microscope. The limitations of the somite infection model with the current equipment were not known beforehand, and only became to light after extensive use of this microscope. One of the reasons for continuation with this microscope was to not introduce variation by changing the image acquisition method. Furthermore, the Elks confocal microscope was adequate for the experiments performed in this thesis. However, certain experiments, such as identification of transient swarms, may necessitate a change in equipment, which should be considered in the experimental design.

Analysis of the time lapses of neutrophil swarming is very time-consuming, and specialised software, like cell counters, could significantly decrease manual labour involved in the analyses. There are many options for automated cell counting available, and new alternatives to existing options are constantly being investigated. ImageJ offers a variety of free tools through downloadable plugins that could support cell counting. These plugins generally use thresholding of pixel intensity to distinguish individual cells from each other (Beretta et al.,

2023; Handala et al., 2019; O'Brien et al., 2016). With swarms this might present an issue, as the cells that make up the swarm tend to blend together with high pixel brightness. Part of this effect may be offset by lowering the laser power or exposure time, but this would in turn decrease visibility of dimmer cells and push their pixel intensity value below the threshold. There is also variation in fluorescence intensity of neutrophils between fish, resulting in the same problem. Over the years, these difficulties with segmentation of overlapping cells has continued to be investigated, with new systems such as Cellpose and CellSAM offering improvements to segmentation accuracy (Han et al., 2023; Israel et al., 2024; Pachitariu & Stringer, 2022; Stringer et al., 2021). Unfortunately, automated three-dimensional cell count quantification methods are even further behind than two-dimensional options (Y. Guo et al., 2022). Recent efforts, such as the segmentation algorithm CellSNAP, have made improvements to available cell segmentation methods, but still struggle accurately distinguishing clumped-up cells from each other (Raj et al., 2023). In the future, a combined effort of improved imaging acquisition methods with better segmentation algorithms may provide trust-worthy automated options for cell counting in swarms.

To conclude, this chapter describes a new model to study neutrophil swarming dynamics in response to infection. Using live-imaging, neutrophil behaviours within the somite can be followed in real-time. This allowed for characterisation of the swarms observed in response to *Mm* infection, which has not been previously described *in vivo* in literature. While only used in conjunction with *Mm* infection in this thesis, this model may potentially be used in other diseases as well. The zebrafish toolbox comprises many possible approaches to altering gene expression and visualisation of cells and bacteria, which could be applied in the somite infection model to study genetic or pathogenic implications on the neutrophil swarming process.

4. Neutrophil swarming in zebrafish is partially dependent on *lta4h* signalling and can be modulated through knockdown of swarming mediators

4.1. Introduction

Neutrophil swarming is a complex behaviour that may involve many different regulators, but the full molecular mechanisms underpinning swarming remain unclear (Lämmermann, 2016). This thesis chapter explores a variety of potential swarming mediators extrapolated from existing literature and investigates the effect of their modulation on the swarming response to localised *Mm* infection.

4.1.1. LTB4

One of the most well-described swarming mediators is leukotriene B4 (LTB4), which has been shown to be particularly important in the secondary response during neutrophil swarming in mice, where distant neutrophils are recruited towards the swarm (Lämmermann et al., 2013). During swarming, neutrophils are the main producer of LTB4, as *Alox5* knockout neutrophils, which are deficient in LTB4 production, significantly abrogates recruitment of distant neutrophils towards the swarm (Lämmermann et al., 2013). LTB4 signalling is also involved in different processes of the swarming response. The eventual exclusion of *Ltb4r1* neutrophils from competitive swarms with wildtype neutrophils indicates this leukotriene plays a role in neutrophil aggregation during swarming as well (Lämmermann et al., 2013). Additionally, GPCR desensitisation during swarming is important for swarming neutrophil arrest at sites of infection and improves bacterial clearance (Kienle et al., 2021). This limits swarm size and prevents neutrophils from responding to persistent recruitment signals, including LTB4, and suggests attenuation of LTB4 signalling may be important during swarm resolution (Kienle et al., 2021). LTB4 signalling has since been confirmed to be involved in the neutrophil swarming response in zebrafish as well (Isles et al., 2021; Poplimont et al., 2020). Activation of LTB4 signalling was shown to occur in clustering neutrophils at a ventral fin wound site (Poplimont et al., 2020), and late-stage neutrophil recruitment to a tailfin transection was significantly impaired in zebrafish with knockdown of leukotriene A4 hydrolase (*lta4h)*, an LTB4-generating enzyme (Isles et al., 2021). Finally, LTB4 has been described as both host-protective (Peres et al., 2007) and host-destructive (Bafica et al., 2005; Sorgi et al., 2020) during *Mtb* infection. Investigation of LTB4 in the somite infection model may therefore further understanding of its functions in neutrophil swarming and its role in *Mm* infection.

4.1.2. CXCR2

The CXC chemokine receptor 2 (CXCR2) is one of the major chemokine receptors expressed by neutrophils, and is one of the main receptors involved in neutrophil trafficking (Metzemaekers et al., 2020). CXCR2 can also activate the pro-inflammatory NF-κB pathway, which results in the production and release of additional neutrophil-recruiting cytokines (Capucetti et al., 2020). Binding of chemokines to the CXCR2 receptor on neutrophils activates the cell, which is required for subsequent extravasation out of the bloodstream into damaged or infected tissue (Kolaczkowska & Kubes, 2013). In swarming, CXCR2 is involved in recruitment of neutrophils, but is not the sole driving force behind this process (Lämmermann et al., 2013), and instead works in tandem with receptors such as LTB4R and CXCR1 (Reátegui et al., 2017). Furthermore, knockout of *cxcr2* was shown to significantly decrease neutrophil aggregation during swarming (Lämmermann et al., 2013). Similarly, inhibition of CXCR2 chemokine CXCL1 significantly abrogated neutrophil aggregation to fat-associated lymphoid clusters (Jackson-Jones et al., 2020). In TB, CXCR2 and its ligands are a key component of the response to infection (Slight & Khader, 2013). Inhibition of CXCR2 was shown to reduce neutrophil-mediated lethal inflammation in mice with a specific polymorphism that increases susceptibility to *Mtb* infection (Dorhoi et al., 2013). However, mice do not have a homolog for one of the most important CXCR2-specific cytokines called CXCL8 (Slight & Khader, 2013), although human CXCL8 is able to activate mouse CXCR2 (Del Prete et al., 2017). In contrast, CXCL8 and CXCR2 are both conserved in zebrafish (van der Vaart et al., 2012). CXCR2 is therefore an attractive target for modulation in the somite infection model.

4.1.3. CXCR4

Signalling involving the CXC chemokine receptor 4 (CXCR4) and its ligand cxc motif chemokine ligand 12 (CXCL12) play a key role in retaining neutrophils in the bone marrow (De Filippo & Rankin, 2018). There is also growing evidence of CXCR4 being responsible for neutrophil clearance, but this has only been substantiated in mouse models (De Filippo & Rankin, 2018). The CXCL12/CXCR4 signalling axis was also found to retain neutrophils at the site of inflammation in zebrafish (Isles et al., 2019), possibly implicating it in the neutrophil swarming process. Here, pharmacological inhibition of CXCR4 signalling with AMD3100 reduced neutrophil numbers at the site of inflammation during inflammation resolution (Isles et al., 2019). CXCR4 expression has been shown to be increased in patients with TB, particularly on macrophages, and increased susceptibility to HIV during TB (Hoshino et al., 2004). CXCR4 was also reported to promote granuloma formation through angiogenesis in zebrafish, but this was neutrophil-independent (Torraca et al., 2017). During early *Mm* infection, CXCR4 may instead contribute to neutrophil swarming by increasing neutrophil retention.

4.1.4. NETosis

A previous study involving the Elks lab implicates NETosis as a potential player in neutrophil swarm formation, as pioneer neutrophils were found to release NET-like structures (Isles et al., 2021). Inhibition of NET component NE significantly reduced swarming prevalence in response to a tailfin wound (Isles et al., 2021). Such a reduction was also seen after inhibiting NET-formation with the drug LDC7559 (Isles et al., 2021), which was thought to inhibit NETosis dependent on the NADPH oxidase NOX2 through gasdermin D (Amara et al., 2021). However, more recently, this drug was found to inhibit NOX2-dependent NETosis independently from gasdermin D, and instead acts through inhibition of the glycolytic enzyme phosphofructokinase-1 liver type (PFKL) (Amara et al., 2021). Since then, a study using mouse neutrophils showed NET formation is independent of gasdermin D (Stojkov et al., 2023). Regardless, LDC7559 inhibition does inhibit NET formation (Amara et al., 2021), and significantly reduced swarming prevalence in response to tailfin transection (Isles et al., 2021). Using cell cultures, NET release has been shown to occur more frequently in neutrophil clusters or neutrophil swarms (P. Deng et al., 2024). Additionally, NET release in neutrophil swarms in microarray experiments restricted fungal growth of *Candida albicans* (Hopke et al., 2020). However, NETs have been reported to merely restrict *Mtb*, and are unable to kill the bacteria (Cavalcante-Silva et al., 2023; Filio-Rodríguez et al., 2017; Ramos-Kichik et al., 2009). Targeting NET formation may further elucidate their involvement in the swarming response to *Mm* infection.

4.1.5. Hypothesis and aims

A zebrafish model to investigate neutrophil swarming in response to infection has now been successfully established (see Chapter 3), but much about the mechanisms that drive this response is still unclear. Investigation of available literature and previous work carried out by our labs has provided a number of targets which modulation may provide more insight in the mechanisms behind neutrophil swarming.

I hypothesise that neutrophil swarming to *Mm* requires Ltb4 and other swarming mediators.

To investigate this hypothesis, the selected swarming mediators were modulated in the somite infection model and the effect of their modulation was assessed in the context of neutrophil swarming to address the following aims:

- Achieving knockdown of *ltb4* using CRISPR-Cas9
- Determining the effect of *lta4* knockdown on neutrophil swarming in response to *Mm*

- Examining the effect of inhibition of potential swarming mediators on neutrophil swarming in response to *Mm*

4.2. Materials and Methods

4.2.1. Drug treatment of swarming mediators

Drug treatment of zebrafish differed slightly per experiment. For experiments that measured neutrophil clustering at 100 mpi, fish were pre-treated with the desired drug at 4 hours before infection (hbi), and were treated after infection until the time of imaging. For experiments that examined bacterial burden at 1 dpi or later, fish were treated with the desired drug immediately after infection. All drugs were administered in Costar 6-well plates (Corning) in 3 ml E3 with the desired drug concentration. Control treatments were done with an equal volume distilled water or DMSO (Sigma-Aldrich) to the volume of drug used. All drugs used in this Chapter can be found in Table 3.

Table 3 | Swarming mediator drugs

Drug target	Drug name (Supplier)	Treatment Concentration	Control
LTA4H	Bestatin	100 µM	DMSO
CXCR2	SB225002	5 µM	DMSO
CXCR4	AMD3100	10 µM	Distilled water
Net Formation	LDC7559	10 µM	DMSO

4.2.2. CRISPR targeting of *cxcr2*

Preparation- and injection of CRIPSR-Cas9 mixes to target *cxcr2* was performed as described in 2.2.3. CRISPR-Cas9 injections, and 2.3. CRISPR-Cas9. The guide RNA (IDT) and primers (IDT) used for knockdown of *cxcr2* are described in Table 4.

Table 4 | *cxcr2* CRISPR guide RNA and primer sequences.

Name CRISPR	Guide RNA (5'-3')	Forward primer (5'-3')	Reverse primer (5'-3')	Enzyme
Cxcr2 original	TACCCTTATGACTCCTTGCCCGG	GAGGTCCTGAATTTCTGAGGTG	GCAAGCTCAGGCAAAAGACTAT	BsaI
Cxcr2 14	CATTAGCATCGACCGCTACATGG	CATGTGCAAAATGATCTCTGGT	GCACCATAACCGGAAGAGATAA	MwoI
Cxcr2 26	AAAGGATCCCGCTCCATATCTGG	ACATAGACGTGGCTTTGTATGC	CTTTAAACCTACCACACCTGCC	BamHI
Cxcr2 Start	CAAGGAGTCATAAGGGTAAATGG	GTGTTTCTCCCTCCACAGCT	CCAGGTGCATCAGGTAAAGG	HinfI

Cxcr2 Middle	GAGTATGACGGATAATGCGACGG	TCTCTTCCGGTTATGGTGCA	TGATAAAAGCCAGCACCACG	FokI
Cxcr2 End	TTCAGAGCTCTGGAAGCACTCGG	CATCCTGTACGCCTTCATCG	ACTCTACCCTACCCTCACACT	SacI

4.3. Results

4.3.1. Inhibition of LTA4H with bestatin does not affect neutrophil numbers and clusters

The importance of LTA4H signalling in the recruitment of distant neutrophils towards the swarm during neutrophil swarming has been well-described in mice (Lämmermann, 2016; Lämmermann et al., 2013). In zebrafish, knockdown of *lta4h* did not affect early neutrophil recruitment at 3 hpw to a tailfin wound, but significantly reduced recruitment at 6 hpw (Isles et al., 2021). To examine if pharmacological knockdown of LTA4H could exert an effect on neutrophil swarming in the somite model, fish were treated with the drug bestatin or a DMSO control 4 hours before infection with *Mm* (Figure 4.1 A). Bestatin inhibits LTA4H, and thereby LTB4, in a dose-dependent manner (Orning et al., 1991). The drug has previously been used in zebrafish studies to study macrophage aggregation in response to *Streptococcus iniae* infection (Vincent et al., 2017), and to examine the effect of LTA4h inhibition on systemic *Mm* infection (Tobin et al., 2010). Fish were imaged at 100 mpi, when swarms are most likely to occur in this model (Figure 3.11), and were examined on neutrophil clustering and number within the somite. Clustering was defined as 3 or more neutrophils in close contact with each other at a single time point. Neutrophil clustering in bestatin-treated fish did not differ from those treated with DMSO (Figure 4.1 B). Additionally, there was no difference in neutrophil number within the somite between the treatments (Figure 4.1 C). This indicates knockdown of LTA4H with bestatin does not affect neutrophil clustering and numbers in response to *Mm* infection.

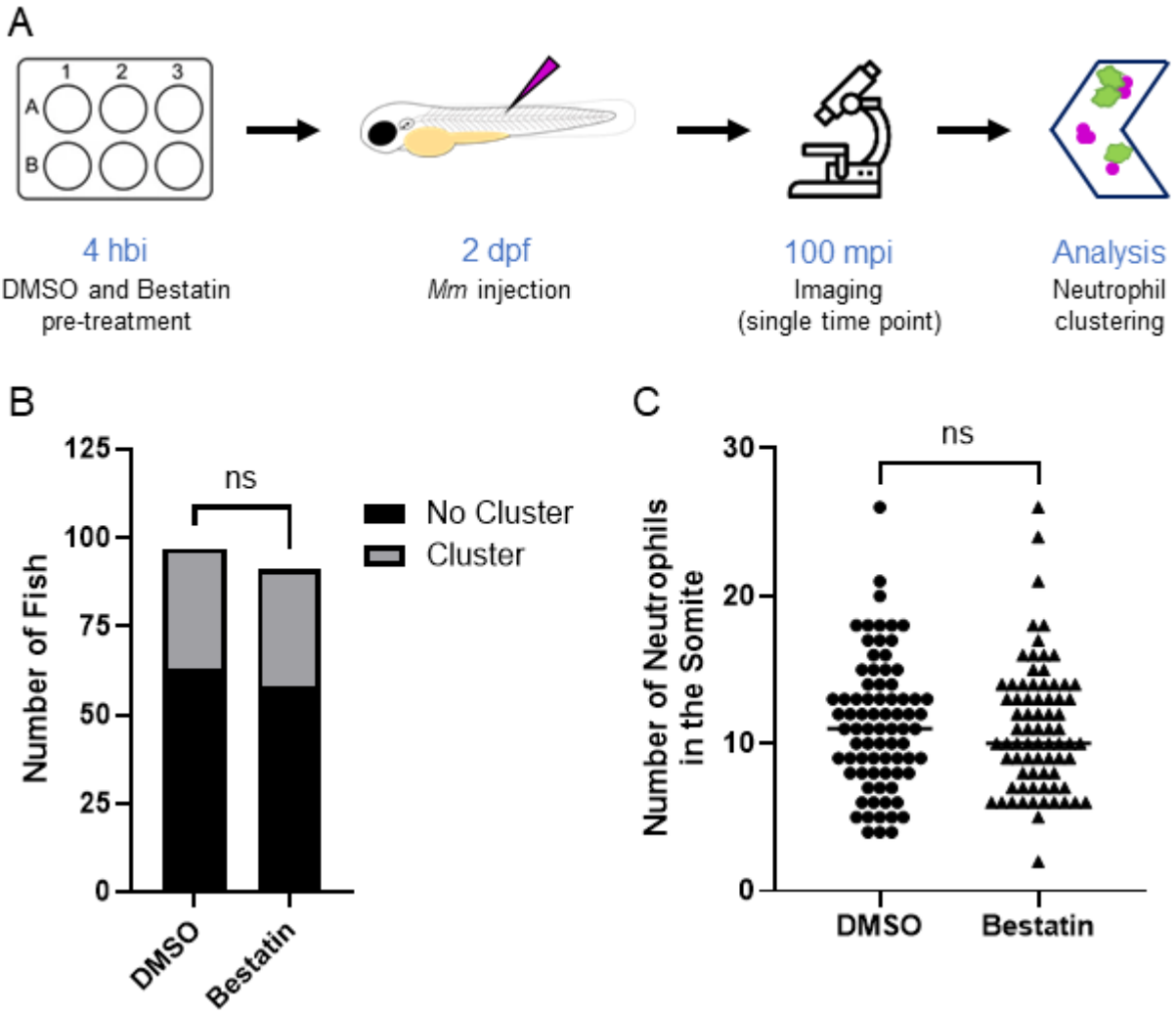


Figure 4.1 | Inhibition of LTA4H with Bestatin has no effect on neutrophil clusters and numbers within the somite. A) Zebrafish were pre-treated with DMSO and bestatin 4 hours before somite infection with *Mm* at 2 dpf. Fish were then imaged at 100 mpi and time lapses were analysed for neutrophil clustering and numbers within the somite. B) There was no difference in swarming prevalence between bestatin-treated fish and DMSO-treated fish ($p = 0.8800$, Fisher's exact test) at 100 minutes post *Mm* infection. C) Similarly, there was no difference in the number of neutrophils within the somite after LTA4H knockdown compared to the DMSO control ($p = 0.7073$, unpaired t-test). Error bars are mean ± SEM, N= 91, 97 fish in the DMSO group and bestatin group respectively from 2 experimental repeats.

4.3.2. Bestatin treatment does not reduce *Mm* bacterial burden in systemic infection

Lta4h knockdown with bestatin did not appear to affect neutrophil clustering in the swarming model, which raises the question if Lta4h is not crucial for cluster formation in this model or if the drug is not functioning. Inhibition of LTA4H with bestatin in a systemic *Mm* infection model has previously been shown to increase bacterial burden at 4 dpi (Tobin et al., 2010). Therefore, zebrafish were treated with bestatin immediately after systemic *Mm* infection at 1 dpf (Figure 4.2 A) to examine if the drug used here functions similarly to previously reported data. Fish were imaged at 4 dpi and analysed for bacterial burden (Figure 4.2 B and Figure 4.2 C). There was no significant difference of bestatin and DMSO treatment on bacterial burden ($p = 0.0674$). However, both repeats showed a similar non-significant decrease in bacterial burden in the bestatin group compared to the DMSO control, which combined with a third repeat may have pushed into significance. Because these 2 repeats showed similar results that may not be in accordance with the finding that bestatin increases systemic *Mm* infection (Tobin et al., 2010), the drug was not used for further experimentation and genetic knockdown approaches were used instead.

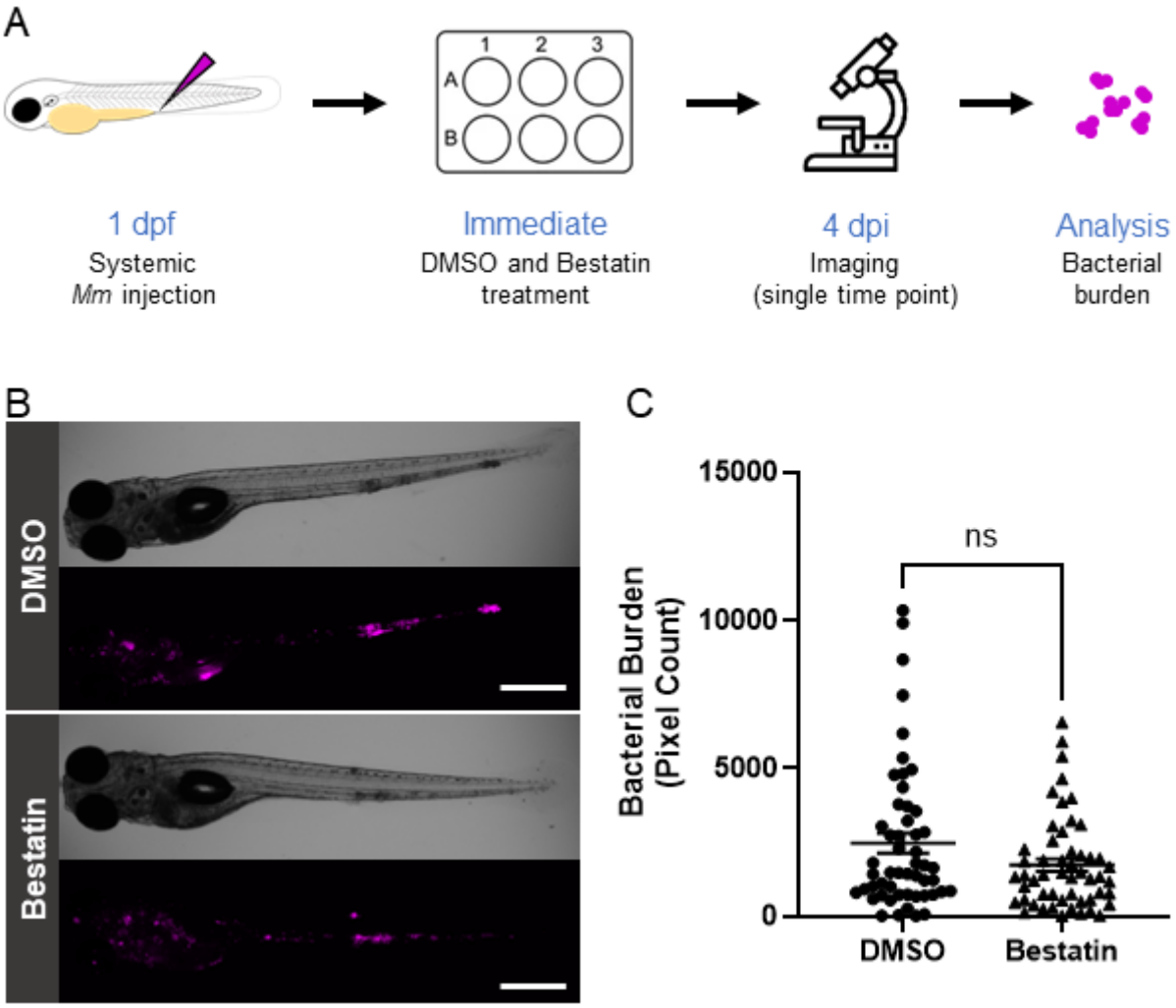


Figure 4.2 | Pharmacological knockdown of LTA4H does not affect systemic *Mm* bacterial burden. A) Zebrafish were infected systemically with *Mm* through caudal vein injections at 1 dpf. Immediately after all fish were injected, they were pooled and distributed between DMSO and bestatin treatment. Fish were imaged at 4 dpi and bacterial burden was quantified. B) Representative images of *Mm* infections at 4 dpi for both the DMSO and bestatin group. Scale bars are 500 µm. C) There was no significant difference in bacterial burden between fish treated with DMSO and with bestatin ($p = 0.0674$). Statistics were performed with an unpaired t-test on 52 DMSO-treated and 53 bestatin-treated fish over 2 experimental repeats. Error bars are mean ± SEM.

4.3.3. Validation of CRISPR-mediated knockdown of *lta4h*

Knockdown of *lta4h* with CRISPR-cas9 has previously been described (Isles et al., 2021). This study used a high-resolution melt (HRM) analysis to detect indels in the DNA created by successful CRISPR knockdown (Isles et al., 2021), a technique that requires special equipment. To avoid the requirement of using this equipment, successful knockdown of *lta4h* was validated with a gel electrophoresis-based approach instead. First, primers had to be designed to cut out a DNA fragment of ~250 bp around the CRISPR target site (Figure 4.3 A). The CRISPR target site (blue square) was already available from the previous study (Isles et al., 2021) and contains a HinfI restriction site. A temperature gradient was used to assess the optimal annealing temperature of the primers in the PCR protocol, and was determined to be 57 °C (Figure 4.3 B). This was done for all primers in this thesis, but will not be individually discussed from hereafter. Zebrafish were injected with the *lta4h* CRISPR or Cas9 control into the cell at the single cell stage and gDNA was collected a day later (Figure 4.3 C). The PCR protocol, including the correct annealing temperature, was performed on the gDNA and part of the resulting PCR products were digested with the HinfI restriction enzyme. A few tubes for both the Cas9 control and the *lta4h* CRISPR group were left undigested. All PCR products were then used for gel electrophoresis (Figure 4.3 D). An undigested control and CRISPR band was expected to be 247 bp, and a digested control was expected to be 179 bp. Successful deletion of the CRISPR target site results in the loss of the HinfI restriction site, meaning a successful CRISPR procedure results in a band of similar size as the undigested control. In this case, the *lta4h* CRISPR appears to have successfully deleted the target site in 4 out of 5 fish. One of these bands (white arrow) appears to indicate the PCR of the gDNA of this one larva was unsuccessful. Together, these results show that the target site in the *lta4h* gene can be successfully deleted through CRISPR-Cas9 and that this can be validated by restriction enzyme digestion and gel electrophoresis.

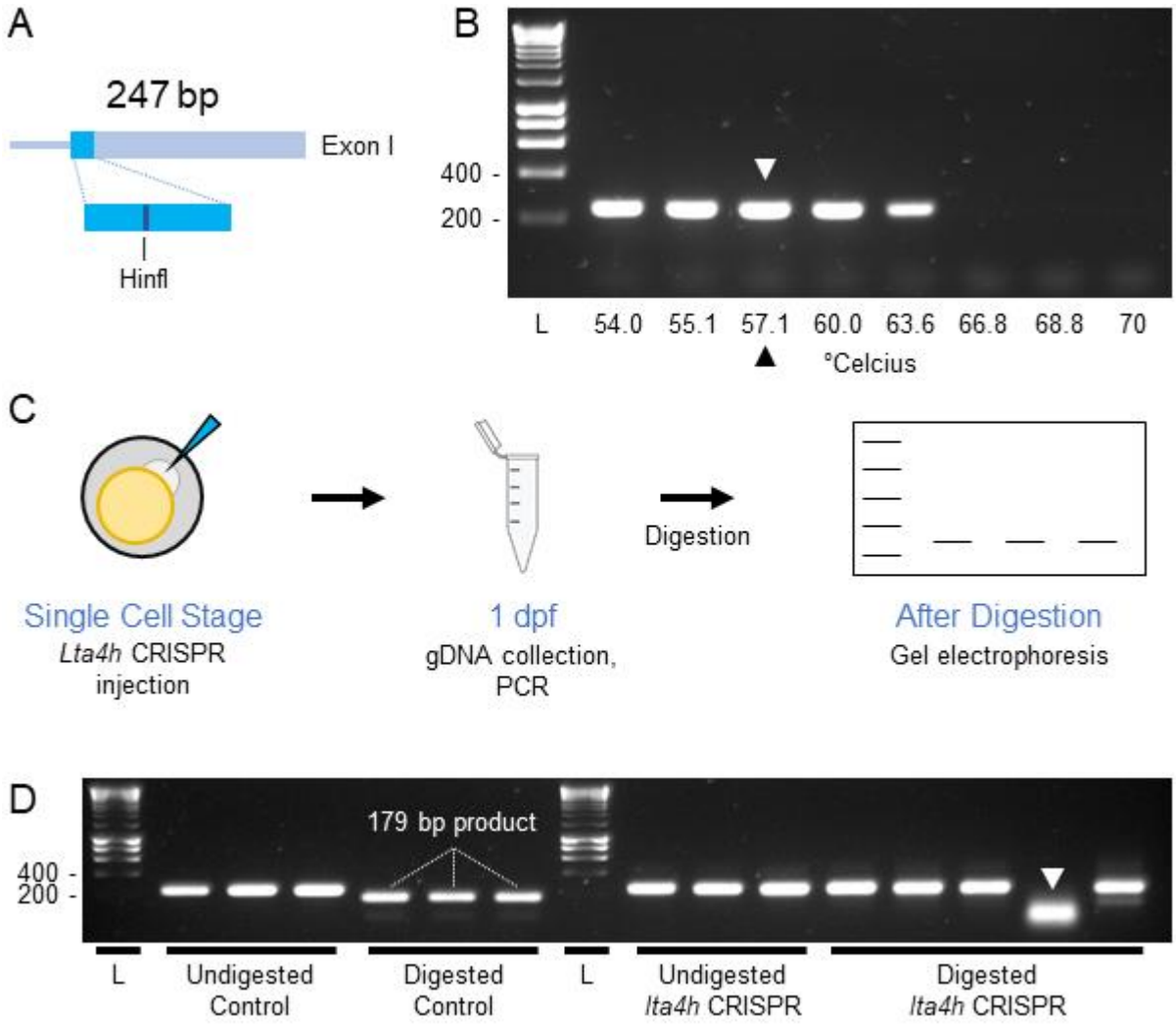
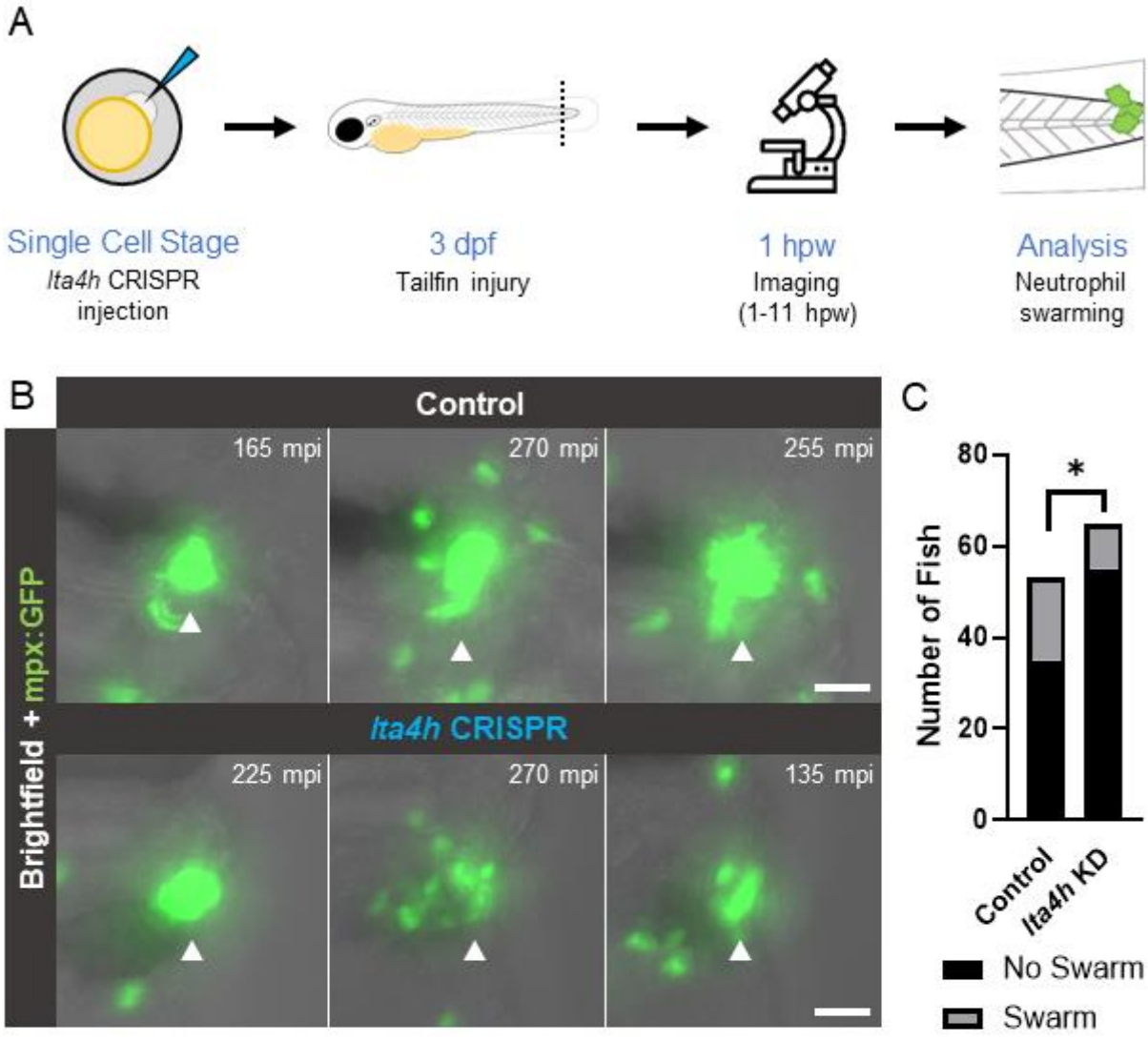


Figure 4.3 | *lta4h* CRISPR validation. A sequence targeting the *lta4h* gene has previously been described (Isles 2021), but a validation method using gel electrophoresis was not yet available. A) The PCR product that results from the primers paired with the crRNA used to target *lta4h*. The crRNA targets a sequence (Blue square) at the start of the first exon (big blue bar) and contains a restriction site for HinfI (dark blue line). Relative sizes are to scale. B) Gel showing PCR products when the annealing step in the PCR protocol was set to a gradient. "L" indicates the ladder. The primers fully lost effectiveness at temperatures of 66.8°C and higher. Band size shows they were seen to anneal similarly effectively for 55.1°C and 57.1°C, and the latter was chosen as the preferred annealing temperature (black and white arrows) for this PCR. C) The process of validating the CRISPR. The *lta4h* CRISPR components and a Cas9 control missing the Cas9 protein were injected into the cell at the single cell stage of zebrafish eggs. gDNA was collected at 1 dpf according to the "Gradient PCR for primer testing and PCR protocol". PCR was performed and the PCR products were digested with HinfI, with 3 products remaining undigested in both the control and CRISPR groups. All PCR products were then loaded on a gel for gel electrophoresis. D) Results of gel

electrophoresis on undigested and digested PCR products of the control and CRISPR groups. “L” indicates the ladder. Digestion of the control resulted in a 179 bp DNA band, compared to a 247 bp undigested band. Successful deletion of the HinfI restriction site by CRISPR results in a band of similar size compared to the undigested PCR products, indicating the CRISPR has been successful. This appeared to be the case in 4 out of 5 CRISPR-injected larvae. The PCR failed in the remaining larva (white arrowhead).

4.3.4. Genetic knockdown of *lta4h* significantly reduced swarming prevalence in a tailfin injury model

With an easily accessible and reliable method to validate knockdown of *lta4h*, its effect could be assessed in the context of neutrophil swarming. Zebrafish were injected with the *lta4h* CRISPR or a Cas9 control into the cell at the single cell stage (Figure 4.4 A). Zebrafish tailfins were transected at 3 dpf like previously described (Isles et al., 2021) and were imaged until 6 hours post wounding (hpw). Time lapse movies were analysed for neutrophil swarming. Neutrophils were present at the wound site from the start of the time lapse, and more neutrophils can be seen migrating towards the wound over time. Neutrophils in both *lta4h* knockdown (KD) larvae and Cas 9 controls were seen to form stable swarms over time (Figure 4.4 B). When compared to the control group, neutrophil swarming in the *lta4h* CRISPants was found to occur significantly less ($p = 0.0286$) with 18 swarms found in the control fish and 10 in the *lta4h* CRISPants (Figure 4.4 C). All of these swarms were initiated during the timeframe of the time lapses, with the earliest cases at 135 mpi (Figure 4.4 D). There was no difference in swarm starting points between the control and the *lta4h* CRISPR groups ($p = 0.5464$). Endpoints of swarms in the *lta4h* CRISPants (mean 295.8 minutes) did not differ from those in control fish (mean 343.6 minutes) ($p = 0.0565$) (Figure 4.4 E). Finally, total durations of swarms observed in the time-lapses was similar in both groups (mean 111.5 minutes vs mean 149.4 minutes, $p = 0.3113$) (Figure 4.4 F). Taken together, these results indicate knockdown of *ltb4* significantly reduced the number of swarms present in fish after tailfin transection, but no effect on swarming time points and durations was found.


A
Single Cell Stage
lta4h CRISPR injection
3 dpf
Tailfin injury
1 hpw
Imaging (1-11 hpw)
Analysis
Neutrophil swarming
B
Control
165 mpi
270 mpi
255 mpi
lta4h CRISPR
225 mpi
270 mpi
135 mpi
Brightfield + mpx:GFP
C
Number of Fish
80
60
40
20
0
*
Control
lta4h KD
No Swarm
Swarm

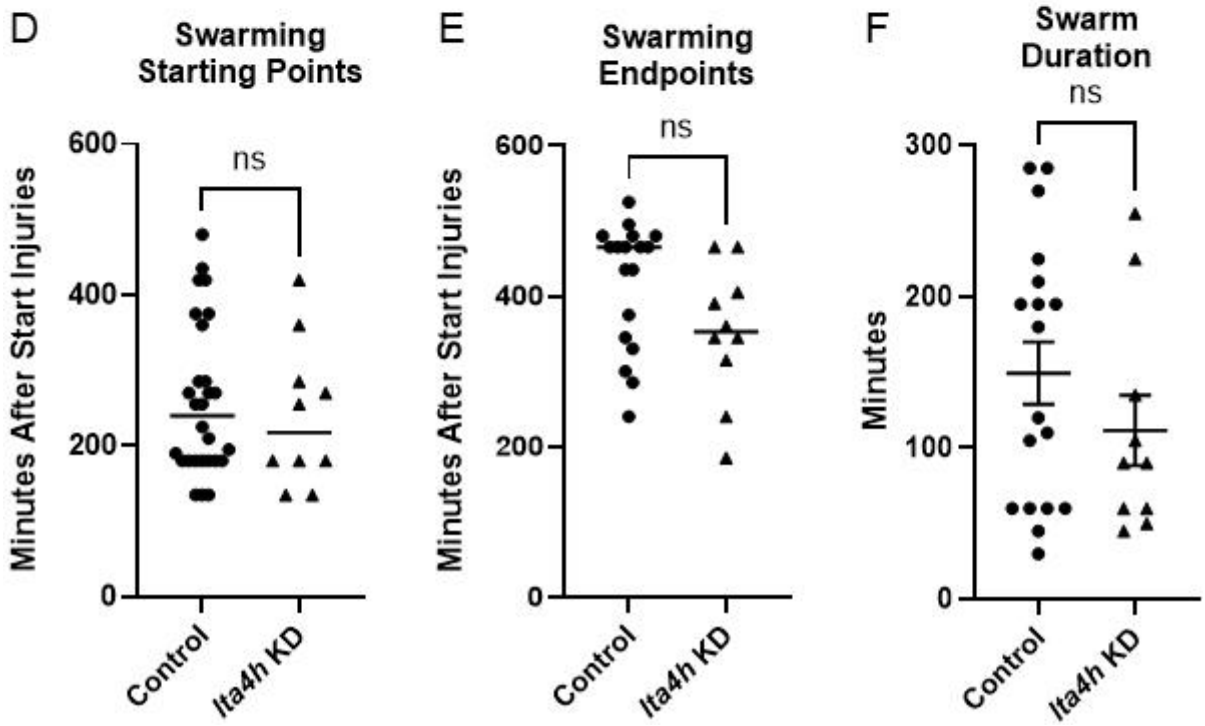

D
Swarming Starting Points
Minutes After Start Injuries
600
400
200
0
ns
Control
lta4h KD
E
Swarming Endpoints
Minutes After Start Injuries
600
400
200
0
ns
Control
lta4h KD
F
Swarm Duration
ns
Minutes
300
200
100
0
Control
lta4h KD

Figure 4.4 | Knockdown of *lta4h* significantly reduced neutrophil swarming prevalence at the wound after tailfin transection. A) Timeline of the experimental procedure. Zebrafish were injected with the *lta4h* CRISPR or Cas9 control into the cell at the single cell stage. Tailfin transection was performed at 3 dpf as previously described (Isles et al., 2021). Imaging commenced at 1 hpw for the first and third repeats, but had to be delayed to 2 hpw for the second repeat due to microscope issues. The time lapse movies were analysed for neutrophil swarming at the tailfin wound. B) Examples of neutrophil swarms at the tailfin wound in both the control and *lta4h* CRISPants groups. C) There was a significant decrease in the prevalence of neutrophil swarming in the *lta4h* CRISPant group compared to the control ($p = 0.0286$, Fisher's exact test) after tailfin transection. D) There was no difference in the starting points of neutrophil swarms between the control group and the *lta4h* CRISPant ($p = 0.5464$, Mann-Whitney test). E) Knockdown of *lta4h* also did not affect endpoints of swarms compared to the control ($p = 0.0565$, Mann-Whitney test). F) There was no effect of *lta4h* knockdown on swarming durations compared to controls ($p = 0.3113$, Mann-Whitney test). Scale bars are 25 µm and error bars are mean ± SEM. N = 53 and 65 for the control group and *lta4h* CRISPR group respectively from 3 experimental repeats.

4.3.5. Knockdown of *lta4h* did not affect neutrophil numbers at the wound area at 3 or 6 hpw

It was previously shown that neutrophil numbers in the wound area were significantly decreased after knockdown of *lta4h* compared to controls at 6 hpw (Isles et al., 2021). To examine if these data could be corroborated, neutrophils in a consistent area around the wound (Figure 4.5 A) were counted over time (Figure 4.5 B). This dataset was gained from tailfin transections performed at 2 dpf, but any other tailfin transection experiments were done at 3 dpf to utilise an increased number of whole-body neutrophils and to be consistent with the previous study (Isles et al., 2021). In control fish, neutrophil numbers present at the wound increased from 2 hpw until reaching a plateau at 5 hpw (Figure 4.5 B). Neutrophil counts in *lta4h* CRISPants appeared to be consistently lower than in the control group over time, but no statistics were performed on this single experimental repeat. From these data, 3 hpw and 6 hpw were chosen as the time points at which to count neutrophil numbers present at the wound site. While the data show a marginally bigger difference in neutrophil counts between the experimental groups at 7 hpw, 6 hpw was chosen to remain fully consistent with the previous study (Isles et al., 2021).

To properly assess neutrophil counts in the wound area at 2 separate time points, zebrafish were injected with the *lta4h* CRISPR or Cas9 control into the cell at the single cell stage (Figure 4.5 C). Tailfin transection was performed at 3 dpf, followed by imaging as early as 1 hpw. Time lapse movies were analysed for neutrophil counts in the wound area (purple box) at 3 hpw and 6 hpw (Figure 4.5 D). Neutrophil counts increased significantly from 3 hpw to 6 hpw in both groups ($p < 0.0001$). There was no statistically significant difference between the groups at both 3 hpw ($p = 0.8370$) and 6 hpw ($p = 0.1527$). Taken together, these results show *lta4h* knockdown does not significantly reduce neutrophil numbers within the wound area at both 3 hpw and 6 hpw.

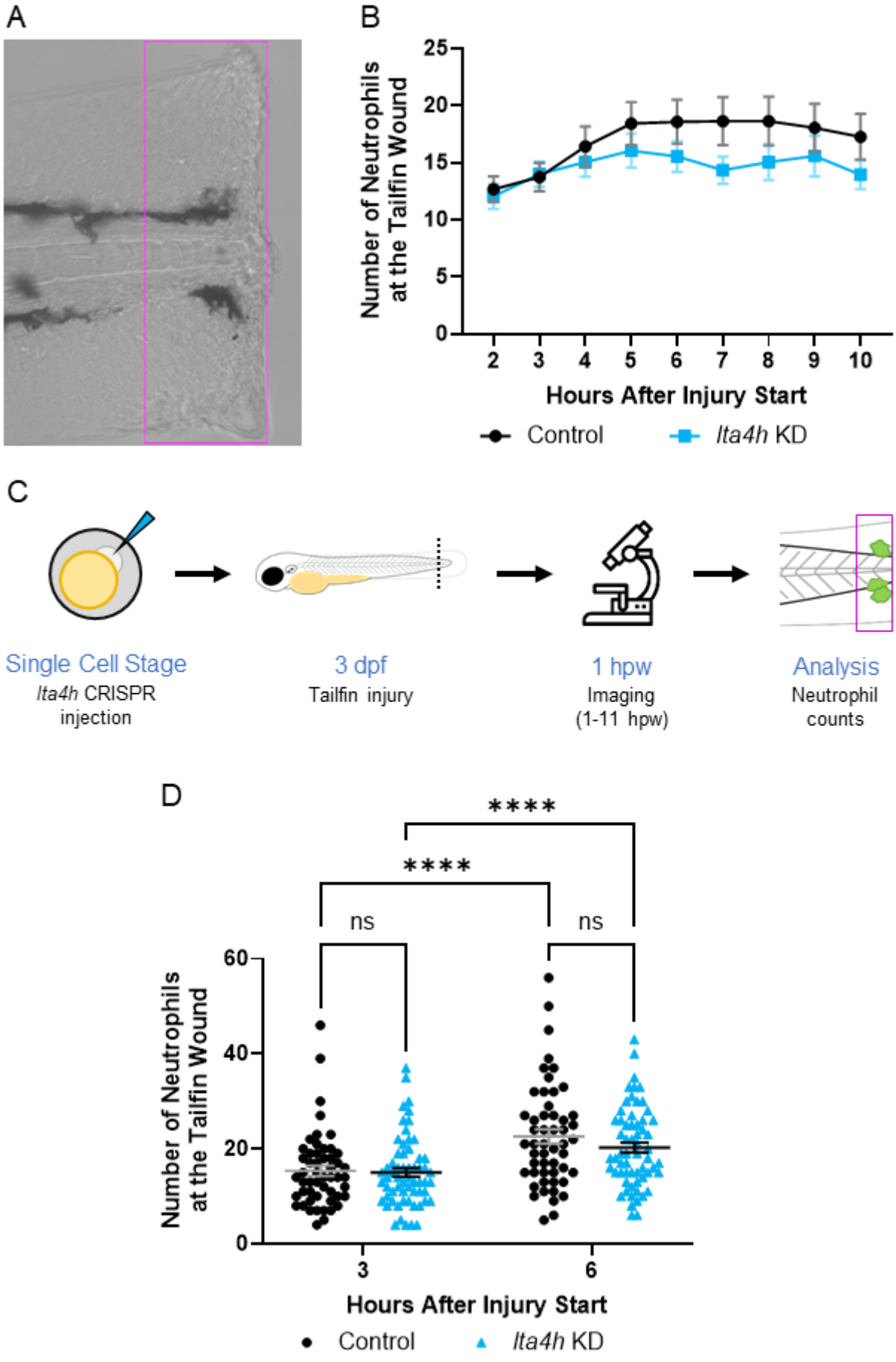

A
B
Number of Neutrophils at the Tailfin Wound
25
20
15
10
5
0
2
3
4
5
6
7
8
9
10
Hours After Injury Start
Control
lta4h KD
C
Single Cell Stage
lta4h CRISPR injection
3 dpf
Tailfin injury
1 hpw
Imaging (1-11 hpw)
Analysis
Neutrophil counts
D
****
****
ns
ns
Number of Neutrophils at the Tailfin Wound
60
40
20
0
3
6
Hours After Injury Start
Control
lta4h KD

Figure 4.5 | Knockdown of *lta4h* does not significantly reduce neutrophil numbers within the wound area. A) The area around the tailfin wound in which neutrophil counts were quantified. B) Neutrophil counts within the wound area over time in Cas9 control fish and *lta4h* CRISPR fish at 2 dpf. N = 19, 20 for the control group and CRISPR group respectively from 1 experimental repeat. C) Schematic overview of the experimental procedure. Zebrafish were injected into the cell at the single cell stage with the *lta4h* CRISPR or a Cas9 control. Tailfin transection was performed at 3 dpf and imaging commenced as early as 1 hpw: 2 repeats at 1 hpw and 1 repeat at 2 hpw due to microscope issues. Time lapse movies were assessed on neutrophil counts within the tailfin wound area. D) Neutrophil counts within the wound area at 3 hpw and 6 hpw between the control group and *lta4h* CRISPants. In both groups, neutrophil counts increased from 3 hpw to 6 hpw ($p < 0.0001$). There was no significant difference in neutrophil numbers between the control group and *lta4h* CRISPants at 3 hpw ($p = 0.8370$) and 6 hpw ($p = 0.1527$). Statistics were performed with a multiple comparisons two-way ANOVA. N = 52, 61 for the control group and *lta4h* KD group respectively from 3 experimental repeats.

4.3.6. Genetic knockdown of *lta4h* reduced swarm duration and area, but not prevalence, in response to localised *Mm* infection

The *lta4h* CRISPR was used in the somite infection model to investigate the effect of *lta4h* knockdown on neutrophil swarming in response to infection. Zebrafish were injected with the *lta4h* CRISPR or Cas9 control into the cell at the single cell stage (Figure 4.6 A). Imaging followed an hour after infection with *Mm* at 2 dpf and time lapses were analysed for neutrophil swarming. *Mm* injection into the somite resulted in formation of neutrophil swarms in both *lta4h* CRISPants and control-injected fish (Figure 4.6 B), with no difference in prevalence between both groups ($p = 0.8025$) (Figure 4.6 C). There also was no difference ($p = 0.7708$) in the swarming endpoints of the swarms that were observed (Figure 4.6 D). When examining swarm durations of swarms that can be seen commencing and resolving during the time-lapse, 10 for the control group and 8 for the *lta4h* CRISPant group, there was a significant reduction in swarm duration in the *lta4h* CRISPants compared to the controls ($p = 0.0145$) (Figure 4.6 E). Considering the endpoints of the swarms were not affected by *lta4h* knockdown, these reduced durations may indicate that swarms in the CRISPR group may start later, have reduced stability, or both. Swarm areas of swarms in *lta4h* CRISPants were decreased compared to those in control fish ($p = 0.0145$) (Figure 4.6 F), suggesting *lta4h* KD may result in smaller, less stable swarms. Together, these data show that *lta4h* does not appear to be involved in swarming prevalence in response to localised *Mm*, but that it may play a role in swarm stability.

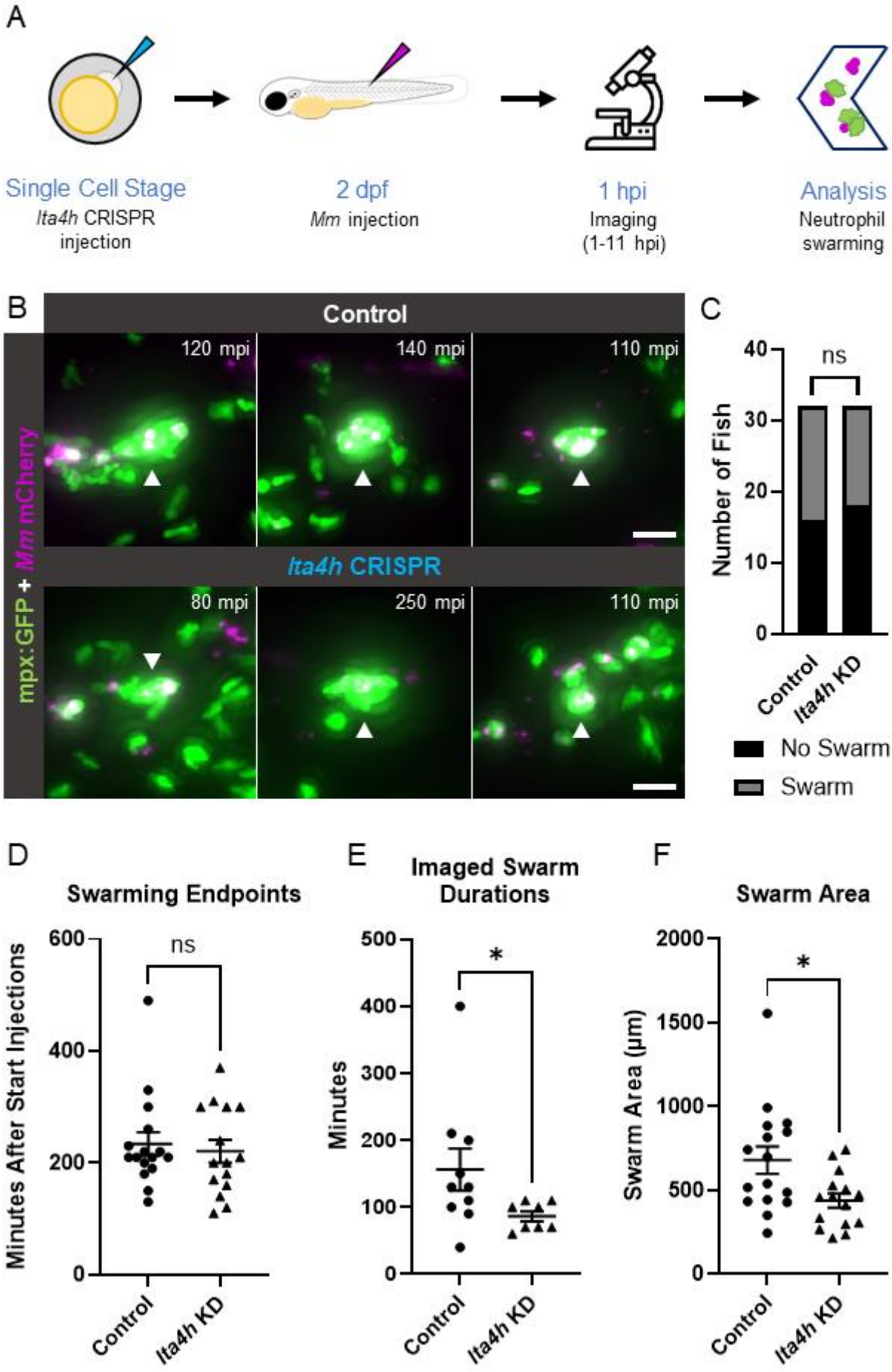
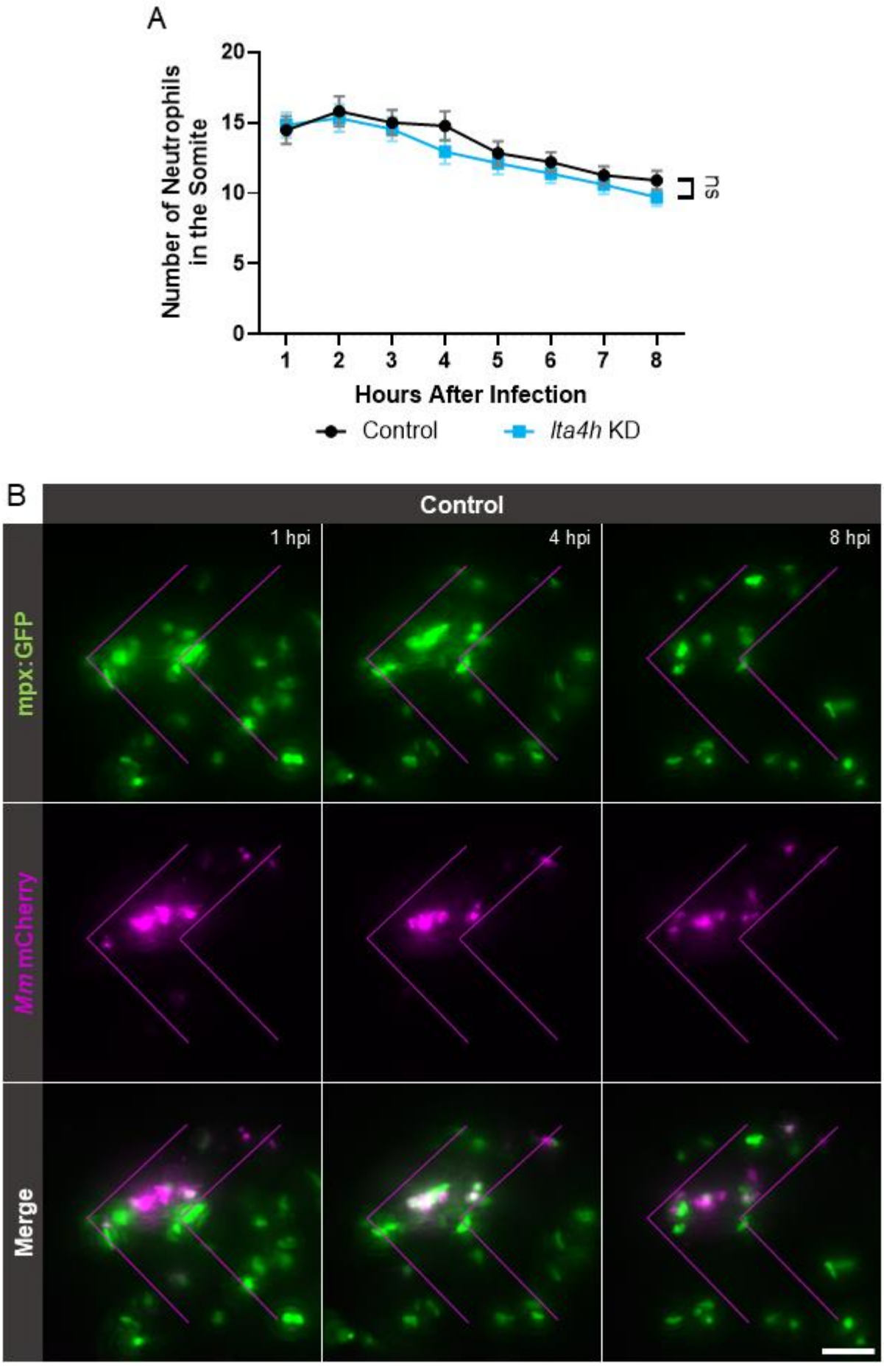

A
Single Cell Stage
lta4h CRISPR injection
2 dpf
Mm injection
1 hpi
Imaging (1-11 hpi)
Analysis
Neutrophil swarming
B
Control
120 mpi
140 mpi
110 mpi
lta4h CRISPR
80 mpi
250 mpi
110 mpi
mpx:GFP + Mm mCherry
C
Number of Fish
ns
Control
lta4h KD
No Swarm
Swarm
D
Swarming Endpoints
Minutes After Start Injections
ns
Control
lta4h KD
E
Imaged Swarm Durations
Minutes
*
Control
lta4h KD
F
Swarm Area
Swarm Area (µm)
*
Control
lta4h KD

Figure 4.6 | CRISPR-mediated knockdown of *lta4h* reduced neutrophil swarm duration and swarm area. A) Zebrafish were injected with a Cas9 control or *lta4h* CRISPR into the cell at the single cell stage of zebrafish eggs. *Mm* was injected into the somite at 2 dpf and larvae were imaged from 1-11hpi. Time lapses were analysed for neutrophil swarming in response to infection. B) Examples of neutrophil swarms (white arrowheads) observed within the infected somite of both control fish and *lta4h* KD fish. C) There was no difference in swarming prevalence after *lta4h* knockdown compared to controls ($p = 0.8025$, Fisher's exact test). D) There was no difference in swarming endpoints of larvae injected with the *lta4h* CRISPR (15 swarms) compared to the control (16 swarms) ($p = 0.7708$, Mann-Whitney test). F) Durations of 10 swarms in control fish and 8 swarms in *lta4h* CRISPants that could be seen forming and resolving within the timeframe of the time lapse. Knockdown of *lta4h* resulted in a significant reduction of neutrophil swarm durations compared to swarms found in control fish ($p = 0.0314$, Mann-Whitney test). F) Areas of swarms in *lta4h* KD fish were significantly decreased compared to swarms in control fish ($p = 0.0145$, unpaired t-test). N = 32 per group, 3 repeats. Error bars are mean ± SEM and scale bars are 25 µm.

4.3.7. The number of neutrophils within the somite over time is not affected by knockdown of *lta4h*

Considering *lta4h* knockdown resulted in reduced swarm duration and swarm area, a reduced neutrophil availability in the somite could be a cause of this. Therefore, in addition to analysis of neutrophil swarming in fish injected with the *lta4h* CRISPR or Cas9 control, neutrophil numbers in the somite were counted every hour from 1 to 8 hpi in both injection groups (Figure 4.7 A, B, C). There was no significant difference in the number of neutrophils over time between the 2 groups ($p = 0.5126$), indicating the reduced swarm duration and area is not due to reduced neutrophil accumulation within the somite.


A
Number of Neutrophils in the Somite
20
15
10
5
0
1
2
3
4
5
6
7
8
Hours After Infection
ns
Control
lta4h KD
B
Control
1 hpi
4 hpi
8 hpi
mpx:GFP
Mm mCherry
Merge

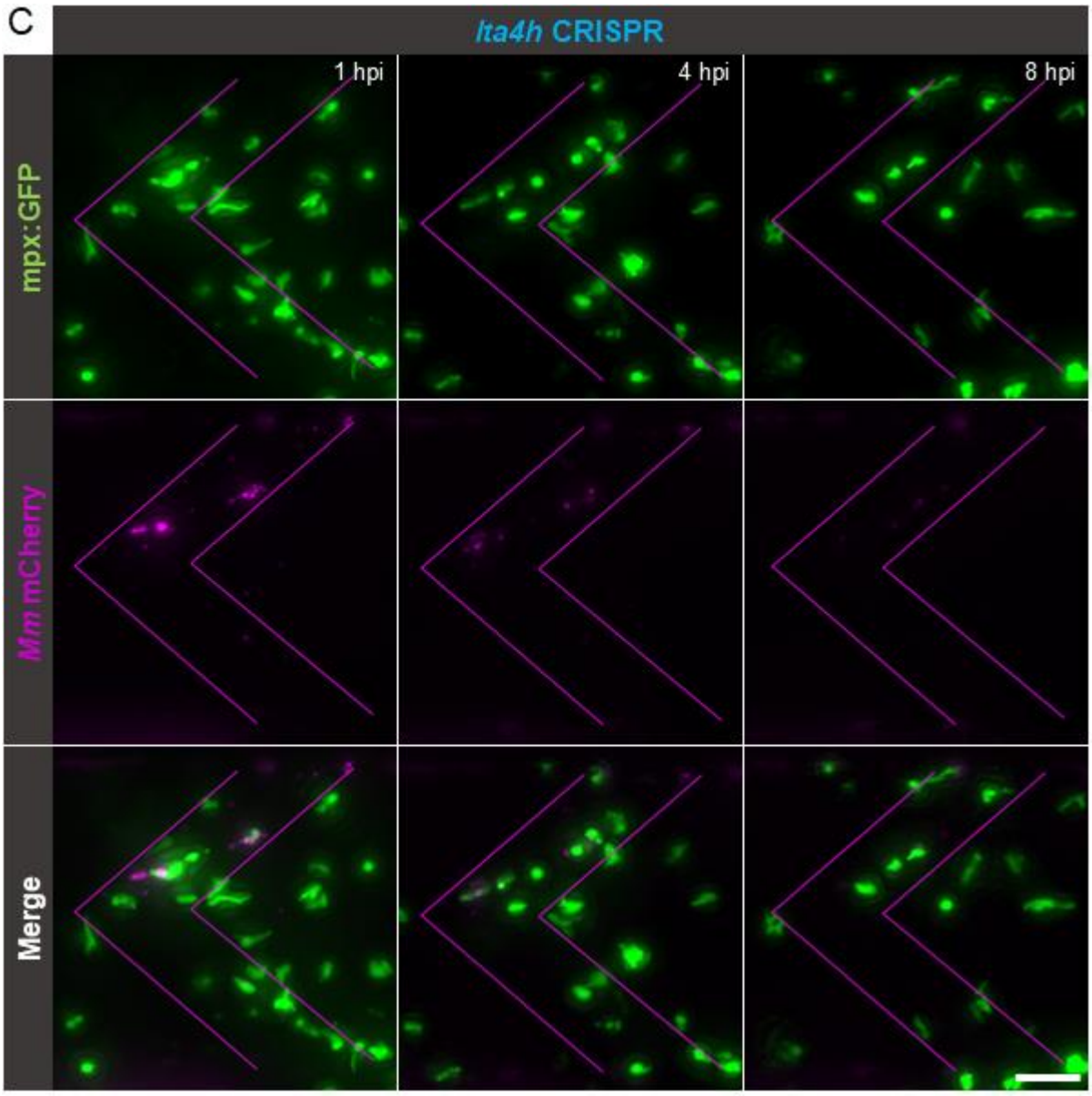


Figure 4.7 | *Lta4h* knockdown does not affect neutrophil numbers within the somite after *Mm* infection. A) Neutrophil numbers within the somite over time did not differ between the *lta4h* CRISPR group and the Cas9 control group (*p = 0.4772,* two-way ANOVA). B) Images of neutrophils within the somite over time in a single Cas9 control larva with neutrophil numbers close to the mean of the curve. C) Images of neutrophils within the somite over time in a single Lta4h CRISPR larva with neutrophil numbers close to the mean of the curve. Scale bars are 50 µm and N = 32 fish per group, 3 repeats.

4.3.8. Genetic knockdown of *lta4h* may increase *Mm* bacterial burden in the somite model

As *lta4h* knockdown may have an effect on neutrophil swarming to *Mm*, this could mean it might also have an effect on clearance of bacteria. LTA4H inhibition with bestatin has previously been shown to significantly increase bacterial burden at 4 dpi in a systemic infection model (Tobin et al., 2010). To examine if a similar effect can be observed in the somite model, zebrafish were injected with the l*ta4h* CRISPR or Cas9 control into the cell at the single cell stage (Figure 4.8 A). Larvae were then injected with 200 CFU, compared to 500 CFU for the other experiments, into the somite at 2 dpf to avoid overburdening the fish with an uncontrollable infection. Imaging was performed at 24 hpi and the images were analysed for bacterial burden within the somite (Figure 4.8 B). Pixel counts on the images showed there was a significant increase in the amount of *Mm* bacterial burden within the somite of fish in the Lta4h CRISPR group compared to the control ($p = 0.0274$, unpaired t-test) (Figure 4.8 C). These results indicate *lta4h* plays a role in the early defence against *Mm* infection.

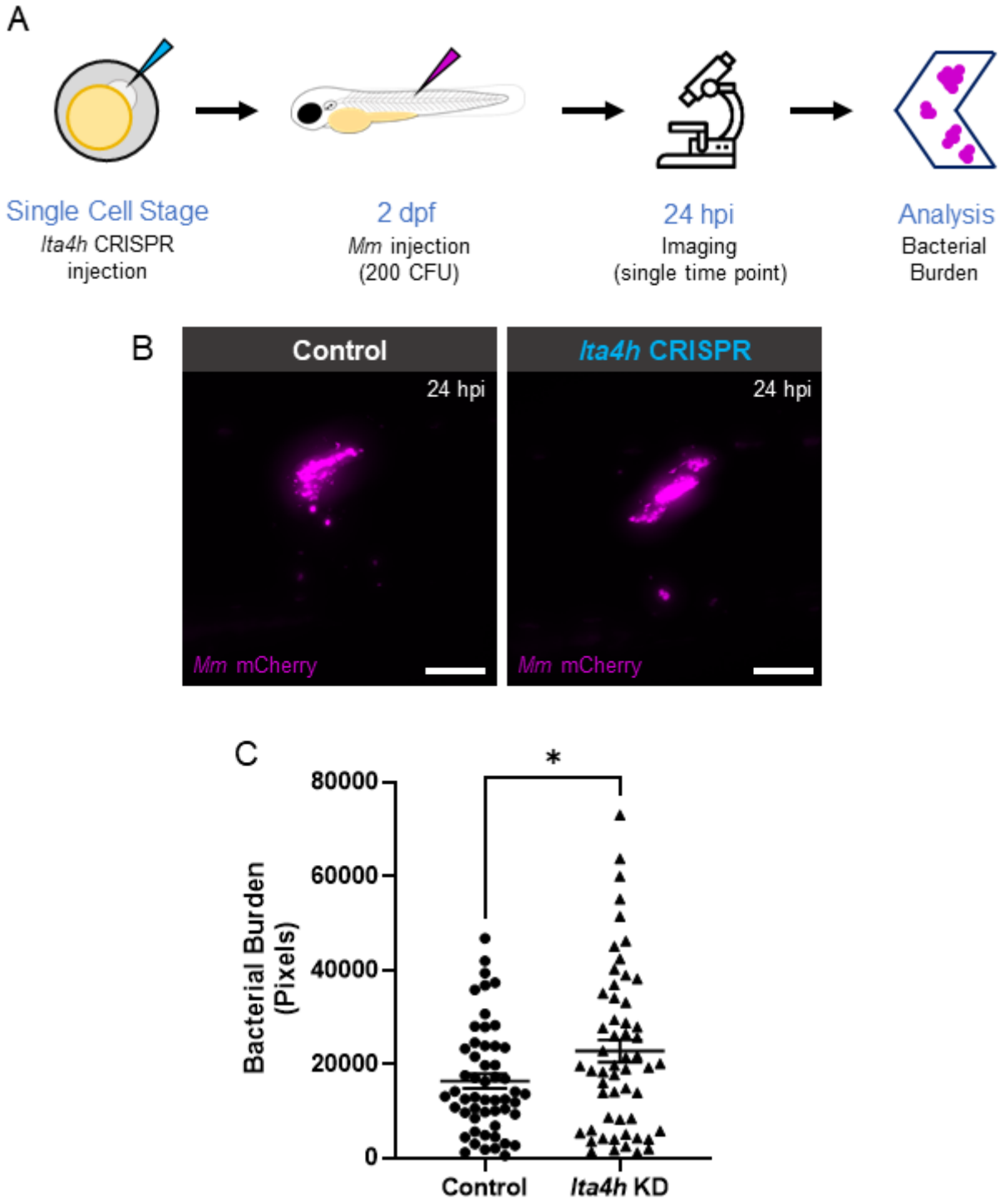


Figure 4.8 | *lta4h* knockdown increases *Mm* bacterial burden in a localised infection. A) Timeline of the experiment. Zebrafish eggs were injected with the *lta4h* CRISPR or a Cas9 control into the cell at the single cell stage. Larvae were injected with 200 CFU *Mm* at 2 dpf and were imaged at 24 hpi. The images were analysed for bacterial burden using pixel counting software. B) Representative images of *Mm* infections in the control group and *lta4h* CRISPants. Scale bars are 100 µm. C) Bacterial burden was significantly increased in the CRISPR group compared to the control ($p = 0.0274$, unpaired t-test). Error bars are mean ± SEM and N = 53, 55 for the control group and CRISPR group respectively over 3 repeats.

4.3.9. *cxcr2* could not be inhibited by targeting individual CRISPR-Cas9 target sites

Cxcr2 is a chemokine receptor that is prominent on neutrophils, and is involved in a variety of human diseases, such as asthma, cystic fibrosis (Stadtmann & Zarbock, 2012), and TB (Slight & Khader, 2013). Despite being a well-studied chemokine receptor, there is no available CRISPR for *cxcr2* described in literature. CHOPCHOP (Montague et al., 2014) was used to develop a CRISPR to knock down *cxcr2* genetically (Figure 4.9). In total, 6 different CRISPR target sequences were used in an attempt to knock down *cxcr2* in this thesis (Figure 4.9 A-F). Figure a-f depicts the different target sites, their locations in the gene, the associated digestion site and enzyme, and the expected bp band size on a gel. All CRISPRs targeted the first exon of the gene, which is the only exon present in the *cxcr2* gene. There were 2 target sequences chosen at the start of the exon (Figure 4.9 A, D), in the middle of the gene (Figure 4.9 B, E), and at the end of the exon (Figure 4.9 C, F). To test these CRISPRs, they were injected alongside a Cas9 control in the single cell during the single cell stage (Figure 4.9 G). gDNA was collected at 1 dpf and was followed by PCR. After digestion of the PCR products with the associated restriction enzyme, all PCR products were run on a gel. Figure 4.9 H shows representative bands of the tests done with the first batch of CRISPR targets, being Cxcr2 Original, Cxcr2 14, and Cxcr2 26. Figure 4.9 I shows the representative bans of the second batch, being Cxcr2 Start, Cxcr2 Middle, and Cxcr2 End. Digested PCR products are indicated with a scissor icon. To reiterate the read-out of these gels as discussed before, the efficacy of the CRISPR can be determined by comparing the bands in a digested CRISPR PCR product to the control bands of both the undigested control and CRISPR band. If the band in the digested CRISPR matches the control bands, it indicates the CRISPR has high efficacy in creating an indel in the gene. If there is also a band present similar to the digested control, it indicates the CRISPR is less efficient. CRISPR Start, Middle, and End all seem to lack a band comparable to the undigested controls (Figure 4.9 I), indicating these CRISPRs are unsuccessful in creating an indel in the DNA. Cxcr2 Original, 14, and 26 appear to have a very faint band which is comparable to the undigested controls, indicating there is a low prevalence of indel induction present. All the CRISPR targets were tested thrice, but never showed any significant prevalence of indels. Taken together, these results indicate that none of the chosen CRISPR targets are successful in knocking down *cxcr2* on their own.

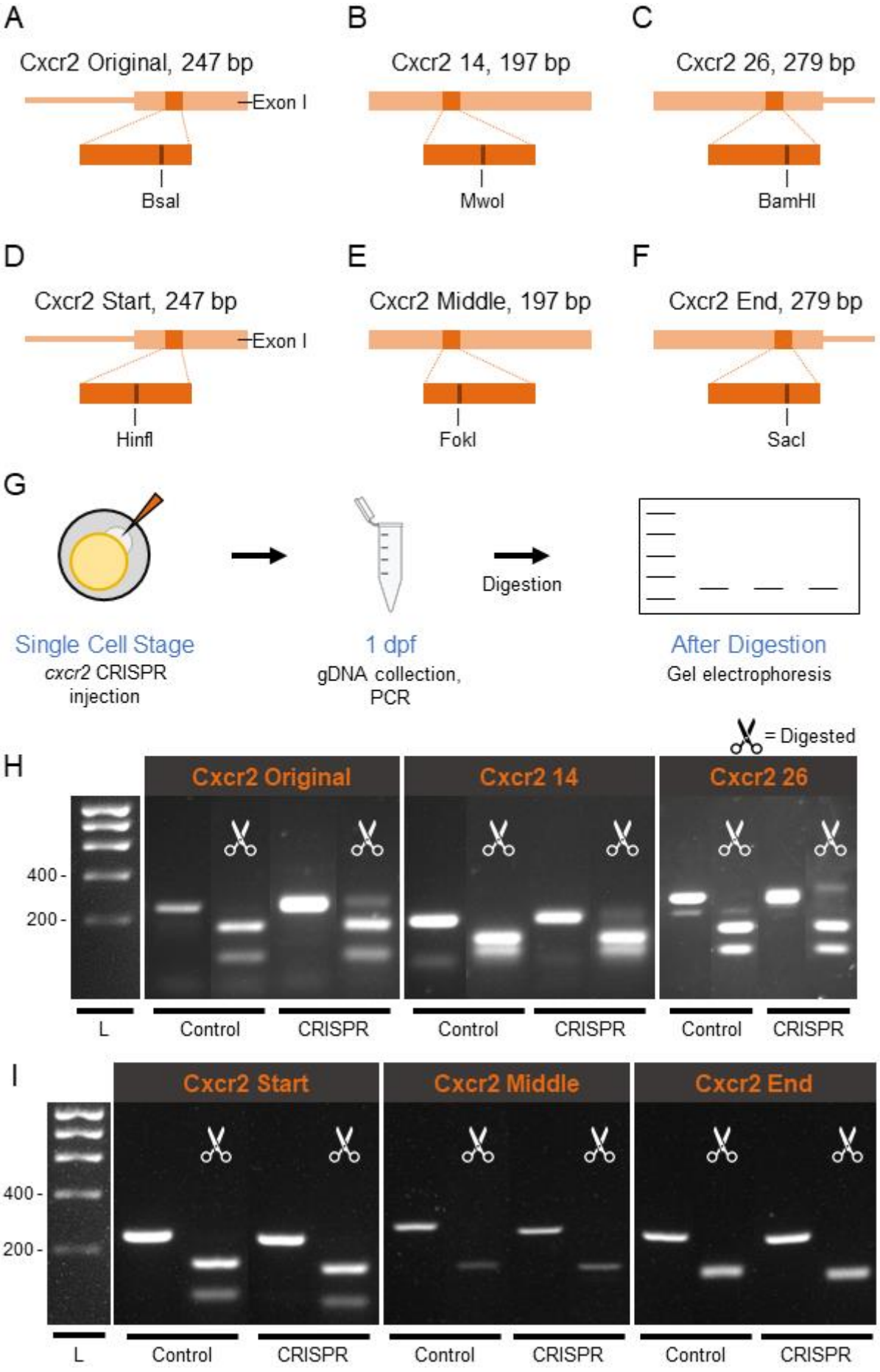

A
Cxcr2 Original, 247 bp
Exon I
BsaI
B
Cxcr2 14, 197 bp
MwoI
C
Cxcr2 26, 279 bp
BamHI
D
Cxcr2 Start, 247 bp
Exon I
HinfI
E
Cxcr2 Middle, 197 bp
FokI
F
Cxcr2 End, 279 bp
SacI
G
Digestion
Single Cell Stage
cxcr2 CRISPR injection
1 dpf
gDNA collection, PCR
After Digestion
Gel electrophoresis
= Digested
H
Cxcr2 Original
Cxcr2 14
Cxcr2 26
400 -
200 -
L
Control
CRISPR
Control
CRISPR
Control
CRISPR
I
Cxcr2 Start
Cxcr2 Middle
Cxcr2 End
400 -
200 -
L
Control
CRISPR
Control
CRISPR
Control
CRISPR

Figure 4.9 | Design and validation of *cxcr2* CRISPR targets. CRISPR target sequences and associated primers were designed using CHOPCHOP (Montague et al., 2014). A-F) The 6 different target sequences in Exon 1 of the *cxcr2* gene, the associated restriction site and enzyme, and their expected PCR product size. A and D target the start of the exon, B and E target the middle of the exon, and C and F target the end of the exon. G) Timeline of the injection and validation of the CRISPRs. Each CRISPR was injected alongside a Cas9 control group into the cell during the single cell stage. gDNA was collected at 1 dpf and was followed by PCR. These PCR products were digested with the appropriate restriction enzyme and all PCR products were subsequently put through gel electrophoresis. H) Representative bands of the Cxcr2 Original, Cxcr2 14, and Cxcr2 26 CRISPRs and their controls from 3 repeated tests. Digested PCR products are indicated by a scissor icon, “L” indicates the ladder. All 3 digested CRISPR targets show a faint band similar to the undigested control bands, indicating these CRISPRs are inefficient at creating indels in the *cxcr2* gene. I) Representative bands of the Cxcr2 Start, Cxcr2 Middle, and Cxcr2 End CRISPRs and their controls. Digested PCR products are indicated by a scissor icon, “L” indicates the ladder. A distinct lack of a band similar to the undigested controls in all of the digested CRISPR target PCR products indicates these CRISPRs are unsuccessful in creating indels in the *cxcr2* gene.

4.3.10. *Cxcr2* could not be inhibited by combining multiple CRISPR targets

Individually, the CRISPR targets for *cxcr2* were unable to reliably cause indels in the target gene. A potential way to increase the effectiveness of these CRISPRs is to combine them in a single mix and target the gene with multiple CRISPRs at the same time (Kroll et al., 2021). This is evident from previous literature, which described a significant increase in the efficacy of gene knockdown after combining 3 crRNAs that target a gene involved in eye pigmentation (Kroll et al., 2021). Therefore, the Cxcr2 Start, Middle, and End CRISPRs were injected together in a mix and were injected into the cell at the single cell stage. gDNA collection, PCR, and gel electrophoresis was performed as described above. The 3 different PCRs associated with the 3 CRISPR targets were performed on the gDNA of the same fish to assess the presence of indels close to any of the target sites. Cas9 controls can be found in Figure 4.9 H, I, but were omitted from this Figure. There were no undigested control-like bands present in the digested PCR products of the Cxcr2 Start and Cxcr2 Middle CRISPRs (Figure 4.10 A, B). There appeared to be a variety band sizes present in both the undigested and digested Cxcr2 End PCR products (Figure 4.10 C). This could indicate that there has been a shift in the DNA present that is not necessarily caused by the Cxcr2 End CRISPR itself and, instead, may have been caused by a combination of the other 2 targets. Taken together, these results indicate that combination of the Cxcr2 CRISPRs does not result in significant gene disruption, and efforts to knock down *cxcr2* with CRISPR-Cas9 were discontinued.

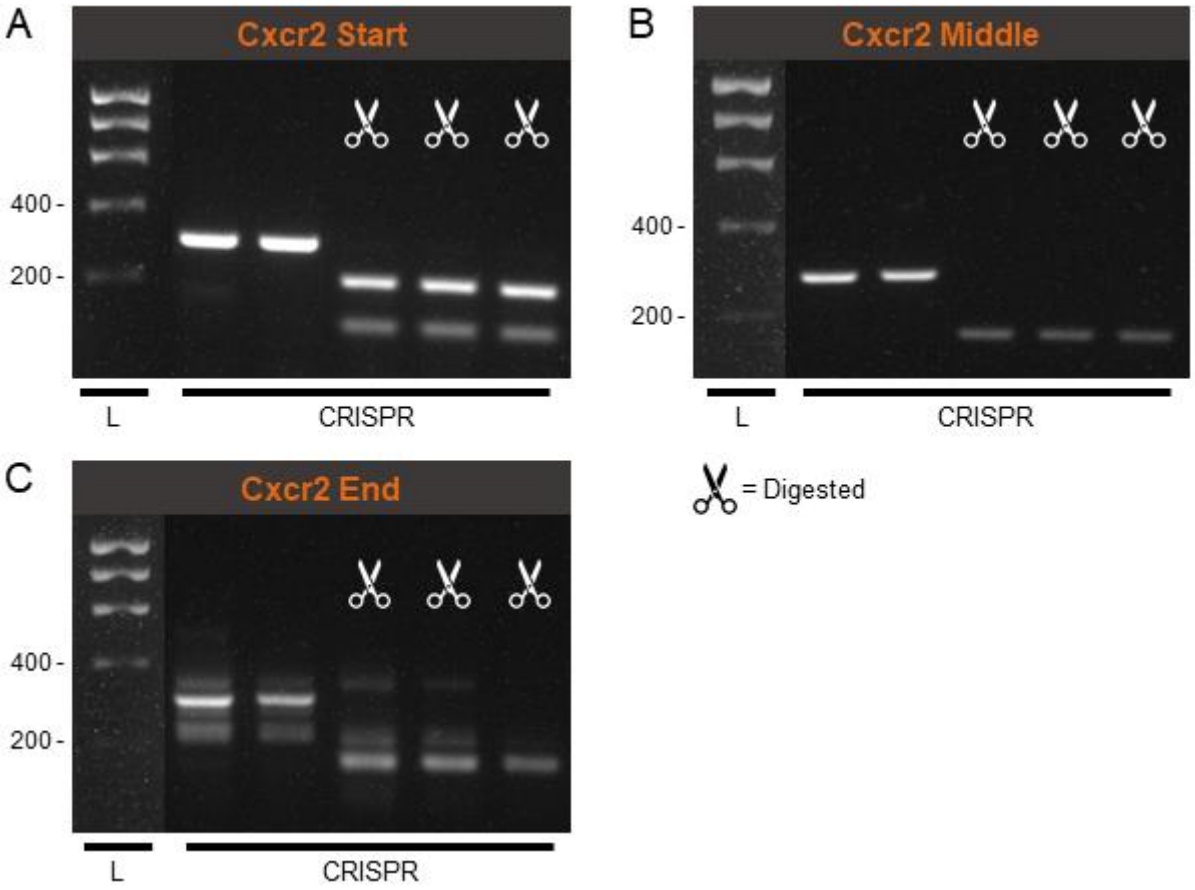


Figure 4.10 | Combination of CRISPR target sites does not increase *cxcr2* knockdown efficacy. Zebrafish eggs were injected with a combination of the 3 CRISPRs Cxcr2 Start, Cxcr2 Middle, and Cxcr2 End into the cell at the single cell stage. PCR on the gDNA of each larva was performed 3 times, once per CRISPR target. A) Gel electrophoresis of PCR products of the Cxcr2 Start CRISPR, showing (un)digested PCR products. B) Gel electrophoresis of PCR products of the Cxcr2 Middle CRISPR, showing (un)digested PCR products. The bands seen in the undigested controls in both A and B do not appear in the digested PCR products, indicating there have been no deletions in the target sequences. C) Gel electrophoresis of PCR products of the Cxcr2 END CRISPR, showing (un)digested PCR products. A variety of bands are present in both the undigested and digested PCR products, indicating there may have been a range of deletions present outside of the target sequence, resulting in different sizes of the PCR products.

4.3.11. Pharmacological inhibition of CXCR2 with SB225002 significantly reduces neutrophil clustering and numbers within the somite after *Mm* infection

Instead of CRISPR-Cas9 knockdown of *cxcr2*, the CXCR2 antagonist SB225002 may be used to examine the effect of CXCR2 signalling disruption on neutrophil swarming in the somite model. Fish were pre-treated with SB225002 and were subsequently infected with *Mm* into the somite (Figure 4.11 A). Analysis of images taken at 100 mpi revealed a significant reduction in the number of neutrophil clusters observed in the SB225002 group compared to the DMSO control ($p = 0.0168$) (Figure 4.11 B). There was also a significant reduction in the number of neutrophils within the somite in fish treated with SB225002 compared to those treated with DMSO ($p < 0.0001$) (Figure 4.11 C). Taken together, these results indicate that inhibition of CXCR2 signalling significantly impairs neutrophil clustering. However, this is paired with a reduction in neutrophil numbers within the somite, potentially meaning impaired neutrophil recruitment is implicated in the reduction in clustering. The objective of manipulating swarming mediators is to find genes that affect clustering without affecting recruitment towards the somite. Inhibition of CXCR2 signalling was therefore not pursued further.

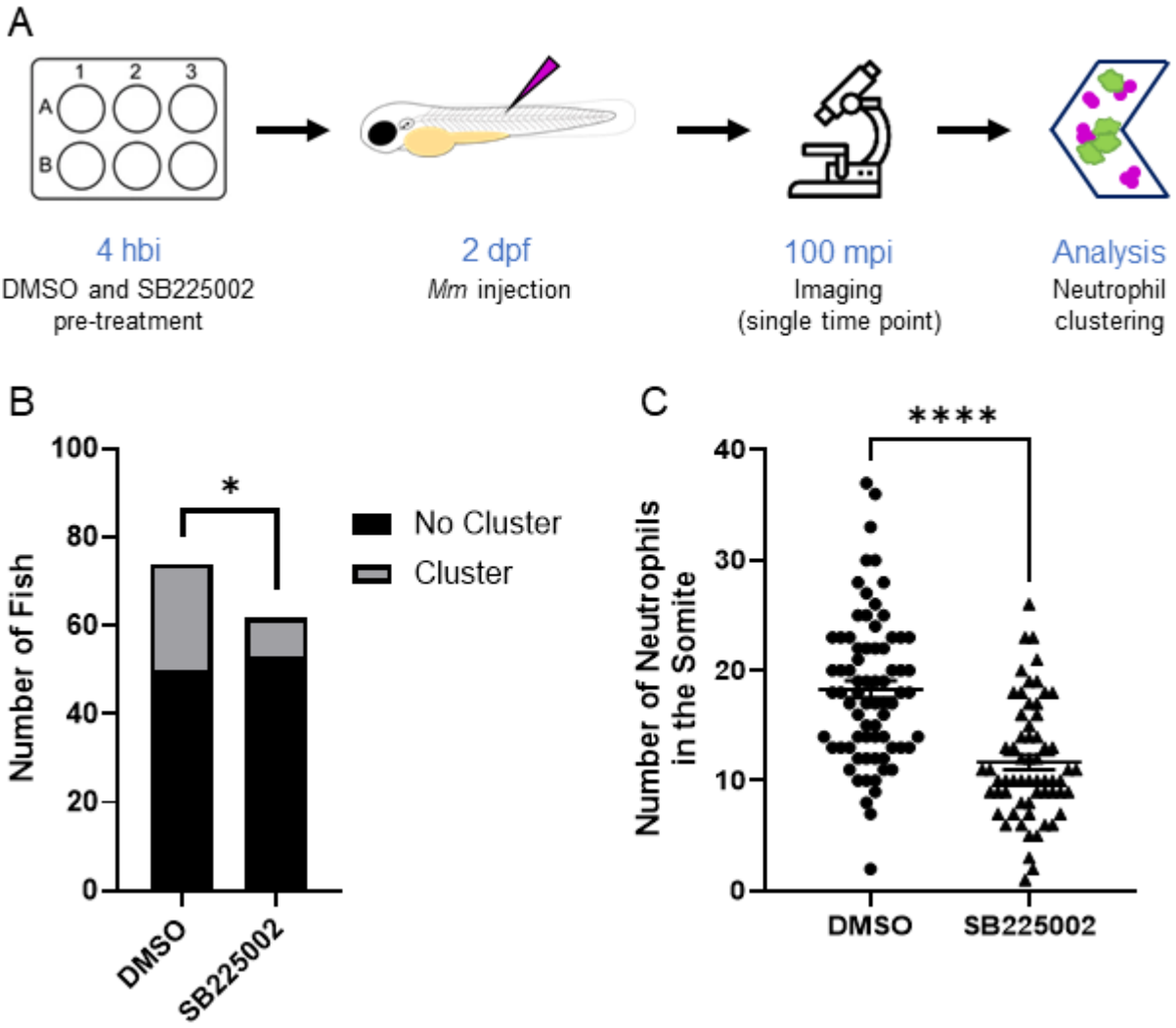


Figure 4.11 | Inhibition of CXCR2 with SB225002 significantly reduces neutrophil clustering and neutrophil numbers in infected somites. A) Schematic of the experimental procedure. Zebrafish were pre-treated with DMSO or SB225002 4 hbi and *Mm* was injected into the somite at 2 dpf. Imaging was performed at 100 mpi and images were analysed for neutrophil clustering and numbers within the somite. B) There was a significant decrease in the number of clusters found in infected somites in the SB225002 group compared to DMSO controls ($p = 0.0168$, Fisher's exact test) at 100 mpi. C) There were significantly fewer neutrophils found within infected somite of fish treated with SB225002 compared to fish treated with the DMSO control ($p < 0.0001$, unpaired t-test) at 100 mpi. N = 74, 62 for DMSO and SB225002 respectively over 2 repeats.

4.3.12. Pharmacological inhibition of CXCR4 with AMD3100 does not affect neutrophil clustering and numbers in response to *Mm* somite infection

CXCR4 and its major ligand CXCL12 are involved in neutrophil signalling during inflammation, and inhibition of CXCR4 with AMD3100 has been shown to increase inflammation resolution and neutrophil reverse migration in a zebrafish tailfin injury model (Isles et al., 2019). Zebrafish were pre-treated with AMD3100 or a ddH_2O control at 4hpi and were infected with *Mm* into the somite at 2 dpf to test the effect of CXCR4 inhibition on neutrophil clustering (Figure 4.12 A). Images captured at 100 mpi were analysed for neutrophil clustering and neutrophil numbers within the somite. Prevalence of neutrophil clustering did not differ between larvae treated with AMD3100 compared to ddH_2O ($p = 0.5111$) at 100 mpi (Figure 4.12 B). Additionally, there was no difference in neutrophil counts within the somite between the treatment groups ($p = 0.4814$) at this time point (Figure 4.12 C). Together, these results indicate there is no effect of pharmacological CXCR4 inhibition on neutrophil clustering and numbers within the somite, and AMD3100 was not used for further experimentation.

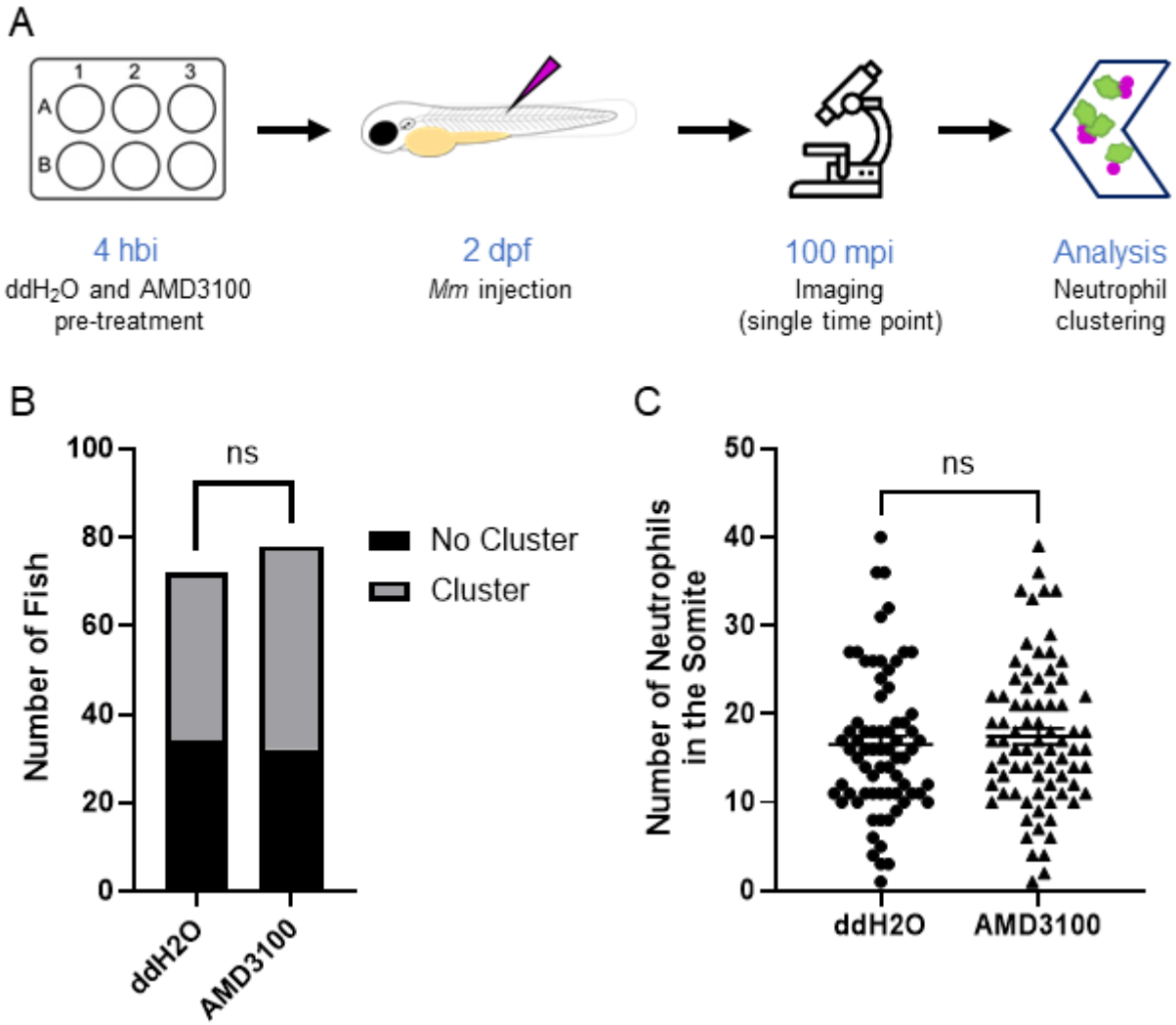


Figure 4.12 | CXCR4 inhibition with AMD3100 does not affect neutrophil clustering and numbers within the infected somite. A) Timeline of the experiment. Zebrafish were pre-treated with AMD3100 or a ddH_2O control and were injected with *Mm* into the somite at 2 dpf. Images captured at 100 mpi were analysed for neutrophil clusters and numbers within the infected somite. B) There was no significant difference in clustering prevalence between the AMD3100 group and the ddH_2O group ($p = 0.5111$, Fisher's exact test) at 100 mpi. C) There was no significant difference in neutrophil numbers within the infected somite between the treatment groups ($p = 0.4814$, unpaired t-test) at 100 mpi. N = 71, 78 for the ddH_2O and AMD3100 group respectively from 2 experimental repeats.

4.3.13. Pharmacological inhibition of NET formation with LDC7559 does not affect neutrophil numbers and clusters in response to *Mm* infection

Previously, inhibition of NETosis with LDC7559 resulted in a significant reduction of neutrophil swarming after a tailfin injury (Isles et al., 2021). Since then, this drug has been described to inhibit NET formation by inhibiting NOX2, rather than gasdermin D (Amara et al., 2021). LDC7559 was used in the somite model to examine if NETs contribute to neutrophil swarms that are observed after localised *Mm* infection. Larvae were pre-treated with DMSO or LDC7559 4 hours before infection and were injected into the somite with *Mm* at 2 dpf (Figure 4.13 A). Imaging was performed at 100 mpi and images were analysed for neutrophil clusters and numbers in the somite. At 100 mpi, there was no difference in the prevalence of neutrophil clustering between DMSO-treated and LDC7559-treated larvae ($p = 0.4173$) (Figure 4.13 B). There was also no significant difference in neutrophil numbers ($p = 0.6127$), with a mean of 15.46 neutrophil in the somite in the DMSO group and 16.00 in the LDC7559 group (Figure 4.13 C). Taken together, inhibition of NETosis through inhibition of NOX2 has no effect on neutrophil clustering or numbers within the somite, and therefore the drug was not used for further experimentation.

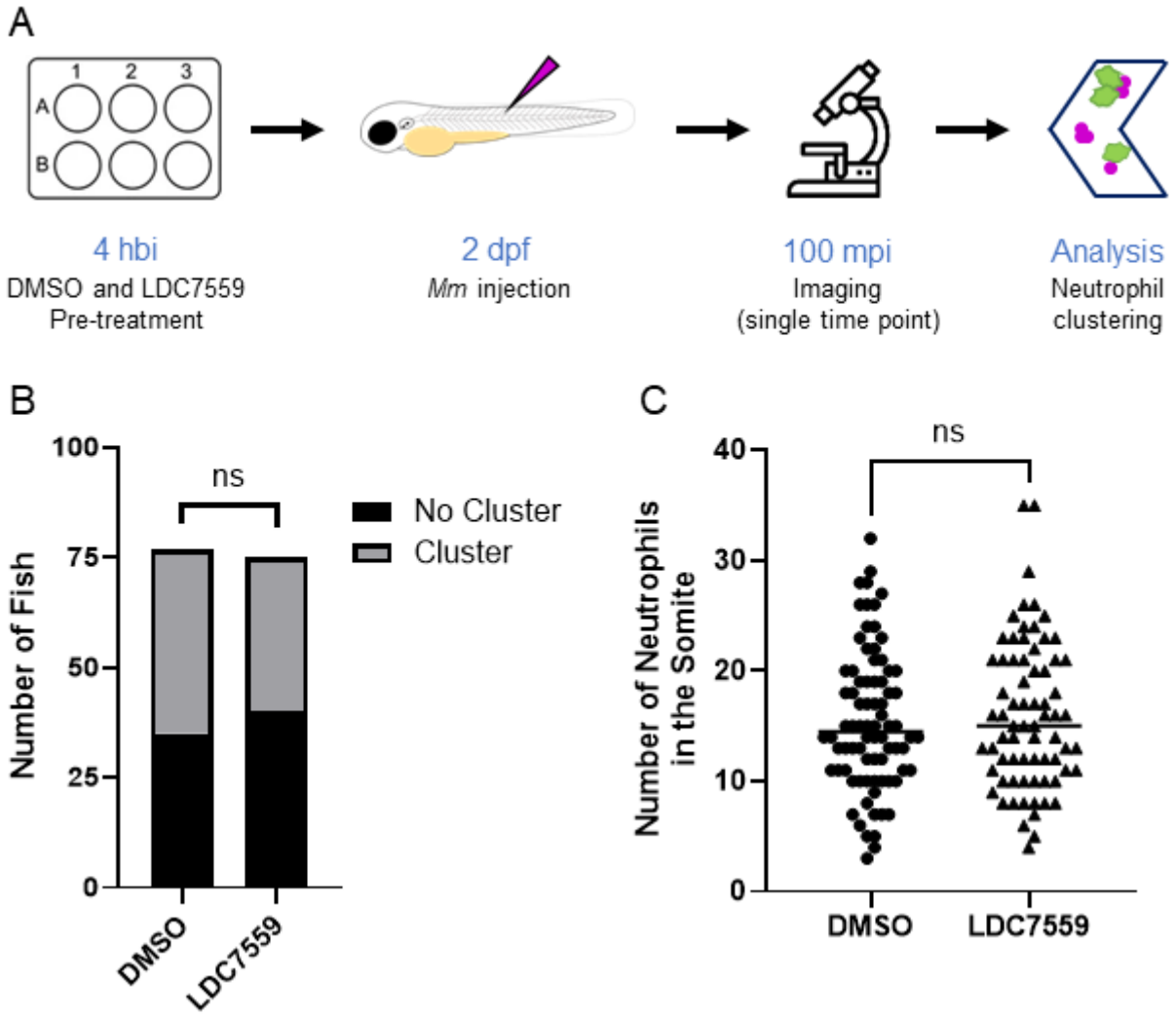


Figure 4.13 | Inhibition of NETosis with LDC7559 does not affect neutrophil clusters or numbers in the infected somite. A) Experimental overview. Zebrafish were treated with LDC7559 or a DMSO control at 4 hbi and were infected with *Mm* into the somite at 2 dpf. Images taken at 100 mpi were analysed for neutrophil clustering and numbers within the infected somite. B) There was no significant difference between the LDC7559 group and the DMSO group ($p = 0.4173$, Fisher's exact test) in terms of clustering prevalence at 100 mpi. Additionally, there was no significant difference found in neutrophil numbers within the infected somite between the treatment groups ($p = 0.6127$, unpaired t-test). N = 80, 72, for the DMSO group and LDC7559 group respectively from 2 experimental repeats.

4.4. Discussion

Neutrophil swarming is a complex neutrophil behaviour that is affected by a variety of signalling pathways, including LTB4 (Lämmermann, 2016). This leukotriene has been shown to be essential for distant neutrophil recruitment towards the neutrophil swarm in the secondary swarming response (Lämmermann et al., 2013). Its involvement has also been confirmed in a tailfin injury model in zebrafish, contributing to the finding that neutrophil swarming is conserved in zebrafish (Isles et al., 2021). This chapter further expands on the involvement of LTB4 in the swarming response in zebrafish, and explores the effect of knockdown of swarming mediators on neutrophil swarming in response to infection. Knockdown of *lta4h* significantly reduced the prevalence of swarming in response to injury, but not infection. Knockdown of *lta4h* reduced swarm duration and area, but did not affect neutrophil numbers within the somite over time. Pharmacological knockdown of LTA4H, CXCR4, and NETosis did not affect neutrophil clustering and numbers within the infected somite. Lastly, genetic inhibition of *cxcr2* was unsuccessful, and pharmacological inhibition significantly decreased neutrophil clustering and numbers within the infected somite. This establishes the swarming model as a means to investigate disruption of swarming mediator signalling on the neutrophil swarming process in response to infection as well as injury.

LTB4 signalling has been shown to be an essential component of neutrophil swarming in both mice and zebrafish (Isles et al., 2021; Lämmermann et al., 2013). Pharmacological knockdown of LTB4 with bestatin in a zebrafish *Mm* infection model has previously been described (Tobin et al., 2010), and was applied to the somite model to investigate its effect on neutrophil clustering. Bestatin was not found to have an effect on neutrophil clustering and neutrophil numbers in the somite in response to infection. In contrast, knockdown of LTA4H signalling by targeting *lta4h* and *blt1* significantly decreased neutrophil numbers at the tailfin wound at 6 hpw in a zebrafish injury model, but its effect on swarming was not described (Isles et al., 2021). Knockdown of LTA4H signalling in mice, both genetically and with bestatin, also showed a reduction in neutrophil accumulation towards the ear skin at 24 hpw (Oyoshi et al., 2012). These results bring into question whether the drug used in the clustering experiments was functional. Therefore, fish were treated with bestatin after systemic infection to assess its effect on bacterial burden at 5 dpf, as previous work has shown that bestatin treatment significantly increased bacterial burden in systemic infections (Tobin et al., 2010). Remarkably, bestatin treatment did not significantly affect bacterial burden in the present study. Additionally, it potentially has the biological effect of reducing bacterial burden compared to DMSO-treated larvae, which directly opposes the findings in the previous study (Tobin et al., 2010). If this discrepancy can be explained by biological variation, it would imply

the drug had no effect. In the present study, the drug was applied at 1 dpf directly after infections and the water was not refreshed daily. The previous study used infections on 2 dpf and refreshed the water daily (Tobin et al., 2010), therefore providing a more constant flow of bestatin and reducing the possibility of the drug losing efficacy over time. However, there may also be a biological reason for the observed reduction in bacterial burden. Research in an *Mtb* mouse model showed that LTB4 production was induced by *Mtb* in a 5-lipoxygenase-dependent manner, and that 5-lipoxygenase-deficient mice lungs contained significantly reduced *Mtb* bacterial burden (Bafica et al., 2005). This suggests LTB4 may play paradoxical roles in *Mtb* and *Mm* infections, and further complicates the interpretation of the results of bestatin treatment. For these reasons, bestatin treatment was not continued and LTB4 signalling was targeted genetically instead.

Genetic inhibition of *lta4h* to target LTB4 signalling in zebrafish has previously been described, but its effect on swarming prevalence was not discussed (Isles et al., 2021). A similar wounding experiment was carried out to further examine the role of LTB4 signalling in the neutrophil swarming response to injury, and found that swarming prevalence was significantly decreased in *lta4h* knockdown fish. Interestingly, this did not coincide with a statistically significant reduction in neutrophil numbers present at the wound site, indicating that the reduction in swarming prevalence did not result from a lack of neutrophil abundance near the wound. Increased swarming prevalence has also been described in other models, as an increase in neutrophil clustering prevalence in response to zymosan was shown to be dependent on LTB4 and BLT1 in a mouse cell culture model (Song et al., 2020). Another study described similar results, as photoactivation of calcium signalling in neutrophils induced neutrophil clustering, which was significantly abrogated after treatment with BLT1 and BLT2 antagonists (Khazen et al., 2022). This may be due to increased directional migration of neutrophils towards the swarm, orchestrated by LTB4 signalling. In a mouse ear dermis wound model, recruitment of relatively distant neutrophils towards the wound was significantly impaired in *Ltb4r1* knockout neutrophils compared to wildtype neutrophils (Lämmermann et al., 2013). Additionally, swarms with a higher number of neutrophils are more persistent than those with a relatively low number (Kienle & Lämmermann, 2016). A decrease in cluster size after disruption of LTB4 signalling has been described in response to a *Pseudomonas aeruginosa* footpad infection model with an *Ltb4r* knockout (Lämmermann et al., 2013), and a cell culture model of mouse neutrophils with pharmacological inhibition of 5-lipoxygenase with MK886 (Khazen et al., 2022). If a higher number of neutrophils in a swarm results in more stable swarms, and disruption of LTB4 signalling results in smaller swarms, this could in theory have contributed to the higher swarming prevalence found in the tailfin transection model.

Neutrophil recruitment towards the wound site at 6 hpw did not appear to be statistically affected by *lta4h* knockdown. This is not in accordance with previous results that showed a significant decrease in neutrophil numbers after disruption of LTB4 signalling with *lta4h* and *blt1* knockdown (Isles et al., 2021). Disruption of *Lta4h* in mice has been shown to significantly increase neutrophil numbers in lungs of influenza-infected mice (Snelgrove et al., 2010). This is thought to result from the inability of *Lta4h*-deficient mice in degrading proline-glycine-proline that is released from extracellular matrix collagen breakdown, which is aids in neutrophil chemotaxis (Snelgrove et al., 2010). In contrast, treatment with bufexamac, an LTB4 inhibitor, inhibited LTB4-release by neutrophils, inhibited neutrophil chemotaxis in a transwell assay, and constrained neutrophil infiltration into lungs of mice with acute lung injury (Xiao et al., 2016). Additionally, inhibition of *Ltb4r1* in a mouse ear skin wounding model showed a significant reduction in neutrophil numbers near the wound (Oyoshi et al., 2012). Finally, neutrophil swarming to live *Candida albicans* clusters was absent altogether in 5-lipoxygenase-deficient mice neutrophil cell cultures, which could not be rescued by LTB4 treatment (Hopke et al., 2022). These findings exemplify how disruption of LTB4 signalling may affect neutrophil recruitment, but they do not explain the discrepancy in neutrophil numbers observed in the present study compared to the previous results (Isles et al., 2021). Instead, there may have been a methodological difference in performing the tailfin transections. Neutrophil recruitment can be initiated by DAMPs that originate from the wound area (de Oliveira et al., 2016). Tailfin transections can be done with or without including part of the zebrafish notochord, with the former introducing a stronger neutrophil response (Xie et al., 2021). It is possible that the tailfin injuries from the present study included transecting a larger part of the notochord, therefore introducing an even bigger initial neutrophil response that is dependent on DAMPs.

Swarm duration in response to infection was significantly negatively impacted by knockdown of *lta4h*. This coincided with reduced swarm areas in *lta4h* CRISPants, yet neutrophil numbers within the somite remained unaffected over time. LTB4 signalling has been reported to be important for neutrophil cluster stability (Lämmermann et al., 2013). Knockout of *ltb4r1* in mice significantly reduced neutrophil cluster persistence in response to a *Pseudomonas aeruginosa* footpad infection (Lämmermann et al., 2013). Additionally, swarms are more likely to persist when a relatively high number of neutrophils present within the swarm (Kienle & Lämmermann, 2016). The reliance on LTB4 for swarming persistence is further evidenced by the mechanisms of swarming stop signals. Both Lipoxin A4 (LXA4) and Resolvin E3 (RvE3) have been unearthed as stop signals for swarming (Reátegui et al., 2017). LXA4 inhibited LTB4 release in human neutrophil cell cultures (Conti et al., 1990), and was shown to be increasingly released by human neutrophils during late swarming (Reátegui et al., 2017).

RvE3 may antagonistically bind to BLT1 and thereby suppress activity of BLT1 induced by LTB4 (Sato et al., 2019). Unlike LXA4, RvE3 release by human neutrophils appears to peak early at 30 after the start of swarming, and is continuously released at a stable rate from 1 hour until 3 hours after the start of swarming (Reátegui et al., 2017). Finally, ω-OH-LTB4 is a product of LTB4-oxidation by omega-hydroxylase and competes with LTB4 for binding to BLT1 (Song et al., 2023). ω-OH-LTB4 elicits a much weaker neutrophil response and thereby acts as a natural inhibitor of LTB4 (Golenkina et al., 2022). In response to *Salmonella enterica* Typhimurium and subsequent fMLP stimulation, the ratio of LTB4 to ω-OH-LTB4 increased in the favour of LTB4 when the ratio of bacterial burden per present human neutrophil increased (Golenkina et al., 2022). This may suggest that LTB4 is instead inhibited through conversion to ω-OH-LTB4 in the event of a plentiful neutrophil response at the site of infection (Song et al., 2023). The reduction in swarm duration and swarm area in response to *ltb4* knockdown, combined with the findings that *ltb4* knockdown does not affect swarming prevalence and neutrophil numbers in the somite over time, suggests LTB4 signalling is required for swarm stability, and that other mediators are more important for the formation of swarms in the somite infection model.

One such mediators is CXCR2, which is one of the main chemokine receptors involved in neutrophil trafficking (Metzemaekers et al., 2020), and which plays a pivotal role in neutrophil aggregation during swarming (Lämmermann et al., 2013). To knock down *cxcr2*, multiple sites within the singular exon of the gene were targeted for CRISPR-Cas9 gene editing. CXCR2 has been extensively studied in decades of research, but there is a distinct lack of available CRISPRs targeting this gene in zebrafish in literature despite its popularity. In hindsight, it is not surprising that all 6 CRISPR targets were unable to cause considerable deletions in the gene. Even combination of multiple crRNAs in a single injection mix, which has been shown to increase knockdown efficiency (Kroll et al., 2021), did not appear to introduce heightened knockdown efficiency. The lack of CRISPR-mediated *cxcr2* knockdown in existing literature despite the well-studied status of the gene may indicate that attempts to knock the gene down with CRISPR have been unsuccessful and therefore remained unpublished. It is unclear why these CRISPR targets were not able to be knocked down. It is possible that the crRNAs included protospacer adjacent motifs (PAMs) that targeted inaccessible regions of the DNA (Christie & Kleinstiver, 2021). These PAM sequences are added to the crRNA sequence and are required for proper functioning of the Cas9 protein (Christie & Kleinstiver, 2021). The gene may potentially reside within a nucleosome, which protects the target DNA from digestion by Cas9, instead of being located on the more easily accessible edge of a nucleosome (Handelmann et al., 2023). Regardless of the underlying mechanisms, the failure of the

multiple CRISPR targets to knock down *cxcr2* indicated pharmacological knockdown may be a more successful avenue.

The drug SB225002 has extensively been used to study the effect of CXCR2 knockdown in zebrafish (de Oliveira et al., 2013; Q. Deng et al., 2013; Gratacap et al., 2017; Kaveh et al., 2020; Zuñiga-Traslaviña et al., 2017). In the somite infection model, pharmacological knockdown of CXCR2 resulted in a significant reduction in both neutrophil clustering prevalence and neutrophil counts within the infected somite. The latter is in accordance with previous results that show a significant reduction in the number of neutrophils that reach a tailfin injury after CXCR2 inhibition with SB225002 (Zuñiga-Traslaviña et al., 2017). A problem with the reduction in neutrophil numbers in the infected somite is that the reduction in swarming prevalence may be a direct consequence of the reduced neutrophil availability. It then becomes very difficult to examine the effect of CXCR2 knockdown during neutrophil swarming, as swarming in fish with CXCR2 knockdown becomes exceedingly rare. CXCR2 was thus not considered for further examination.

The significant reduction of clustering prevalence and neutrophil numbers within the somite after CXCR2 inhibition, and not *lta4h* knockdown, suggest non-LTB4 neutrophil recruitment signals may be more relevant than LTB4 during the initiation of the swarming response to infection in the somite model. Needle injury paired with infection induces both DAMPs and PAMPs that may in turn recruit neutrophils towards the somite (de Oliveira et al., 2016). LTB4 signalling has been shown to be important for recruitment of more distant neutrophils, and its knockdown did not affect recruitment of neutrophils patrolling relatively close to the wound centre in a mouse ear injury model (Lämmermann et al., 2013). *Ltb4r1* knockout also induced smaller clusters, which are associated with less stable swarms (Lämmermann et al., 2013). Investigations of zymosan-induced neutrophil swarming showed LTB4R works in tandem with CXCR1 and CXCR2 signalling in the recruitment of distant neutrophils towards the swarm (Reátegui et al., 2017). Blocking of CXCR1 and CXCR2 did not affect neutrophil recruitment, but blocking CXCR1, CXCR2, LTB4R1, and LTB4R2 significantly impaired neutrophil recruitment, which was an exacerbated effect compared to LTB4R1 and LTB4R2 blocking alone (Reátegui et al., 2017). Inversely, the CXCR2-dependent recruitment may be dependent on LTB4 signalling, as CXCR1/CXCR2 ligands CXCL1 and CXCL5 were not able to induce neutrophil recruitment to knee joints of mice with Rheumatoid Arthritis when only LTB4 was knocked down (Grespan et al., 2008). Considering inhibition of CXCR2, but not *lta4h*, in the somite infection model significantly abrogated initial neutrophil recruitment and clustering prevalence, it stands to reason that CXCR2 may be the more dominant factor in the initial recruitment to infection in this model. A potential explanation for this is the distance of the site

of challenge from the CHT, which is considerable smaller in the somite infection model compared to the tailfin injury model. CXCR2, and potentially DAMPs and PAMPs, may therefore be primarily responsible for consistent neutrophil influx into the somite over time, while LTB4 further directs neutrophils towards swarms and acts as a retention signal. In the somite infection model, neutrophils may not be as reliant on LTB4 signalling and instead are more influenced by DAMPs, PAMPs, and CXCR2 signalling.

While CXCR2 was excluded from further examination in this thesis, it is of interest for future research. This may instead focus on knocking down CXCR2 signalling later during the swarming response instead of 4 hours before infection. For instance, infected fish can be assessed for neutrophil swarming at the same time point used here. The group of swarming fish could then be treated with SB225002 or with DMSO and subsequently used for time lapse imaging to examine how CXCR2 knockdown affects neutrophil swarming in response to *Mm*. CXCR2 was previously reported to significantly inhibit neutrophil recruitment to infection after 1 hour of pre-treatment and 1 hour of treatment post infection (Q. Deng et al., 2013), suggesting the drug swiftly exerts its effect. Unfortunately, these experimental changes may result in data with a substantial amount of variation, and the experimental procedure would have to be swiftly executed in order to apply treatments within the swarming window. A small window of time in-between subsequent images of each fish will also be required to properly assess swarm progression and potential early resolution, but this will significantly impact the maximum sample size per experimental repeat. Further experimentation with CXCR2 in the somite swarming model may therefore prove difficult.

CXCR4 is another chemokine receptor that was of interest for its potential involvement in the swarming process. Pharmacological inhibition with AMD3100 did not lead to differences in neutrophil clustering prevalence and neutrophil numbers within the infected somite at 100 mpi. These data suggest that CXCR4 signalling does not play a role in neutrophil clustering and accumulation in response to *Mm* infection. However, previous research in a zebrafish tailfin injury model showed CRISPR-mediated knockdown of *cxcr4b* and *cxcl12a* led to significantly increased neutrophil inflammation resolution (Isles et al., 2019). This was also the case with pharmacological inhibition with AMD3100, which decreased the number of neutrophils at the wound during late inflammation and which increased reverse migration of neutrophils (Isles et al., 2019). Similarly, CXCR4 inhibition with AMD3100 in mice reduced neutrophil retention in mice lungs after inhalation of crystalline silica in dust (Q. Sun et al., 2022). In infection, miR-206-depleted zebrafish infected with *Mm* into the trunk showed increased neutrophil numbers and longer-lasting neutrophil response at the site of infection (K. Wright et al., 2021). Finally, CXCR4 is important for neutrophil release from the bone marrow, but this can be circumvented

with the use of AMD3100, which was shown to not affect whole body neutrophil counts in zebrafish at 24 hours after administration (Isles et al., 2019). Taken together, these published findings offer strong evidence for potential involvement of CXCR4 signalling in neutrophil swarming, but evidence to support this was not found in the localised *Mm* infection model.

The time point of observation for neutrophil clusters and counts was at 100 mpi based on previous experiments. Throughout the thesis, most swarms under normal conditions start resolution after 200 mpi, and it was theorised that a loss of CXCR4 signalling would bring this time forward. However, the effect may have been overestimated, resulting in an imaging time point that was too early to recognise any effect CXCR4 knockdown may have had on swarming resolution. These experiments may be redone with the inclusion of extra time points to further pinpoint the time at which swarm resolution occurs.

Finally, inhibition of NETosis with LDC7559 was previously reported to significantly reduce swarming prevalence in a zebrafish tailfin transection model (Isles et al., 2021), but the drug had no effect on cluster prevalence and neutrophil numbers within the somite in response to *Mm* infection. NET release has been shown to increase with cell density in neutrophil cell cultures, and NETotic neutrophils in neutrophil plaques are able to induce NETosis in naïve neutrophils surrounding the plaque (P. Deng et al., 2024). Swarm-initiating pioneer neutrophils at zebrafish tailfin wounds were also found to release NET-like structures, and swarming neutrophils were seen expelling chromatin (Isles et al., 2021). Additionally, NET release in neutrophil swarms in microarray experiments restricted fungal growth of *Candida albicans* and contributed to swarm expansion (Hopke et al., 2020). It is unclear if NETosis occurred in response to *Mm* infection in the somite model, even less if swarming neutrophils perform NETosis. The virulent bacterial strain *Mtb* H37Rv elicited NET release in human neutrophil cell cultures by 180 mpi, and NET levels were further increased by 380 mpi (Ramos-Kichik et al., 2009). This indicates *Mtb* is capable of inducing rapid NET formation, but it is unclear how this translates to *Mm* infection in zebrafish.

While the lack of effect of LDC7559 suggests NETs are not involved in neutrophil clustering and neutrophil accumulation in the *Mm*-infected somite, this cannot be ruled out completely. While this drug targets NADPH oxidase-dependent NET formation (Amara et al., 2021), there is another pathway that can activate NETosis independently of NADPH oxidase (Rosazza et al., 2020). This pathway instead occurs after activation of neutrophils and subsequent calcium fluxes, but the mechanisms of the remainder of the pathway are unclear (Rosazza et al., 2020). Due to the involvement of multiple pathways of NET formation, future research should attempt to target multiple facilitators of NETosis to rule out its involvement in *Mm* in the somite

infection model. Additionally, transgenic zebrafish lines can confirm presence of NET-like structures in swarms or neutrophils near infection sites. One such line, T*g(mpx:GFP)i114;Tg(lyz:h2az2a-mCherry,cmlc2:GFP)sh530*, labels neutrophils and neutrophil histone H2az2a (H2A) and has previously been used to determine swarming neutrophils release NET components (Isles et al., 2021). This line was used in conjunction with *Mm* Crimson to assess swarm formation in response to *Mm* infection (data not shown), but considerable fluorescence bleed-through issues caused by *Mm* massively complicated determination of NET formation, and further experimentation was suspended. Instead, knockdown of other NETosis mediators can be explored. NE is important in NADPH oxidase-dependent NET release (Rosazza et al., 2020), and its inhibition with MeOSu-AAPV-CMK significantly reduced swarming prevalence in a zebrafish tailfin transection model (Isles et al., 2021). MPX, called MPO in humans, inhibition in this model was achieved through CRISPR-Cas9-mediated knockdown, but did not affect swarming prevalence (Isles et al., 2021). Nevertheless, this is still a target of interest as its inhibition or deficiency results in the abrogation of NET production during *Candida albicans* infection in human neutrophil cultures and mice (Guiducci et al., 2018; Metzler et al., 2011). Finally, DNase I treatment is used in mice and cell cultures to break down NETs (Ramos-Kichik et al., 2009; Veras et al., 2023), but DNase I treatment of zebrafish by submersion is toxic (Isles et al., 2021). In short, future research should explore NET inhibition through the mediators described above to fully examine the involvement of NET formation during neutrophil swarming to *Mm* in zebrafish.

A limitation of the somite infection model to investigate neutrophil swarming that is evident in this chapter is the low statistical power resulting from low sample sizes. Many experiments performed here introduced up to 2 groups per experimental repeat, meaning the sample size per group was significantly reduced compared to the majority of experiments in Chapter 3. Additionally, swarming prevalence is around 50%, meaning the sample size is further reduced when examining swarm-specific parameters such as duration and area. Power calculations could not always be performed as the zebrafish somite infection swarming model and its mediation in response to *Mm* has not previously been described. Instead, post-hoc power calculations were done to assess power of the experiments. These calculations were done with the following formula: 2*7.9*(SD control group*SD control group)/(20% expected difference*20% expected difference). The swarm duration of the injury experiments in Figure 4.4 F are used to serve as an example. The required sample size for 80% confidence is 135 fish, calculated with an SD of 87.24 and 20% expected difference of 29.88. The sample sizes during these experiments were 53 and 65 for the groups individually, but only 18 swarms were present in control fish compared to 10 in CIRSPants. Statistical analyses involving swarm-

specific parameters, like duration and area, therefore suffer additionally from limited sample sizes.

This may be partially resolved by changing experimental procedures, but there are a variety of limiting factors in the experimental setup. Neutrophil swarming in response to *Mm* infection often commences within an hour after starting infections. In order to increase the likelihood of capturing all swarms during early infection, all experimental procedures must be finished within this hour. Increasing the sample size means increasing the time it takes to infect, mount, and prepare fish for imaging. It is unlikely that this process can be significantly improved without the addition of another person performing the methodology. Alternatively, the initiation of imaging could be pushed back if the experiment allows it, like in late swarming investigations. Unfortunately, the increased sample size resulting from these solutions means the time it takes for each to be imaged in a cycle is increased, which forces increasing the cycle time during time lapse imaging. Using the current hardware, transgenic neutrophil line, and fluorescent bacteria, a cycle time of 10 minutes guaranteed enough time to image about 24 fish in total. This would reduce the sample size per group to 6 if there were 4 groups present. A possible solution to this problem would be to use better hardware with faster imaging speeds. Additionally, a brighter strain of *Mm* could significantly decrease the exposure time, as this fluorescent channel is responsible for the majority of the imaging time. The drug treatment experiments were performed with the low power of the neutrophil swarming time lapse experiments in mind and significantly increased the number of fish in each experiment, but they also reduced temporal resolution with a single time point. However, addition of a few extra time points with a 20-minute cycle time to these experiments may drastically improve accuracy of clustering neutrophils. Finally, increasing the prevalence of neutrophil swarming could significantly lower sample size requirements if investigating swarm-specific parameters. In the mouse ear dermis injury model, mice underwent skin trauma at 3 to 4 hours to recruit endogenous neutrophils to the dermal interstitium before focal laser injury and subsequent swarming assays (Lämmermann et al., 2013). Alternatively, exogenous neutrophils were injected into the ear dermis prior to swarming assays (Lämmermann et al., 2013). Both of these methods increase neutrophil presence close to the site used for the swarming, and prime the host for an optimal swarming response to subsequent laser injury (Lämmermann et al., 2013). In infection, neutrophils can be primed to enhance their functions, such as phagocytosis, chemotaxis, and granule release, during subsequent immune challenge (Miralda et al., 2017). One such priming agents is LPS, which has been used at low doses to prime neutrophils in a wide range of studies over the last few decades (Condliffe et al., 1998; Miralda et al., 2017). Treatment of zebrafish with *Escherichia coli*-derived LPS has been shown to be well-tolerated, as doses of 50 µg/ml and below did not kill zebrafish at 2 dpf

(Novoa et al., 2009). Low dose treatment of LPS may therefore prime neutrophils and subsequently potentiate the swarming response to *Mm*. In short, the current experimental procedures can be adapted to potentially improve sample sizes and, therefore, statistical power.

In conclusion, this chapter shows the involvement of LTB4 in the neutrophil response to *Mm* infection in the somite infection model. These results further bolster the status of LTB4 as an important mediator in the swarming response and establish the zebrafish as a suitable model for investigation into swarming mediation by LTB4 and other signals. Future investigation into mediation of the swarming response should take into consideration the adaptations that can be made to the experimental setups to further increase their effectiveness.

5. Hif-1α stabilisation is protective against *Mm* and potentiates neutrophil swarming

5.1. Introduction

One of the main regulators of the hypoxia response, Hypoxia-Inducible Factor (HIF), is a transcription factor involved in the modulation of hundreds of genes (J. W. Lee et al., 2019) that target processes such as neutrophil survival and phagocytosis (Kling et al., 2021). The HIF transcription factor has 2 subunits called HIF-α and HIF-β that act as heterodimers, both of which are constitutively expressed (Masoud & Li, 2015). However, during normoxic conditions prolyl-4-hydroxylase (PHD) enzymes hydroxylate HIF-α, which targets HIF-α for proteasomal degradation (J. W. Lee et al., 2019). The activity of these PHD enzymes is suppressed during hypoxia, resulting in the stabilisation of HIF-α (J. W. Lee et al., 2019). It then translocates into the nucleus and bind to the HIF-β subunit to form a transcription complex (Vito et al., 2020). This complex then binds to co-activators and subsequent hypoxia responsive elements to affect activation of HIF target genes (Masoud & Li, 2015).

HIF-1α, has been shown to be important for neutrophil function in inflammation (Tang et al., 2023). Human peripheral blood neutrophils were shown to have increased survival in hypoxic conditions due to HIF-1α-dependent inhibition of apoptosis by NF-κB (Walmsley et al., 2005). Hypoxia was also shown to contribute to the phagocytosis of heat-inactivated streptococci by neutrophils (Walmsley et al., 2006). Increased phagocytosis was also seen in neutrophils from patients with VHL disease, a disease in which there are disruptions in a regulator of HIF-α proteasomal degradation (Walmsley et al., 2006). HIF-1α is essential for anaerobic generation of ATP, and HIF-1α deficiency has been shown to significantly impair myeloid functions such as aggregation, motility, and bacterial killing (Cramer et al., 2003). Such importance of HIF-1α signalling in neutrophils is clearly evidenced by studies examining HIF-1α in disease. Healthy human neutrophils and arthritic mouse neutrophils were shown to induce increased expression of macrophage migration inhibitory factor (MIF) and IL-23, which overexpression lead to arthritis (Nakamura et al., 2024). Inhibition of HIF-1α attenuated or prevented spondyloarthritis pathology in mice (Nakamura et al., 2024). In a mouse *Aspergillus fumigatus* infection model, knockout of myeloid *Hif-1α* significantly reduced granuloma size and increased fungal burden, which was associated with deficient neutrophil recruitment and survival (da Silva-Ferreira et al., 2022). Stabilisation of Hif-1α in zebrafish both pharmacologically and genetically decreased *Mm* bacterial burden in a systemic infection model in a process that was dependent on nitric oxide (Elks et al., 2013). Finally, HIF-1α stabilisation during *Mm* infection significantly increases IL-1β production, which was required

for the aforementioned host-protective effect of the HIF-1α NO response (Ogryzko et al., 2019). These findings indicate manipulation of the HIF pathway can be used to potentiate neutrophil function and potentially aid the host in defending itself against pathogenic threats.

Modulation of hypoxia signalling in zebrafish has previously been performed both pharmacologically and genetically (Elks et al., 2011, 2013). The drug dimethyloxalylglycine (DMOG) stabilises Hif-1α and can be used on zebrafish larvae via immersion (Elks et al., 2011). Genetically, Hif-1α can be stabilised by microinjections at the one cell stage of *hif-1α* RNA that has undergone mutagenesis targeting its hydroxylation sites, resulting in a stable protein that cannot be targeted for hydroxylation (Elks et al., 2011). These methods have since been used in a number of studies to investigate the effect of Hif-1α stabilisation on a variety of neutrophilic behaviours, such as inflammation resolution (Elks et al., 2011; Schild et al., 2020) and *Mm* bacterial clearance (Elks et al., 2013; Schild et al., 2020). Additionally, successful stabilisation of Hif-1α can be visualised with a transgenic zebrafish line that contains GFP-labelled PHD3, one of the PHD enzymes responsible for hydroxylation of Hif-1α (Santhakumar et al., 2012). Visible GFP expression in the body of zebrafish larvae indicates the PHD3 enzyme is expressed in an effort to combat Hif-1α stabilisation, indicating pharmacological or genetic stabilisation of Hif-1α was successful (Santhakumar et al., 2012).

5.1.1. Hypothesis and aims

Stabilisation of Hif-1α has previously been shown to be host-protective in response to *Mm* infection in a zebrafish systemic infection model (Elks et al., 2013). It is unclear if this protective effect of Hif-1α stabilisation can be observed in the somite infection model that has been established in this thesis, and if this potential host-protective effect is dependent on neutrophil swarming.

I hypothesise that Hif-1α stabilisation is protective against *Mm* by potentiating neutrophil swarming.

To investigate this hypothesis, Hif-1α was stabilised both pharmacologically and genetically, and its effect on *Mm* bacterial burden and neutrophil swarming was observed. Knockdown of *lta4h* in conjunction with Hif-1α stabilisation was used to further examine the link between hypoxia pathway modulation and neutrophil swarming. This was done to address the following aims:

- Examine if the host-protective effect of Hif-1α stabilisation is maintained in the somite infection model

- Investigate the effect of Hif-1α stabilisation on neutrophil swarming
- Evaluate the effect of Hif-1α stabilisation on infection outcome

5.2. Materials and Methods

5.2.1. Drug treatment

For the bacterial burden experiments, injected larvae were transferred to a Costar 6-well plate (Corning) at 1 hpi. Each well contained 3 ml clear E3 with either 3 µl DMSO (Sigma-Aldrich) or 3 µl 100 µM DMOG (Enzo Life Sciences), depending on the experimental group. In experiments with FG-4592, fish were treated with a final concentration of 2.5 µM FG-4592 (Selleckchem). Larvae were treated overnight and were transferred to clean petri dishes with clear E3 the morning after initiation of treatment. For time-lapse experiments, fish were pre-treated with DMSO or DMOG at 4 hbi. Treatment continued immediately after infections until mounting. DMSO and DMOG were also added to the LMP agarose (Sigma-Aldrich) that was used to mount the fish, at the same final concentration as used during standard treatment.

5.2.2. Genetic Hif-1α stabilisation

Genetic stabilisation of Hif-1α was achieved by injecting PR or DA1 into the yolk of zebrafish eggs up to the 4 cell stage. DA1 (stock: ~4000 ng/µl) was diluted in 20 µl Phenol Red (1:10 diluted in MiliQ, Sigma-Aldrich) for a final concentration of 50 ng/ml for injection. Combination of Hif-1α stabilisation with CRISPR-mediated knockdown of *lta4h* was done using 4 different mixes, as displayed in Table 5.

Table 5 | Genetic Hif-1α stabilisation and *lta4h* knockdown mixes.

Mix 1	Mix 2	Mix 3	Mix 4
TRACR RNA, 1 µl	TRACR RNA, 1 µl	TRACR RNA, 1 µl	TRACR RNA, 1 µl
crRNA, 1 µl	crRNA, 1 µl	crRNA, 1 µl	crRNA, 1 µl
Cas9 Protein, 1 µl	Cas9 Protein, 1 µl	DEPC, 1 µl	DEPC, 1 µl
PR, 1 µl	DA1 1 µl	PR, 1 µl	DA1 1 µl

5.3. Results

5.3.1. Pharmacological stabilisation of Hif-1α is host-protective in the somite infection model

Stabilisation of Hif-1α with the drug DMOG has previously been shown to be host-protective in a zebrafish systemic infection model, as it significantly decreased whole body bacterial burden compared to the DMSO control group (Elks et al., 2013). The somite infection model used throughout this thesis utilises a much smaller-scale infection and it was unclear if a host-protective effect of Hif-1α stabilisation can be observed here as well. Therefore, fish were infected with *Mm* into the somite at 2 dpf (Figure 5.1 A). Infected fish were pooled, then split randomly into 2 groups treated with DMSO and DMOG overnight immediately after infection. They were imaged at 3 dpi and images (Figure 5.1 B) were analysed for bacterial burden. There was a significant reduction in bacterial burden after treatment with DMOG compared to DMSO ($p = 0.0311$), indicating Hif-1α stabilisation is host-protective in the somite infection model (Figure 5.1 C). However, the infections in these fish often grew substantially over the course of 3 days, resulting in big infections suggesting infection levels that could not be controlled by local leukocytes. The experiments were therefore repeated with at an earlier time point of 1 dpi to avoid extreme bacterial growth (Figure 5.2).

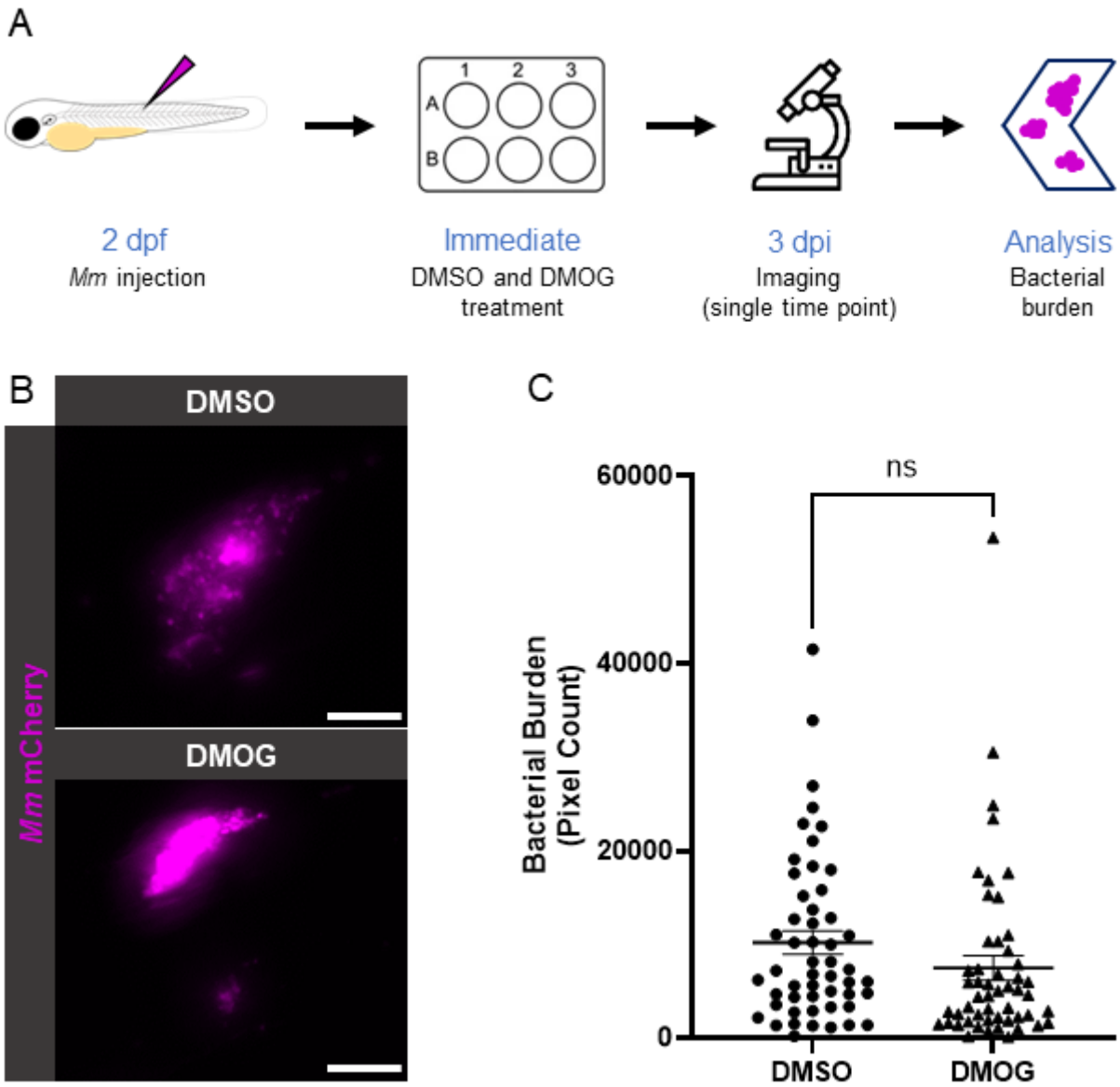


Figure 5.1 | Pharmacological Hif-1α stabilisation is host-protective in *Mm* somite infections at 3 dpi. A) Schematic overview of the experimental procedure. Fish were infected with *Mm* into the somite at 2 dpf. Infected fish were then pooled and immediately treated with DMSO or DMOG overnight. At 3 dpi, fish were imaged and these imaged were analysed for bacterial burden. B) Examples of infections found in DMSO- and DMOG-treated fish close to the mean in C. Scale bars are 200 µm. C) DMOG treatment significantly reduced *Mm* bacterial burden compared to DMSO treatment ($p = 0.0311$, unpaired t-test) at 3 dpi. Error bars are mean ± SEM, and N = 70 per group from 3 experimental repeats.

Fish were injected with *Mm* at 2 dpf and subsequently treated with DMSO or DMOG but were imaged at 1 dpi instead of 3 dpi (Figure 5.2 A). Images (Figure 5.2 B) were analysed for bacterial burden. At this time point, there was no significant reduction in bacterial burden after treatment with DMOG compared to DMSO ($p = 0.1342$) (Figure 5.2 C). Taken together, these results indicate that the host-protective effect of Hif-1α stabilisation found in systemic *Mm* infections can be observed in the somite infection model, but this effect is only substantial at a later time point.

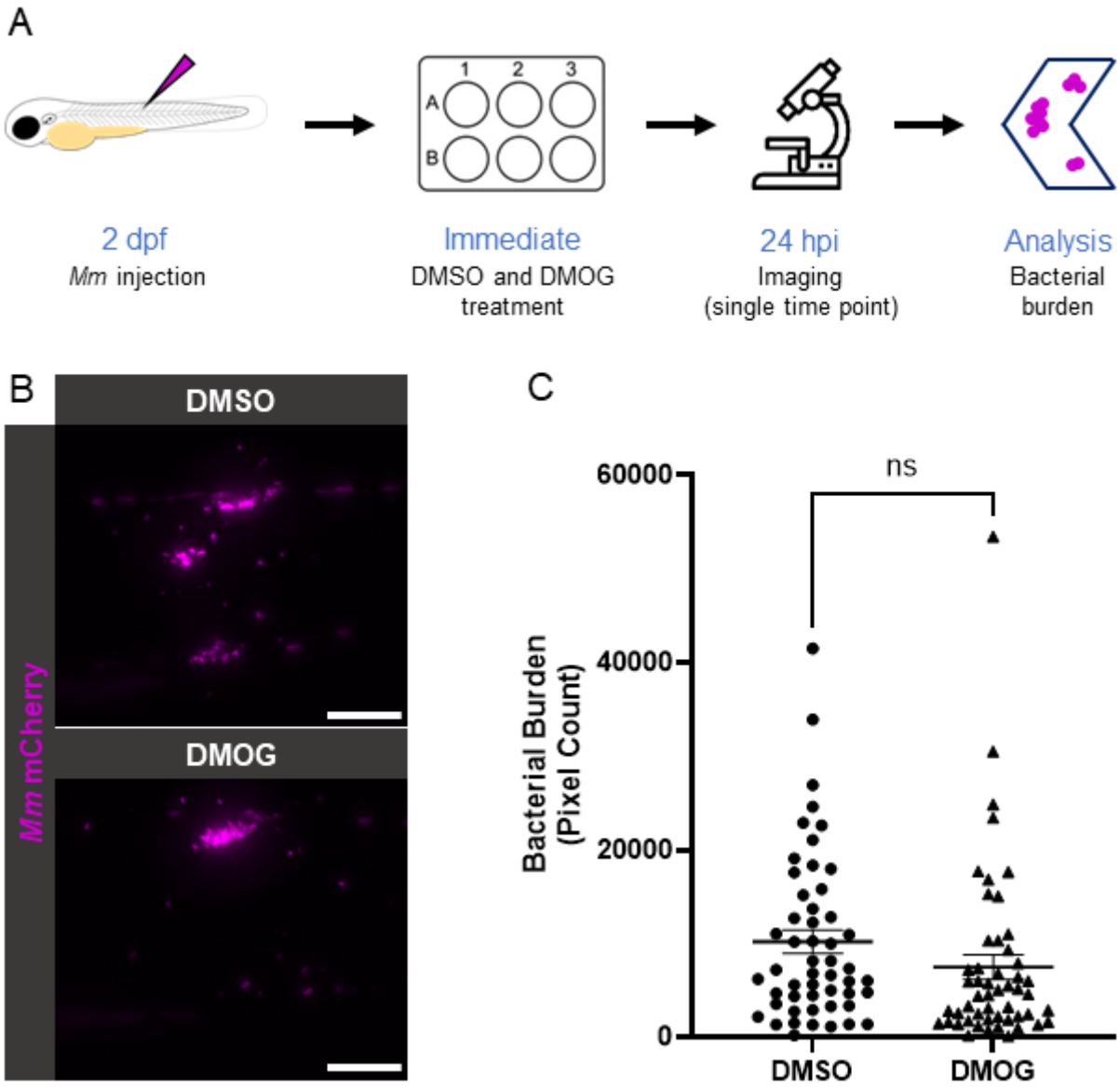


Figure 5.2 | Pharmacological Hif-1α stabilisation is host-protective in *Mm* somite infections at 24 hpi. A) Schematic overview of the experimental procedure. Fish were infected with *Mm* into the somite at 2 dpf. Infected fish were then pooled and immediately treated with DMSO or DMOG. At 24 hpi, fish were imaged and these imaged were analysed for bacterial burden. B) Examples of infections found in DMSO- and DMOG-treated fish close

to the mean in C. Scale bars are 200 µm. C) DMOG treatment did not reduce *Mm* bacterial burden compared to DMSO treatment ($p = 0.1342$, unpaired t-test) at 24 hpi. Error bars are mean ± SEM, and N = 52 per group from 3 experimental repeats.

5.3.2. Pharmacological stabilisation of Hif-1α does not affect neutrophil swarming in response to *Mm* infection

Considering Hif-1α stabilisation with DMOG was host-protective in the somite infection model, it is possible that neutrophil swarming may be partially responsible for this. First, it was examined if DMOG treatment affects neutrophil swarming. Larvae were treated with DMOG 4 hours before infection and were infected with *Mm* into the somite at 2 dpf (Figure 5.3 A). Time lapse imaging followed at 1 hpi and time lapse videos were analysed for neutrophil swarming. Swarms were present in both treatment groups (Figure 5.3 B) and there was no significant difference in swarming prevalence between the groups ($p = 0.7994$) (Figure 5.3 C). Additionally, there was no difference in swarming endpoints in DMOG-treated fish compared to DMSO-treated fish ($p = 0.2197$) (Figure 5.3 D). Finally, treatment did not affect swam durations of 13 DMSO swarms and 8 DMOG swarms that could be seen commencing and resolving within the time-lapses ($p = 0.5567$) (Figure 5.3 E). Taken together, these results indicate that DMOG treatment does not affect neutrophil swarming.

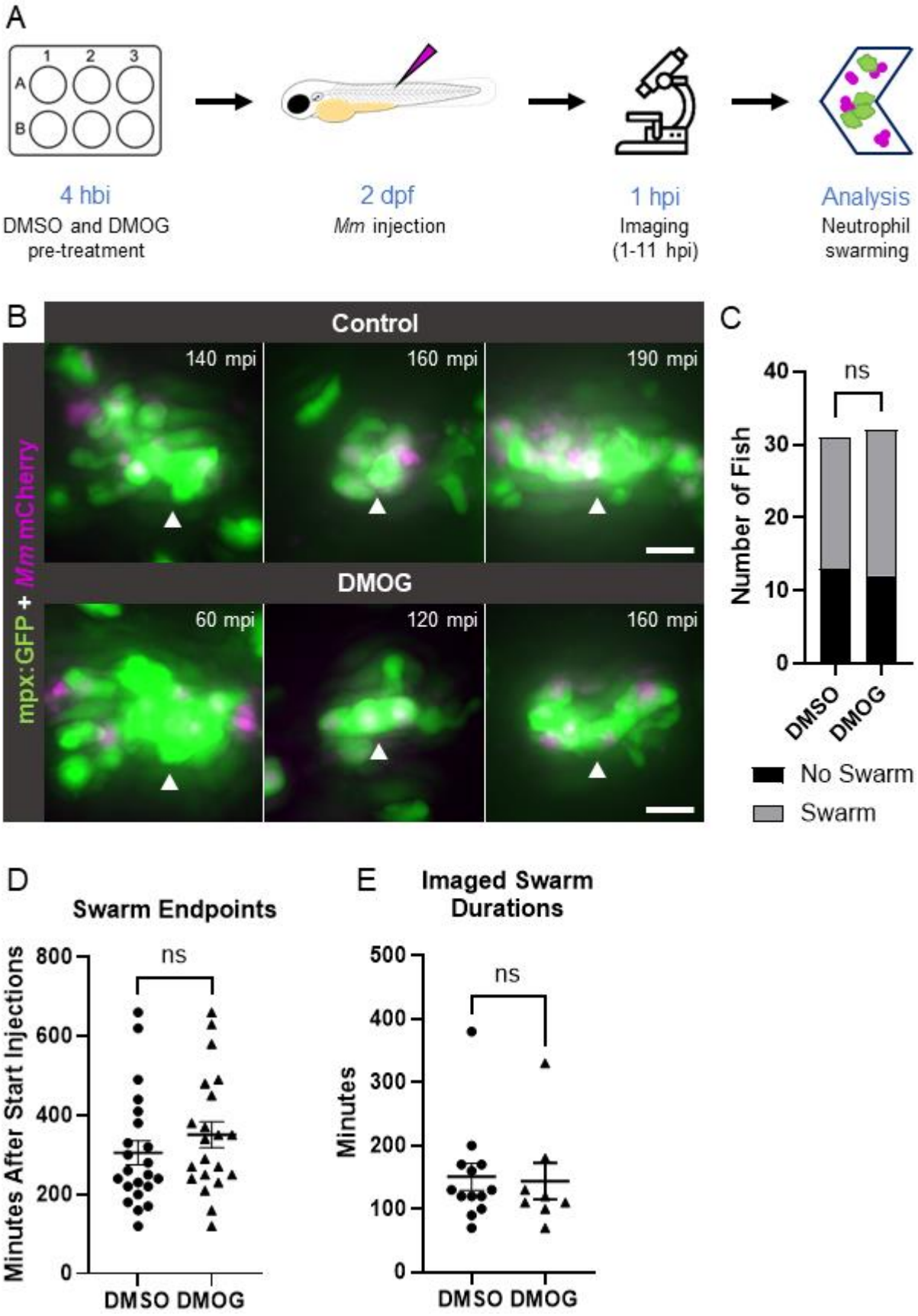


Figure 5.3 | Neutrophil swarming is not affected by pharmacological Hif-1α stabilisation.

A) Timeline of the experimental procedures. At 4 hbi, fish were pre-treated with DMSO or

DMOG and were subsequently infected with *Mm* into the somite at 2 dpf. They were then imaged for 10 hours as of 1 hpi, and time lapse movies were analysed for neutrophil swarming. B) Examples of neutrophil swarms (white arrowheads) found in DMSO-treated fish and DMOG-treated fish. Scale bars are 50 µm. C) There was no significant difference in swarming prevalence between the DMOG group and DMSO group (*p = 0.7994*, Mann-Whitney test), with 22 and 21 swarms in these groups respectively. D) There was no significant difference in endpoints of neutrophil swarms found in DMSO- or DMOG-treated fish (*p = 0.2197*, Mann-Whitney test). E) When only including neutrophil swarms that could be observed commencing and resolving within the time-lapses, there was no difference found in swarm durations between the DMSO group and the DMOG group (*p = 0.5567*). This included 13 swarms in the DMSO group and 8 swarms in the DMOG group. Error bars are mean ± SEM, and N = 31, 32 accumulated from 3 repeats for the DMSO group and the DMOG group respectively.

5.3.3. DA1 achieves easily verifiable and reliable stabilisation of Hif-1α

Injection of dominant active *hif-1α* (DA1) into the zebrafish yolk has previously been shown to stabilise Hif-1α, which decreased *Mm* bacterial burden in a systemic infection (Elks et al., 2013). DA1 was first examined for its efficacy in achieving Hif-1α stabilisation so it could later be used to investigate its effect on neutrophil swarming. A *Tg(phd3:GFP)i144* zebrafish line to validate Hif-1α stabilisation after DA1 injection as previously described (Santhakumar et al., 2012). Mutation of proline hydroxylation sites of DA1 that are normally targeted by prolyl hydroxylases (PHDs) results in stabilisation of Hif-1α, as the PHD enzymes are unable to hydroxylate DA1 (Elks et al., 2011). Injection of DA1 will therefore lead to expression of *phd3:GFP*, which acts as a reporter of Hif-1α activity. Indeed, injection of DA1 into the yolk resulted in clear expression of *phd3:GFP* at 1 dpi, which indicates stabilisation of Hif-1α was successful (Figure 5.4 A). Of all DA1-injected, 12 fish showed no signs of GFP expression, comparable to uninjected fish, indicating a lack of Hif-1α stabilisation. Over 7 experiments, the success rate of Hif-1α stabilisation was 93.6%, which is equal to 175 out of 187 fish (Figure 5.4 B). The lowest success rate in an individual experiment was 87.5% with the highest at 100%. Taken together, these results show that injection of DA1 leads to highly efficient stabilisation of Hif-1α that is easily verifiable with microscopy.

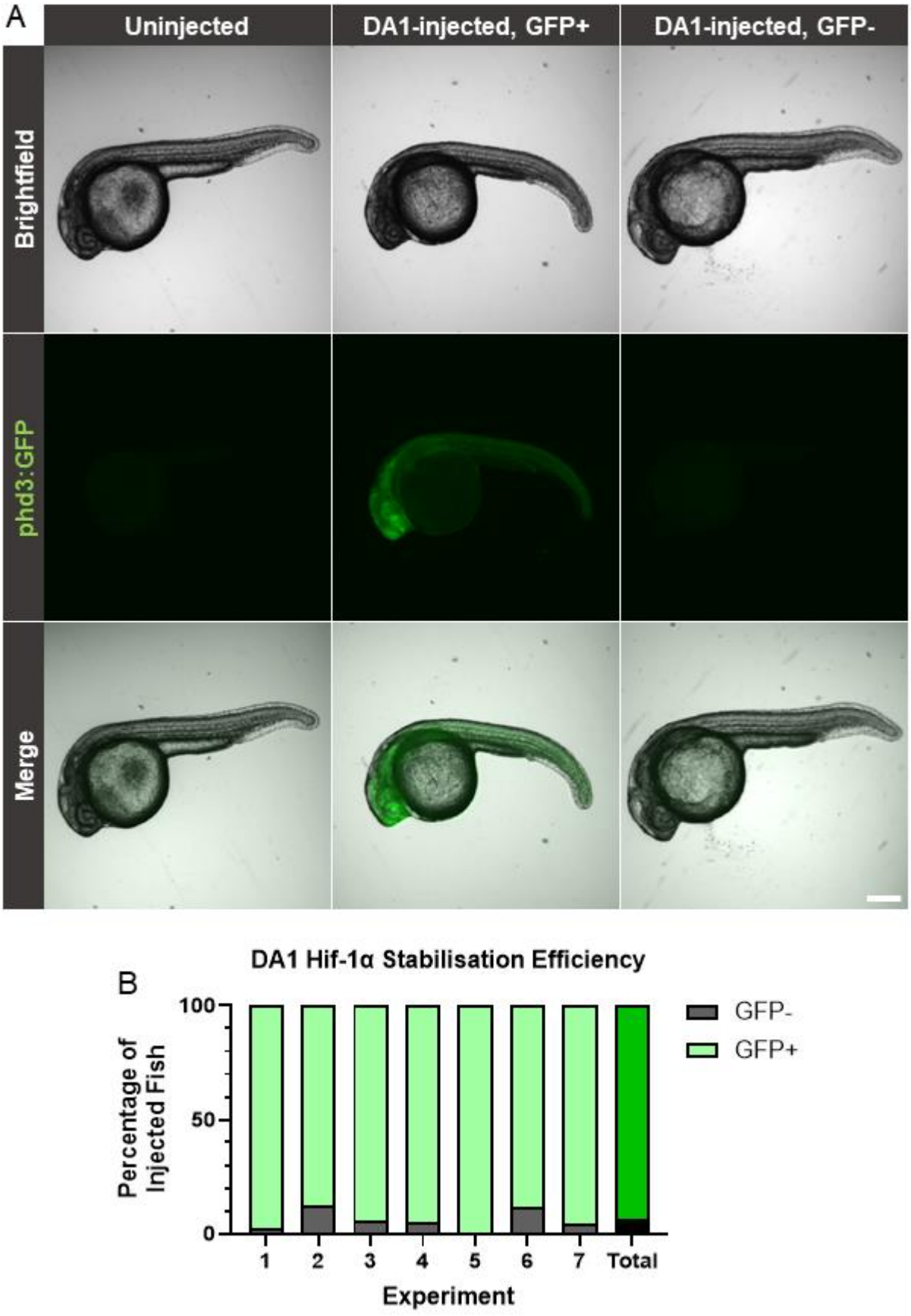


Figure 5.4 | Stabilisation of Hif-1α with DA1 efficiently upregulated *phd3:GFP*. Hif-1α was stabilised by injecting dominant active *hif-1α* (DA1) into the yolk up until the 4 cell stage in

eggs of a phd3:GFP line. Fish were examined for GFP expression, which indicates successful Hif-1α stabilisation, at 1 dpi. A) Examples of uninjected, DA1-injected GFP-positive, and DA1-injected GFP-negative fish. The middle panel shows clear GFP-expression that indicates Hif-1α stabilisation (GFP+). This was clearly absent in uninjected fish and fish where DA1 did not successfully stabilise Hif-1α (GFP-). B) Efficiency of Hif-1α stabilisation induced by DA1 injection over 7 separate experiments. The lowest percentage of GFP+ fish in a single experiment was 87.5%, with the highest at 100%. All experiments combined showed a 93.6% success rate in stabilising Hif-1α. N = 187 fish total.

5.3.4. Stabilisation of Hif-1α significantly increases swarming prevalence in response to localised *Mm* infection

Stabilisation of the hypoxia mediator Hif-1α has previously been shown to increase neutrophil retention at both wound or infection sites (Schild et al., 2020). It also increased neutrophil life-spans and reduced their apoptosis in human neutrophil cell cultures (Walmsley et al., 2005). These findings suggest there may be a potential role of Hif-1α stabilisation in swarming mediation as well. To examine this, dominant *hif-1α* (DA1), dominant negative *hif-1α* (DN1), and a phenol red (PR) control were injected into the yolk before the 4-cell stage of zebrafish eggs (Figure 5.5 A). DN1 RNA is a truncated version of *hif-1α* that competitively inhibits *hif-1α* signalling (Elks et al., 2011). Larvae were injected with *Mm* into the somite at 2 dpf and time lapse imaging followed shortly after from 1 hpi to 11 hpi. Time lapse movies were analysed for neutrophil swarming and neutrophil numbers within the infected somite over time. Stabilisation of Hif-1α significantly increased the prevalence of neutrophil swarming in response to *Mm* infection compared to the PR control ($p = 0.0319$) during the time lapse (Figure 5.5 B). There was no difference between the PR and DN1 groups ($p = 0.2730$), and the DA1 and DN1 groups ($p = 0.2742$). Stabilisation of Hif-1α did not affect swarm endpoints compared to the PR control ($p = 0.9926$) or the DN1 negative control ($p = 0.4550$) (Figure 5.5 C). There was also no difference between the PR group and the DN1 group ($p = 0.5569$). Swarm durations were not assessed due to the lack of swarms that started within the timeframe of the time-lapses. The increase in swarming prevalence after Hif-1α stabilisation suggests this may be due to increased neutrophil numbers within the somite, considering fish with swarms had increased neutrophil numbers within the somite as described in Chapter 3 (Figure 3.12). There was no significant increase in the number of neutrophils within the infected somite in the DA1 group compared to the PR control ($p = 0.0533$) and in the DN1 group compared to the PR control ($p = 0.0583$) over time (Figure 5.5 D). There was no difference between the DA1 and DN1 groups ($p = 0.1571$). Together, these results indicate that stabilisation of the hypoxia mediator Hif-1α increases neutrophil swarming prevalence, but swarm endpoints and neutrophil numbers within the somite remained unaffected.

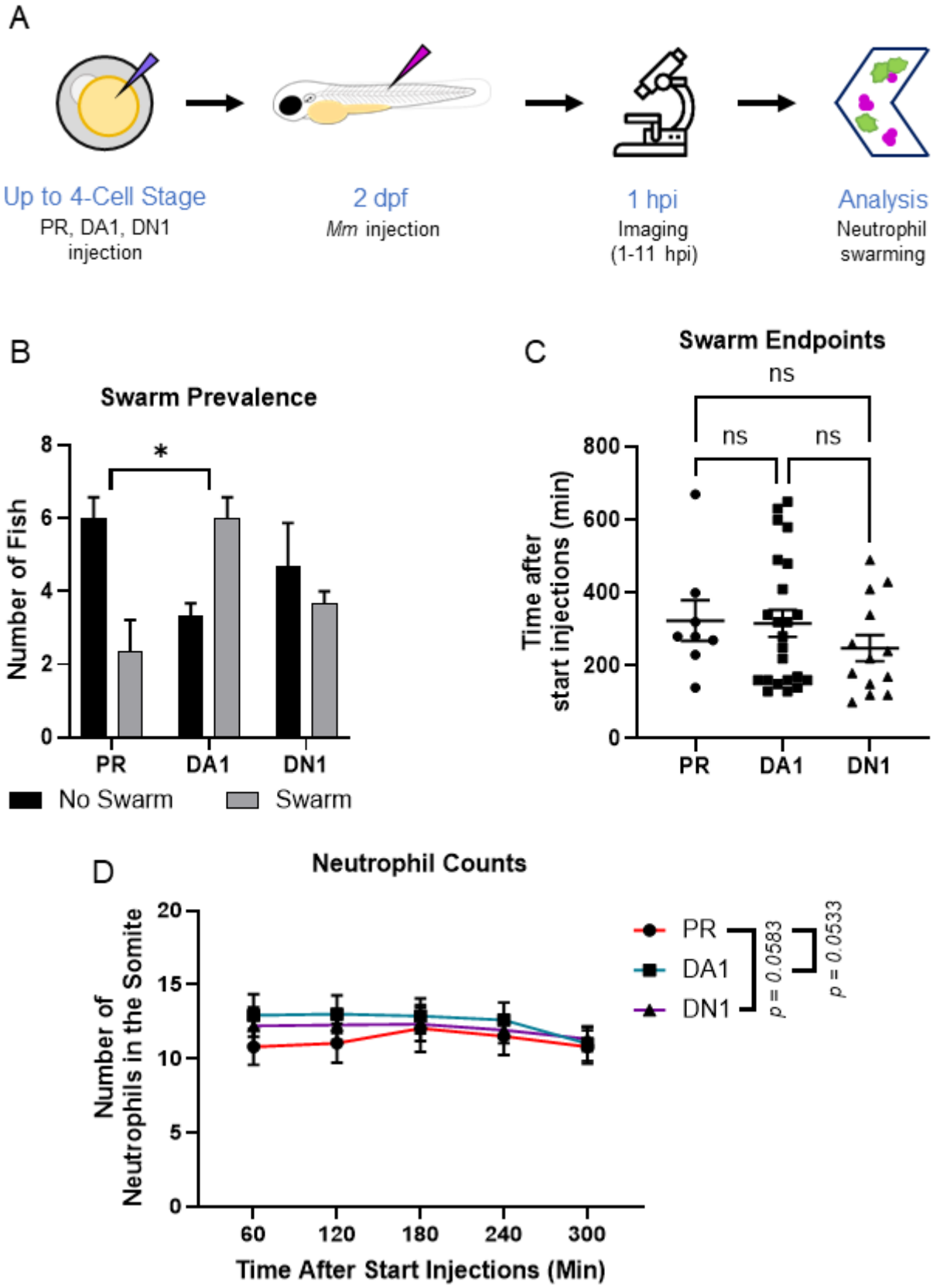


Figure 5.5 | Genetic Hif-1α stabilisation significantly increased neutrophil swarming prevalence in response to localised *Mm* infection. A) Experimental overview. Zebrafish were injected into the yolk with phenol red (PR), dominant active *hif-1α* (DA1), or dominant

negative *hif-1α* (DN1) up until the 4-cell stage. The larvae were injected with *Mm* into the somite at 2 dpf and were imaged from 1 hpi until 11 hpi. Time lapse movies were analysed for neutrophil swarming and neutrophil counts within the somite over time in response to *Mm* infection. B) DA1 injection significantly increased swarming prevalence compared to the PR control ($p = 0.0319$). There was no difference in swarming prevalence between DN1-injected and PR-injected fish ($p = 0.2730$), and between the DN1-injected and DA-1 injected fish ($p = 0.2742$). Statistics were performed on the percentages of fish with swarms observed per experiment using a one-way ANOVA. C) Endpoints of neutrophil swarms in response to *Mm* were not affected by Hif-1α stabilisation, as there was no significant difference between the groups (Mann-Whitney test). D) Neutrophil numbers within the somite over did not significantly differ between the DA1 group compared to the PR control ($p = 0.0533$), and in the DN1 group compared to the PR control ($p = 0.0583$). There was no difference in neutrophil numbers between the DA1 and DN1 groups ($p = 0.1571$). Statistics were performed with a repeated measures one-way ANOVA. N = 25 for the PR and DN1 groups, and 28 for the DA1 group from 3 experimental repeats.

5.3.5. Pharmacological Hif-1α stabilisation does not affect neutrophil numbers and bacterial burden 24 hpi

Previously, it was shown that Hif-1α stabilisation increased retention of neutrophils at the site of a wound or infection at 4 hpw/hpi (Schild et al., 2020). Hif-1α stabilisation with DMOG was also shown to increase neutrophil numbers at the site of injury at 24 hpi in a zebrafish tailfin transection model (Elks et al., 2011). Endpoints of swarms of fish with pharmacological, but not genetic, stabilisation of Hif-1α showed a non-significant trend towards later swarming resolution. Additionally, neutrophils were still seen near *Mm* somite infections at 24 hpi in previous experiments (data not shown). This time point was therefore chosen to further investigate if Hif-1α stabilisation is associated with higher neutrophil numbers within the somite during the later neutrophil response to *Mm*. Fish were infected with *Mm* into the somite at 2 dpf (Figure 5.6 A), and pooled infected fish were immediately treated with DMSO, DMOG, or FG4592, the latter of which also stabilises Hif-1α. Fish were imaged at 24 hpi and were analysed for neutrophil numbers within the somite and *Mm* bacterial burden. There was no significant difference in neutrophil numbers within the somite between any of the groups (Figure 5.6 B). There was also no significant difference in bacterial burden between the treatment groups (Figure 5.6 C). Taken together, this indicates that Hif-1α stabilisation with DMOG and FG4592 does not affect neutrophil numbers within the somite and bacterial burden at 24 hpi.

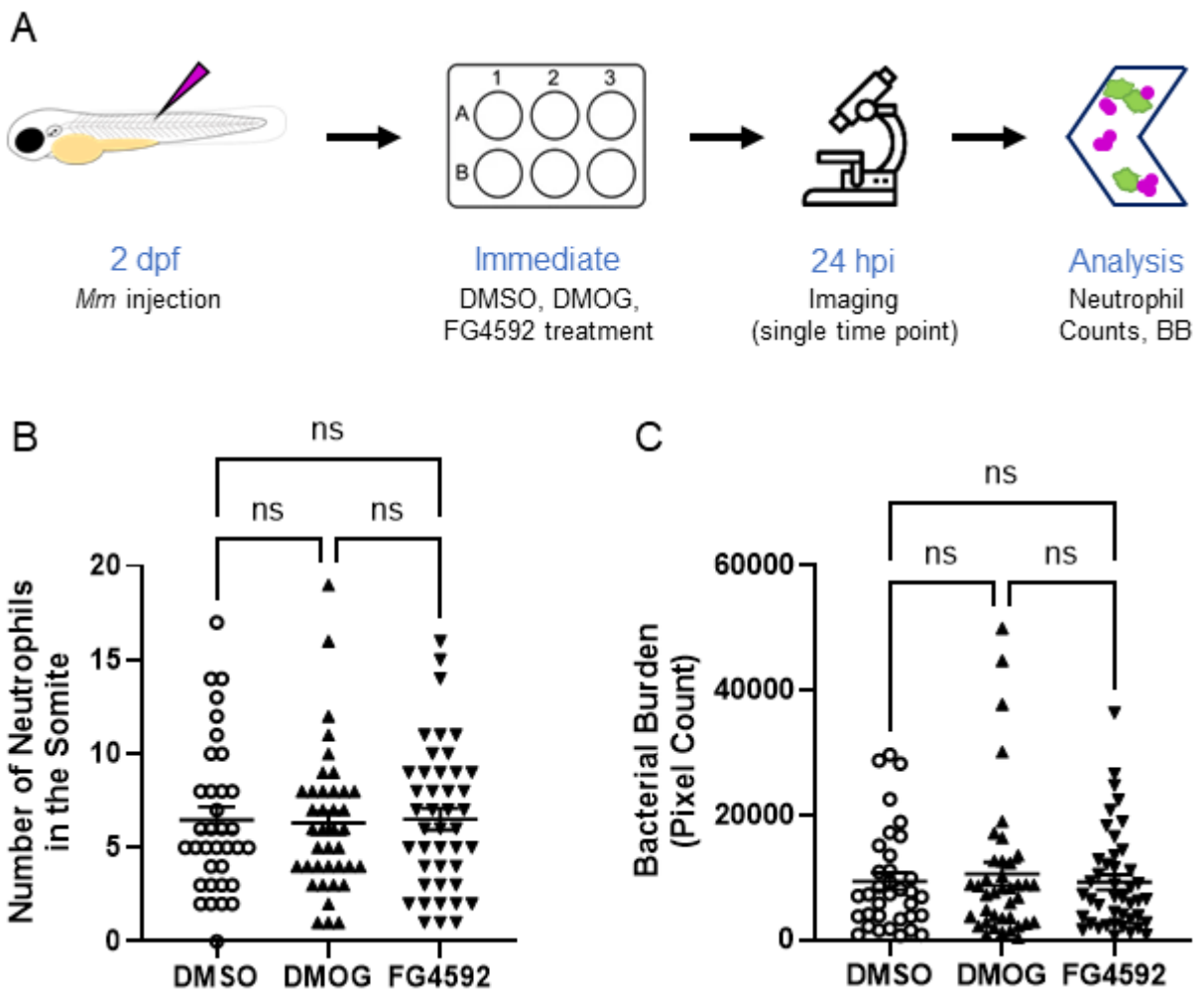


Figure 5.6 | Stabilisation of Hif-1α with DMOG and FG4592 does not affect neutrophil numbers within the somite and bacterial burden at 24hpi. A) Schematic overview of the experiment. Zebrafish were infected with *Mm* into the somite at 2 dpf. Infected fish were pooled and immediately treated with DMSO, DMOG, or FG4592. Imaging was performed at 24 hpi and images were analysed for neutrophil numbers within the somite and bacterial burden. B) Neutrophil numbers within the somite did not differ between any of the groups. C) There was no difference in *Mm* bacterial burden in any of the treatment groups. Error bars are mean ± SEM, and N = 35, 39, 43 in the DMSO, DMOG, and FG4592 group respectively. Statistics were performed with a Kruskal-Wallis test.

5.3.6. Neutrophil clustering does not affect *Mm* bacterial burden

A more simplistic approach was used to investigate if neutrophil clustering affects *Mm* bacterial burden. This approach involved a clustering experiment, which allows for a bigger sample size as fish only need to be imaged at a single time point. The image acquisition process is also significantly shorter than during a time lapse experiment, which means the bacteria are in a 28°C environment that is optimal for growth. Fish were infected with *Mm* into the somite at 2 dpf and were imaged from 50 – 110 mpi to determine fish with neutrophil clusters (Figure 5.7 A). Fish were split in a group without neutrophil clusters and a group with neutrophil clusters. These groups were imaged again at 1 dpi and images (Figure 5.7 B) were analysed for bacterial burden. There was no difference in bacterial burden in fish that showed neutrophil clustering compared to those that did not ($p = 0.5918$) (Figure 5.7 C). This indicates neutrophil clustering does not affect *Mm* bacterial burden.

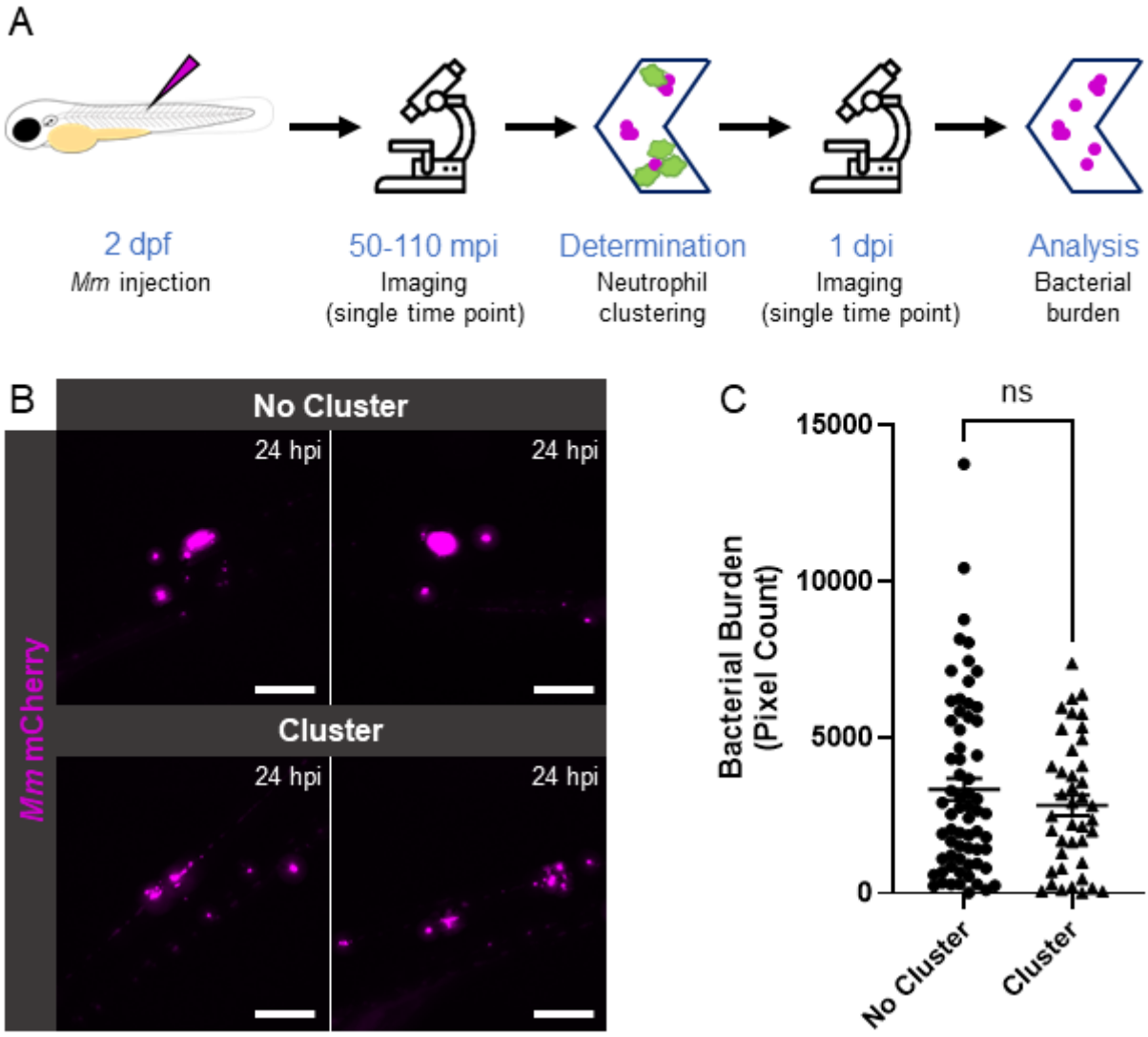
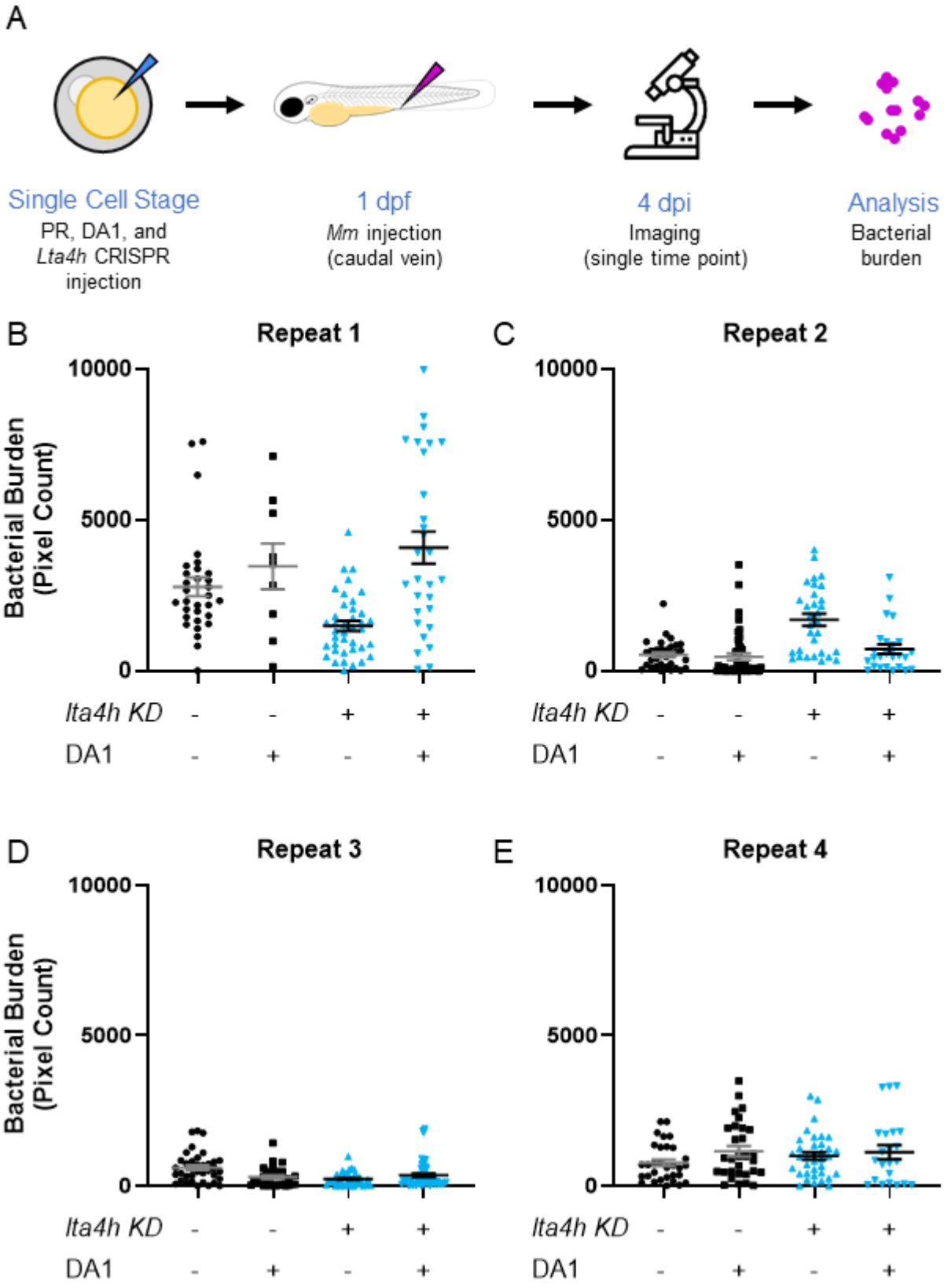


Figure 5.7 | Neutrophil clustering has no effect on *Mm* bacterial burden. A) Schematic overview of the experiment. Zebrafish were injected with *Mm* into the somite at 2 dpf. Fish

were imaged from 50 to 110 mpi to determine fish with neutrophil clustering. At 1 dpi, fish were imaged again and bacterial burden was calculated. B) Examples of infections seen in fish without clusters and with clusters around the means in C. Scale bars are 200 μm. C) There was no difference in bacterial burden found in fish with clusters compared to fish without clusters ($p = 0.5918$, Mann-Whitney test) at 1 dpi. Error bars are mean ± SEM, and N = 65, 41 for the no cluster and cluster group respectively.

5.3.7. Hif-1α stabilisation and *lta4h* knockdown induce varying changes in *Mm* bacterial burden.

Earlier, it was shown that Hif-1α stabilisation using DMOG was host-protective against *Mm* infection in the somite infection model. Stabilisation has also been shown to be host-protective in systemic *Mm* infections (Elks et al., 2013). To further investigate if this host-protective effect is dependent on *lta4h* signalling, and therefore potentially neutrophil swarming, zebrafish eggs were injected with combinations of PR, DA1, Cas9 Control, and the *lta4h* CRISPR (Figure 5.8 A) into the yolk at the single cell stage. At 1 dpf, fish were infected systemically with *Mm* by injection into the caudal vein. Infected fish were imaged at 4 dpi and the images were analysed for bacterial burden. Unfortunately, the experimental repeats suffered from substantial variation, both in the effects of Hif-1α stabilisation and *lta4h* knockdown (KD) as well as overall infection level between repeats (Figure 5.8 B-F). Experimental repeats were not combined for statistical analysis due to this variability, and further experimentation was suspended.


A
Single Cell Stage
PR, DA1, and
Lta4h CRISPR
injection
1 dpf
Mm injection
(caudal vein)
4 dpi
Imaging
(single time point)
Analysis
Bacterial
burden
B
Repeat 1
C
Repeat 2
D
Repeat 3
E
Repeat 4
Bacterial Burden
(Pixel Count)
10000
5000
0
lta4h KD
DA1

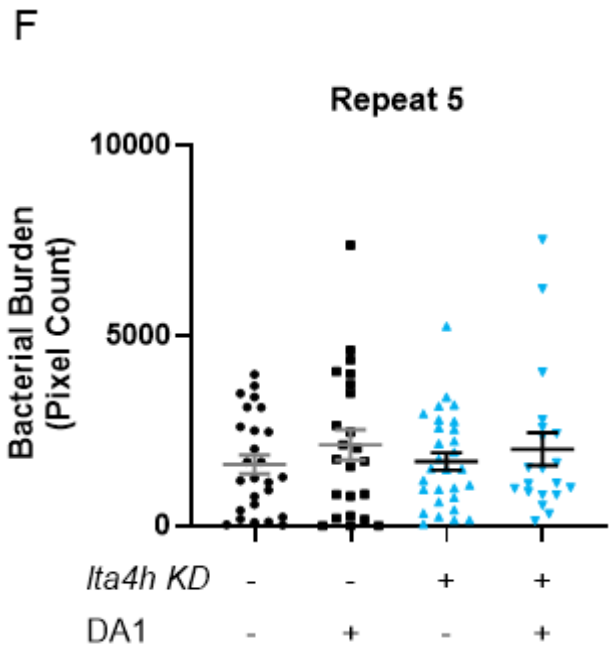


Figure 5.8 | The effect of Hif-1α stabilisation and *lta4h* knockdown on *Mm* bacterial burden is highly variable. A) Timeline of the experiment. Fish were injected with combinations of PR, DA1 and Cas9 control, *lta4h* CRISPR into the yolk at the single cell stage. At 1 dpf, these fish were systemically infected with *Mm* into the caudal vein. Infected fish were imaged at 4 dpi and images were analysed for bacterial burden. B – F) Individual results of 5 different repeats of the experiment. G) Merge of all experimental repeats. There was a significant reduction in bacterial burden in the Cas9 control+DA1 group compared to the Cas9 control+PR group ($p = 0.0176$, Kruskal-Wallis test), and in the Cas9 control+DA1 group compared to the *lta4h* KD+PR group ($p = 0.0059$, Kruskal-Wallis test). There were no significant differences found between the other groups. N = 139-173 accumulated from 5 experimental repeats. Error bars in B – G are mean ± SEM.

5.3.8. The host-protective effect of Hif-1α stabilisation was not linked to neutrophil swarming

Since the host-protective effect of Hif-1α stabilisation at 3 dpi is maintained in the somite infection model, it was hypothesised that this effect was in part due to neutrophil swarming. While DMOG did not affect swarming prevalence or bacterial clearing at 1 dpi, it may affect the ability of swarming neutrophils to affect bacterial clearance, and comparing treated fish with swarms to those without swarms may answer these questions. To further examine these hypotheses, fish were treated with DMSO and DMOG 4 hours before infection and were subsequently infected with *Mm* into the somite (Figure 5.9 A). They were then imaged for 10 hours from 1 hpi and the time lapse movies were analysed for neutrophil swarming. The next morning, the fish were put in an incubator at 28°C until 24 hpi, at which the fish were imaged again. Images of both 1 hpi and 24 hpi (Figure 5.9 B) were analysed for bacterial burden, and the difference between the 2 time points was calculated (Figure 5.9 C). Over the 24 hours of infection, bacterial burden appeared to increase over time, but there was no significant difference in growth between the DMOG-treated fish and DMSO-treated fish ($p = 0.3553$). Upon further inspection, the infection levels appeared to be remarkably low at 24hpi compared to experiments in Figure 5.2, suggesting that bacterial growth during imaging was slowed. To test this a few infected fish were left in the incubator while others were imaged. When imaging both these groups again at 24 hpi, fish left in the incubator appeared to have more developed infections and the fluorescence intensity appeared to be higher in general (Figure 5.9 D). Taken together, these data indicate that the conditions during image acquisition are not suitable for proper infection progression. Therefore, the current methods are not applicable to investigate the involvement of neutrophil swarming in the host-protective effect of Hif-1α stabilisation.

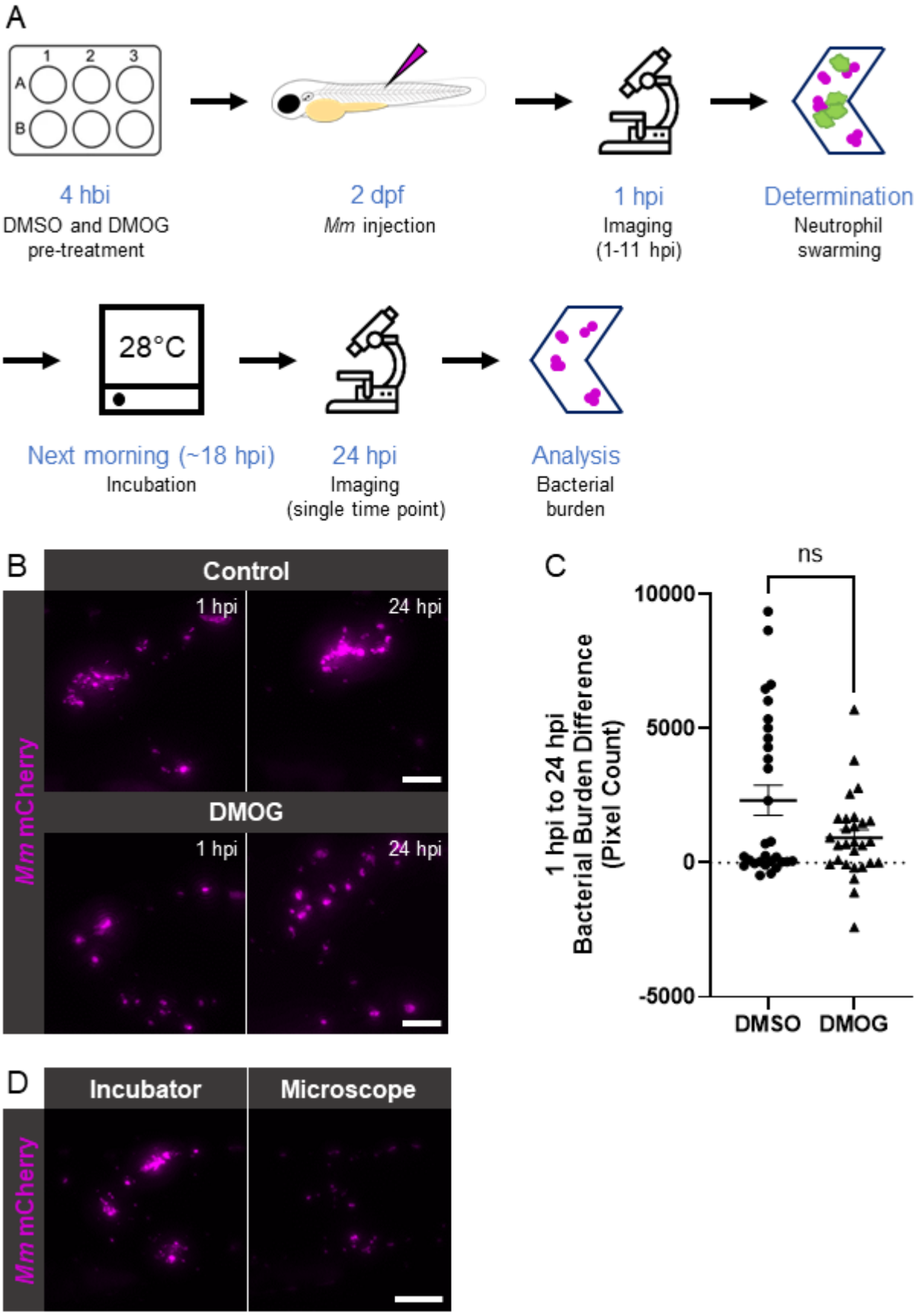


Figure 5.9 | Imaging conditions do not allow for optimal bacterial growth and prevent examination of the effect of neutrophil swarming on bacterial burden using the current

experimental approach. A) Experimental set-up. Zebrafish were treated with DMSO or DMOG 4 hours before infection and were injected with *Mm* into the somite at 2 dpf. Infected fish were imaged for 10 hours as of 1 hpi, and these movies were used to determine which fish contained neutrophil swarming events. After completion of the time lapse, on the next morning, fish were incubated at 28°C until further imaging at 24 hpi. These images were used to determine 24 hpi bacterial burden and were compared to burden at 1 hpi. B) Examples of *Mm* infections of a single DMSO group fish and a single DMOG group fish at 1 hpi and 24 hpi, close to the means in C. Scale bars are 100 μm C) Difference in the bacterial burden at 24 hpi compared to 1 hpi. There was no significant difference in the progression of bacterial burden from 1 hpi to 24 hpi between the DMSO group and the DMOG group ($p = 0.3553$, Mann-Whitney test). D) Representative images of fish in the same experiment that were put in the incubator after injection until 24 hpi (left), or that were imaged under the microscope for a time lapse (right). The fish that had been imaged showed an overall decreased level of fluorescence intensity and stinted growth compared to the overall levels in fish that had been in the incubator (data not shown). Error bars are mean ± SEM, and N = 29, 28 for the DMSO and DMOG groups respectively.

5.4. Discussion

Hypoxia signalling and its neutrophil-activating effect has been well-described in literature, such as increased neutrophil survival and increased phagocytic capabilities (Walmsley et al., 2005, 2006). Stabilisation of one of the hypoxia regulators, Hif-1α, was shown to significantly boost neutrophil-mediated clearance of *Mm* in zebrafish (Elks et al., 2013). The current chapter explored whether Hif-1α affects bacterial clearance through the behaviour of neutrophil swarming. Pharmacological stabilisation of Hif-1α significantly decreased *Mm* bacterial burden in the somite model at 3 dpi, but not at 1 dpi. In contrast to genetic stabilisation with DA1, pharmacological Hif-1α stabilisation did not affect swarming prevalence. Swarming endpoints and durations were affected by neither pharmacological nor genetic stabilisation. Pharmacological stabilisation of Hif-1α with DMOG and FG4592 did not affect neutrophil numbers within the somite or *Mm* bacterial burden at 1 dpi. There was no effect of clusters on *Mm* bacterial burden. Further examination into the dependence of the protective effect of Hif-1α stabilisation on neutrophil swarming was performed, but firm conclusions to these experiments could not be drawn due to issues with variability and disrupted *Mm* growth under prolonged imaging.

Stabilisation of Hif-1α has previously been described to significantly decrease bacterial burden in a systemic infection in zebrafish in a NO-dependent manner (Elks et al., 2013). Considering neutrophils swarm in response to *Mm*, it was hypothesised that this host-protective effect is partially dependent on neutrophil swarming. Therefore, it first needed to be confirmed whether this host-protective effect of Hif-1α stabilisation could be detected on a smaller scale in a localised infection. Using DMOG, this host-protective effect was shown to be retained in the somite infection model, as *Mm* bacterial burden was decreased at 3 dpi, but not 1 dpi. This discrepancy between time points indicates that this protective effect is minor on the short term, but is increased to significance later during infection. It is unclear whether Hif-1α stabilisation decreases systemic *Mm* bacterial burden time points before 4 dpi, as this was not discussed in the previous study (Elks et al., 2013). Regardless, the host-protective effect of Hif-1α stabilisation on localised *Mm* was confirmed, which prompted further investigation into its effect on neutrophil swarming.

During hypoxia, stabilisation of HIF-1α has been shown to increase neutrophil life-span through inhibition of neutrophil apoptosis in a NF-κB-dependent manner (Walmsley et al., 2005). It also retains neutrophils at the site of the wound or infection in a comorbid zebrafish wounding and *Mm* infection model (Schild et al., 2020). It was theorised that manipulation of the hypoxia pathway may have an effect on neutrophil swarming due to its effects on

neutrophils during hypoxia. Stabilisation of Hif-1α through injection of dominant-active RNA, but not stabilisation through DMOG, significantly increased neutrophil swarming prevalence compared to the phenol red control. There was no effect on swarm endpoints or duration. Additionally, there was no difference in swarming prevalence after injection of dominant-negative RNA compared to the Hif-1α stabilised group and the PR control. This DN1 RNA is a truncated version of Hif-1α and competitively blocks Hif-1α signalling (Elks et al., 2011). DN1 injection blocked the effect of pharmacological Hif-1α stabilisation on neutrophil counts at a tailfin wound to numbers similar in DMSO-treated injured fish (Elks et al., 2011). The effect of DN1 on neutrophil swarming was therefore expected to be comparable to the effect of PR injection, but this was not the case. Over the span of many different experiments, *Mm*-infected fish in control treatment groups, which are expected to not be affected by their treatment, show a swarming prevalence of near 50% of fish with swarms. Injection with DN1 resulted in a swarming prevalence of slightly below 50%, which would not be unusual for a (negative) control group. Instead, the outlier in these experiments may be the PR-injected group, in which swarming was observed in about 25% of fish. Phenol red is frequently used as a vehicle for injection to allow for easy monitoring of injections (Houseright et al., 2020; Schoen et al., 2019; Zhang et al., 2017), and is not expected to have a biological effect. It stands to reason that the low swarming prevalence in the PR group may have been the result of an unlikely pattern of variation. Additionally, due to the low sample size, the impact of such unlikely variation can seriously impact the overall results in the group.

If the DN1 group was selected as the more appropriate control, the conclusion of the experiment would shift to a lack of a statistically significant effect on neutrophil swarming by DA1 injection, which was also the result of the pharmacological Hif-1α stabilisation. The lack of statistical power in this experiment is again a problem here. However, there is evidence in literature that suggests there may indeed be a biological effect of Hif-1α stabilisation on neutrophil swarming involving neutrophil recruitment and retention. An increase in neutrophil recruitment facilitated by Hif-1α was previously described in an *Aspergillus fumigatus* mouse infection model (da Silva-Ferreira et al., 2022). Deletion of myeloid *Hif-1α* in mice significantly impaired neutrophil recruitment towards lungs infected with *A. fumigatus* at 3 dpi (da Silva-Ferreira et al., 2022). In another mouse model at the earlier time point of 24 hpi, treatment with PHD-inhibitor ADK-4924, which stabilises HIF-1α, significantly enhanced neutrophil recruitment towards LPS skin injections (Leire et al., 2013). Additionally, Hif-1α increased neutrophil retention at the site of *Mm* infection in a comorbidity zebrafish model (Schild et al., 2020). Pharmacological stabilisation with DMOG retained neutrophils at the site of the wound in a zebrafish tailfin injury model, and this delay in resolution of inflammation introduced by DMOG was blocked by DN1 injection (Elks et al., 2011). However, in the somite infection

model, neutrophil counts within the somite over time in response to *Mm* infection were not affected by genetic Hif-1α stabilisation, and neutrophil counts at 24 hpi were not affected by pharmacological Hif-1α stabilisation. Furthermore, injection of DA1 has been reported to have no effect on whole body neutrophil counts at 30 hpf and 120 hpf (Elks et al., 2013). In other models, a *Streptococcus* infection in HIF-1α-null mice did not appear to affect neutrophil recruitment towards the infection at 6, 12, and 24 hpi (Peyssonnaux et al., 2005). Similarly, treatment with HIF-1α agonist mimosine did not affect infiltrating neutrophil numbers in response to *Staphylococcus aureus* skin infection at 4 dpi (Zinkernagel et al., 2008). Together, this suggests changes in neutrophil recruitment or retention after Hif-1α stabilisation are not responsible for the decrease in burden.

The host-protective effect of Hif-1α stabilisation may instead be a result of boosted neutrophil function. In zebrafish, nitrotyrosine, a marker for NO production, was significantly upregulated in neutrophils after Hif-1α stabilisation. Morpholino inhibition of inducible nitric oxide synthase (iNOS) significantly decreased NO-production in these neutrophils, and both pharmacological and genetic inhibition iNOS abrogated the host-protective effect of Hif-1α stabilisation on systemic *Mm* bacterial burden at 4 dpi (Elks et al., 2013). A follow-up study showed that nitrotyrosine was further significantly upregulated after systemic infection with heat-killed *Mm* in fish with DA1-mediated Hif-1α stabilisation at 1 dpi (Elks et al., 2014). Interestingly, this additional effect was abolished when live *Mm* was injected, indicating *Mm* may actively counteract NO production (Elks et al., 2014). DA1-induced NO production also appears to be partially *il-1β* dependent, as morpholino inhibition attenuated nitrotyrosine levels in a mock injection (Ogryzko et al., 2019). An *il-1β* knockout line and morpholino inhibition completely attenuates the host-protective effect of Hif-1α stabilisation on *Mm* bacterial burden (Ogryzko et al., 2019). However, nitrotyrosine levels of DA1-injected fish were significantly reduced after *Mm* injection compared to PVP injection, further indicating *Mm* reduces NO production (Ogryzko et al., 2019). Considering Hif-1α stabilisation is protective during *Mm* infection, yet *Mm* actively inhibits NO production, the authors suggest the protective effect results from pre-infection Hif-1α stabilisation (Ogryzko et al., 2019). This essentially primes the immune system by upregulating IL-1β and, subsequently, NO, which primes the immune system to better fight infection when it occurs (Ogryzko et al., 2019).

Other neutrophil processes may also be involved in Hif-1α-mediated killing of *Mm*. Cell cultures with neutrophils from patients with a disease that causes inactivation of VHL showed increased phagocytosis of *Streptococcus pneumoniae* compared to neutrophils from healthy volunteers, and matching that of healthy neutrophils in hypoxic conditions (Walmsley et al., 2006). Expression of PHD3 was upregulated in VHL-deficient neutrophils, suggesting activity

of HIF-1α (Walmsley et al., 2006). Neutrophils may even potentiate phagocytosis by driving HIF-1α activation themselves, through a mechanism involving mitochondrial ROS (mROS) (Willson et al., 2022). Neutrophils from healthy volunteers showed increased mROS production during hypoxia compared to normoxic conditions in cell cultures, and inhibition of mROS significantly reduced expression of HIF-1α during hypoxia (Willson et al., 2022). Inhibition of the glycerol 3-phosphate pathway, which links glycolysis to mROS release, significantly reduced both mROS and HIF-1α expression, and abrogated phagocytosis of HK *Staphylococcus aureus* (Willson et al., 2022). It has been suggested that mROS stabilises HIF by directly targeting PHD enzymes, but mROS did not regulate these enzymes in human cell cultures, and was not required for stabilisation of HIF-1α (Chua et al., 2010). Similarly, HIF-1α stabilisation in human neuroblastoma cells during hypoxia was not affected by antioxidant-mediated ROS reductions in cell cultures (Kumar et al., 2021). Evidently, the role of ROS in HIF-1α stabilisation, and therefore phagocytosis, remains unclear. Nevertheless, there are clear reports that HIF-1α potentiates neutrophil phagocytosis and killing of pathogens. Phagocytosis of zymosan particles was significantly increased in human whole blood exposed to systemic hypoxia (Fritzenwanger et al., 2011). Phagocytosis of heat-killed *Staphylococcus aureus* by human neutrophils in cell cultures was also upregulated in hypoxic conditions compared to normoxia (Willson et al., 2022). In contrast, Hif-1α-deficient neutrophils were not hampered in their ability to phagocytose *Aspergillus fumigatus* conidia in mouse cell cultures (Shepardson et al., 2014). Regardless of these findings, it is unclear if phagocytosis of *Mm* by neutrophils would result in bacterial killing as the efficacy of neutrophils in killing *Mtb* through phagocytosis is controversial and has been shown to at times be ineffective (Hilda et al., 2020). Instead, efferocytosis of infected immune cells by other immune cells may play a role, but this has already been discussed in the Discussion of Chapter 3.

Other processes affected by Hif-1α stabilisation are neutrophil degranulation and NET formation (Lodge et al., 2020). HIF-1α stabilisation with DMOG significantly increased secretion of granule components MMP8 and MMP-9 in human cell cultures (Ong et al., 2018). Additionally, inhibition of Hif-1α significantly decreased NET formation in a human neutrophils stimulated with LPS (McInturff et al., 2012). However, NET formation is severely hampered under hypoxic conditions, even when stimulation is involved, suggesting that HIF-1α can only be harnessed for its NETotic effect in normoxic conditions (McGettrick & O'Neill, 2020). The granule populations of neutrophil elastase, myeloperoxidase, lactoferin, and MMP-9 were significantly increasingly released in neutrophil cultures after stimulation with GM-CSF and fMLP in hypoxic conditions compared to normoxia (Hoenderdos et al., 2016). However, this did not appear to be the case after treatment with DMOG, indicating this process was HIF-independent (Hoenderdos et al., 2016). Granules have been shown to be effective in killing

Mtb (Jena et al., 2012; Tan et al., 2006). A study with human neutrophils uncovered a significant reduction in *Mtb* bacterial burden after incubation with a subset of neutrophil granules (Jena et al., 2012). Interestingly, macrophages with phagocytosed *Mycobacterium smegmatis* could internalise these granules into their phagosomes, which further aided bacterial killing (Jena et al., 2012). Similarly, another study with human neutrophils and macrophages found phagocytosis of apoptotic neutrophils by macrophages infected with *Mm* resulted in delivery of neutrophil granules to the phagosome, which coincided with increased bacterial killing (Tan et al., 2006). NETs, however, play a controversial role in *Mtb* disease progression. NETs may capture bacteria but they do not appear to result in the killing of *Mtb* (Cavalcante-Silva et al., 2023). Macrophages may again benefit from NET release through NET-mediated induction of cytokine release and internalisation of NET peptides that may assist in killing of *Mtb* (Cavalcante-Silva et al., 2023). In short, HIF-1α affects a wide range of processes that can be beneficial in fighting *Mm* infection, and many of which may be involved in the swarming response. However, it still remained unclear if swarming was instrumental in their exertion of the protective effect of HIF-1α stabilisation.

Hif-1α stabilisation could be involved in neutrophil directional migration towards the swarm or neutrophil aggregation, perhaps through interaction with LTB4 signalling. Expression of 5-lipoxygenase activating protein, important in LTB4 formation, was significantly increased in response to HIF-1α stabilisation in human cell cultures (Gonsalves & Kalra, 2010). Furthermore, this increase was completely abrogated by HIF-1α inhibition (Gonsalves & Kalra, 2010), implicating HIF-1α signalling in regulation of LTB4 expression. Furthermore, HIF-1α-depleted mice showed a significant reduction in LTB4 release after challenge with *Aspergillus fumigatus* (Caffrey-Carr et al., 2018). Production of LTB4 was also decreased in isolated mouse adipocytes with *HIF-1α* knockout (Y. S. Lee et al., 2014). Recruitment of relatively distant neutrophils towards swarms at the site of injury in a mouse ear dermis injury model was significantly impaired after *Ltb4r1* knockout (Lämmermann et al., 2013). These relatively far distances appear to be around 100 µm and higher from the wound (Lämmermann et al., 2013), but it is unclear how this translates into a zebrafish model. Regardless, this suggests that HIF-1α may increase directional migration towards the neutrophil swarm by inducing LTB4 expression. In addition, LTB4 has been found to be involved in neutrophil aggregation (Song et al., 2020). Inhibition of 5-lipoxygenase and BLT1 significantly decreased neutrophil aggregation in mouse cell cultures, as it introduced a significant reduction in the number of neutrophil clusters per square millimetre compared to controls (Song et al., 2020). In mouse cell cultures, HIF-1α null mouse macrophages showed complete inhibition of self-aggregation compared to wildtype macrophages, which the authors implied to function similarly for neutrophils (Cramer et al., 2003). Considering LTB4 inhibition and Hif-1α stabilisation

individually did not affect neutrophil numbers within the infected somite in the *Mm* somite infection model, Hif-1α stabilisation may have increased swarming prevalence through LTB4-induced neutrophil aggregation. In conclusion, Hif-1α signalling may directly affect neutrophil aggregation, or may induce neutrophil aggregation and directional migration towards the swarm by mediating LTB4 signalling, resulting in increased swarming prevalence in Hif-1α-stabilised fish.

Considering the host-protective effect of Hif-1α stabilisation on *Mm* bacterial burden (Elks et al., 2013), the involvement of HIF-1α signalling in LTB4 signalling (Caffrey-Carr et al., 2018; Gonsalves & Kalra, 2010; Y. S. Lee et al., 2014), and the observed increase in swarming prevalence after Hif-1α stabilisation, multiple experimental setups were dedicated to further investigating if neutrophil swarming is protective. Unfortunately, there have been no reports discussing the effect of neutrophil clustering or swarming on *Mtb* or *Mm* infection, but it has been discussed in another zebrafish infection model. Clearance of *Pseudomonas aeruginosa* at a wound site near the CHT in zebrafish was previously described to rapidly ensue upon initiation of neutrophil clustering, with a peak in bacterial fluorescence intensity occurring within the first 20 minutes post wounding (Poplimont et al., 2020). There were 3 different set-ups dedicated to examine such an effect of neutrophil clustering and swarming on *Mm* bacterial burden. The first investigated bacterial burden outcome in the context of neutrophil clustering. Neutrophil clusters were determined shortly after infection and fish were divided in groups with and without clustering events. Neutrophil clustering was not found to affect bacterial burden. The second included calculating the difference in bacterial burden between 24 hpi and 1 hpi for each fish individually, and separating fish into groups with and without swarming events. Time lapse imaging was required to assess swarming occurrence in each fish and was done overnight. This meant fish were in suboptimal growth conditions for *Mm*, which has an optimal growth temperature of 30°C (Aubry et al., 2017), for the majority of the 24 hours post infection. Consequently, bacteria showed markedly reduced growth in fish that had been used for time lapse imaging compared to those that resided in the incubator from 1 hpi to 24 hpi. The results were therefore deemed untrustworthy. In the final experiment, the effect of simultaneous *lta4h* inhibition and Hif-1α stabilisation was examined to explore if DA1 exerts its host-protective effect on *Mm* bacterial burden through signalling of LTB4. As mentioned above, LTB4 signalling has been shown to be affected by Hif-1α in mice (Gonsalves & Kalra, 2010). Unfortunately, this experiment suffered from large variability and conclusions remained open-ended.

Unfortunately, there was no further available time in this project to explore changes in the aforementioned experiments that could lead to more robust conclusions. In the future, a

microscope with temperature chamber can be used during imaging to maintain the zebrafish at a constant temperature of 28°C, which is the incubation temperature for zebrafish and *Mm* used in this thesis. This may solve the growth issues experienced during microscopy, as *Mm* grows optimally at temperatures surrounding 30°C (Aubry et al., 2017). Furthermore, temperature has effects on neutrophils as well, as neutrophil migration speed in cell cultures was increased at increased temperatures between 30°C and 42°C (Khachaturyan et al., 2022). Neutrophil NET formation in cell cultures was also increased at 40°C compared to 35°C, 37°C, and 42°C in response to both infectious and sterile stimuli, and phagocytosis of *Staphylococcus aureus* and *Escherichia coli* by neutrophils increased significantly at 40°C and 42°C compared to lower temperatures (Janko et al., 2023). A temperature chamber may therefore not only resolve issues with bacterial growth, it may also boost neutrophil function, such as migration. Swarming prevalence may therefore also be increased, which would alleviate sample size issues.

NO production in neutrophil swarms is another area of interest, particularly NO production in swarming neutrophils. Hif-1α stabilisation primes neutrophils by inducing IL-1β and subsequent NO production pre-infection (Ogryzko et al., 2019), allowing them to significantly reduce systemic *Mm* infection in an iNOS and NO-dependent manner (Elks et al., 2013). Furthermore, NO mediates neutrophil recruitment (Benjamim et al., 2000; Saini & Singh, 2019), implicating it in neutrophil swarming. LTB4 installation into the nasal cavity of dogs initiated recruitment of neutrophils, which was abrogated upon inhibition of NO production (Cardell et al., 2008). In cell cultures, stimulation of neutrophils with LTB4 induced production of NO in a concentration-dependent manner (Lärfars et al., 1999). This further suggests NO may play a role in neutrophil swarming. NO production can be visualised by anti-nitrotyrosine staining (Elks et al., 2013; Ogryzko et al., 2019), but this involves fixating, and therefore killing, of the zebrafish embryos. Instead, diaminofluorophore 4-amino-5-methylamino-2′-7′-difluorofluorescein diacetate (DAF-FM-DA) can be used to visualise NO in live zebrafish, and merely requires submersion and subsequent washing in E3 for administration (Lepiller et al., 2007). Unfortunately, labelling with DAF-FM-DA inhabits the same excitation and emission wavelengths as mpx:GFP (Lepiller et al., 2007), requiring a different fluorophore for neutrophil labelling. Use of an *Tg*(*mpx:mCherry*) line (Davis et al., 2016) and *Mm* Crimson circumvents this issue. These methodological advances paired with stabilisation of Hif-1α, either pharmacologically or genetically, could further elucidate the role of Hif-1α and NO during swarming, and how these 2 players are interlinked in the swarming response.

To conclude, this chapter shows the potential of Hif-1α modulation for application within the *Mm* somite infection model characterised in this thesis. In this model, Hif-1α stabilisation is

host-protective and increased swarming prevalence after genetic stabilisation. It was also shown how this model may be used to investigate the effect of different potential swarming mediators in tandem through co-injection. Unfortunately, a number of experiments were inconclusive due to unforeseen circumstances, but clear suggestions have been made for future research to re-evaluate these findings. With the groundwork provided in this chapter, further investigation could elucidate how Hif-1α stabilisation exerts its protective effect against *Mm* infection, and how it might possibly interact with neutrophil swarming.

6. General Discussion

In this thesis I used the zebrafish model to investigate neutrophil swarming in response to localised *Mm* infection, and examined how modulation of potential swarming mediators affected this process. Neutrophils are the first line of defence against pathogens and are crucial to the killing of such invaders by the immune system, but their uncontrolled activation is associated with disease pathogenesis during later stages (Gaffney et al., 2022). The high incidence of drug resistance in TB warrants the search for other treatment avenues (Dartois & Rubin, 2022), one of which is targeting the host instead of the bacterium. Neutrophils are instrumental in host-defence against bacteria, fungi (Segal, 2005), and viruses (Ma et al., 2021). These cells have been shown to reduce the level of infection of a variety of pathogens, including but not limited to: *Pseudomonas aeruginosa* (Kienle et al., 2021; Poplimont et al., 2020), *Candida albicans* (Hopke et al., 2020), and *Staphylococcus aureus* (Payne et al., 2021). Neutrophils can be manipulated for therapeutic benefit, as stabilisation of the hypoxia regulator Hif-1α in zebrafish increases neutrophil production and, thereby, reduces *Mm* bacterial burden during early infection (Elks et al., 2013). However, it remains unclear if neutrophil swarming is involved in the host-defence against mycobacterial infection and if the protective effect of Hif-1α stabilisation is dependent on this neutrophil behaviour. Therefore, I set up a model that can be used to reliably and reproducibly initiate and visualise neutrophil swarming in response to *Mm* infection, and combined this model with genetic and pharmacological means of targeting signalling cascades to examine their involvement in the swarming process. This model might well be suitable to investigate swarming in response to other infections as well.

First, neutrophil swarming to *Mm* had to be characterised to understand how the process unfolds without any intervention of, for instance, inhibitory drugs. This understanding of the swarming response will also be vital to identify any possible treatment avenues. Following *Mm* injection into the somite of zebrafish, neutrophil numbers within the infected somite significantly increased compared to mock injections. Neutrophil swarming rapidly occurred within 60 mpi in 50% of fish, and swarms lasted over 4 hours on average. This may mark the first thorough investigation into neutrophil swarming in response to mycobacterial infections, as similar studies were not found in literature. In addition to swarming to sterile injury in mice and zebrafish (Isles et al., 2021; Lämmermann et al., 2013; Poplimont et al., 2020), neutrophils have also been shown to swarm to *Pseudomonas aeruginosa* in mice (Kienle et al., 2021) and a zebrafish wound/infection comorbidity model (Poplimont et al., 2020), *Candida albicans* on microarrays (Hopke et al., 2020) and in mice (E. K. S. Lee et al., 2018), *Staphylococcus aureus*

in mice (Kamenyeva et al., 2015) and zebrafish (Isles et al., 2021), and *Cryptococcus neoformans* in mouse cell culture (D. Sun & Shi, 2016). In these studies, neutrophil swarming was found to aid clearance of *P. aeruginosa* in mice (Kienle et al., 2021) and zebrafish (Poplimont et al., 2020), and *C. albicans*, but only on microarrays (Hopke et al., 2020). In mice, LTB4 was not required to effectively clear *C. albicans* infection, and LTB4-induced neutrophil swarming was associated with pulmonary haemorrhage and worse disease outcomes (E. K. S. Lee et al., 2018). Neutrophil swarming therefore does not appear to solely be protective against infections, and it is unclear how this process affects TB disease progression. Investigating these questions cannot be ethically done in humans, and therefore first requires studies using model organisms, such as the zebrafish, with similarities in, for instance, the innate immune system to further examine potential therapies that could later be applied to humans.

The role of neutrophils in TB infections is controversial, as they may be host-protective in early infection but then contribute to pathology during late stage TB (Gaffney et al., 2022). Macrophages, either alveolar or monocyte-derived, are considered to be the main intracellular niche of *Mtb* (Lai et al., 2024). However, neutrophils have been shown to be involved in *Mtb* bacterial killing directly (Elks et al., 2013; Hu et al., 2017; Kisich et al., 2002; C.-T. Yang et al., 2012) and indirectly through interaction with other immune cells (Andersson et al., 2020; Tan et al., 2006). This can result in a protective effect in early infection, which is exemplified by rat experiments in which neutrophilia was reduced *Mtb* bacterial burden when induced during early infection, but not when induced 10 days after infection (Sugawara et al., 2004). Another convincing case of evidence comes from zebrafish, in which pharmacological and genetic stabilisation of Hif-1α significantly reduced systemic *Mm* bacterial burden by upregulating neutrophil NO production (Elks et al., 2013). This protective effect was still detectable on a smaller scale in the somite infection model established in this thesis, and Hif-1α stabilisation increased swarming prevalence compared to a PR control. It was therefore hypothesised that Hif-1α stabilisation partially exerts its host-protective effect by boosting neutrophil swarming. Unfortunately, this could not be confirmed due to bacterial growth issues during time lapse imaging and *Mm* culture issues in a Hif-1 stabilisation and *lta4h* inhibition combination experiment. This could be solved in future work by using a microscope with a temperature-controlled sample chamber. However, knockdown of *Lta4h* significantly increased *Mm* bacterial burden compared to the control at 24 hpi. LTB4 signalling is vital to the formation of a proper swarming response by recruiting relatively distant neutrophils to the site of challenge (Lämmermann et al., 2013), which was shown to be conserved in zebrafish (Isles et al., 2021). Previous work has shown that inhibition of Lta4h with bestatin significantly increased *Mm* bacterial burden in a systemic infection (Tobin et al., 2010). This suggests that neutrophil

swarming may be host-protective in an *lta4h*-dependent manner. Such a protective effect of LTB4 signalling has been described in mice, in which knockout of 5-lipoxygenase (5-LO) attenuated LTB4 production and significantly increased *Mtb* bacterial burden within the lungs (Peres et al., 2007). However, this is in exact opposition of another mouse study, in which knockout of 5-LO increased mouse survival (Bafica et al., 2005). This host-destructive effect of LTB4 during *Mtb* infection, and during infection with its close relative *Mm*, has also been described in other studies (Sorgi et al., 2020; Tobin et al., 2013). The exacerbation of infection after *lta4h* knockdown may therefore indicate that LTB4 signalling is protective during early infection. Taken together, these findings suggest that neutrophil swarming is likely to be protective against *Mm* infection, but further research will have to be done to investigate if the presence of swarms is host-protective and if the host-protective effect of Hif-1α stabilisation is dependent on neutrophil swarming. One way to examine this is combining stabilisation of Hif-1α with knockdown of *lta4h* during *Mm* infection and imaging the fish in a temperature-controlled chamber and investigating the reduction in bacterial burden over time between fish without swarms and fish with swarms.

Neutrophil swarming is a complicated process with a wide range of signals that play a role at different stages of infection (Lämmermann et al., 2013), but it is unclear how modulation of these signals affect the swarming response. CXCR2, CXCR4, and NET formation were inhibited to investigate their potential roles in neutrophil swarming. Signalling of the well-established swarming mediator LTB4 was also inhibited (Lämmermann et al., 2013). In this thesis, the lack of effect of *lta4h*, but not CXCR2, on neutrophil swarming and numbers may indicate that CXCR2 is more involved in the initial recruitment towards the infection than *lta4h*. Interestingly, such a disruption of the initial neutrophil recruitment after *Cxcr2* knockout was not seen in a mouse injury swarming model (Lämmermann et al., 2013), meaning there may be a difference in neutrophil recruitment signals towards *Mm* infection compared to a sterile wound, or that there is a difference between neutrophil recruitment in zebrafish compared to mice. The latter may depend on a difference in CXCR2 signalling between zebrafish and mice, as the gene for CXCL2, the main CXCR2 ligand in humans, is only present in zebrafish and not in mice (Zuñiga-Traslaviña et al., 2017). The lack of apparent *lta4h* involvement in swarming may further be explained by the distance of the infection from the CHT, as sites of challenge away from the CHT generally elicit later responses than those close to the CHT (Coombs et al., 2019). LTB4 signalling was shown to be crucial in the recruitment of neutrophils at 200-300 µm distance from the swarm centre in a mouse injury model, while recruitment of relatively close neutrophils was unaffected (Lämmermann et al., 2013). The distance of the neutrophil swarms from the CHT in the somite infection model ranges from 200-500 µm, which is equal to- or greater than in the aforementioned study. The signalling

range of neutrophil recruiters other than LTB4 may be higher in zebrafish than in mice, which might make LTB4 signalling at these distances redundant. Similarly, neutrophil recruitment as a consequence of NET release may be redundant in this model as well, even though inhibition of NET formation and NET-component NE significantly decreased swarming prevalence in a zebrafish tailfin injury model (Isles et al., 2021). This could explain the lack of effect on swarming prevalence and neutrophil counts within the infected somite at 100 mpi after inhibition of NET formation. Finally, CXCR4 inhibition also did not affect these parameters, which could be a consequence of the time of imaging. CXCR4 inhibition was shown to increase inflammation resolution in a zebrafish tailfin injury model (Isles et al., 2019). Neutrophil numbers and swarming prevalence were assessed at 100 mpi, which is considerably earlier than the average swarm resolution time of 257 mpi observed in Chapter 3 (Figure 3.11), meaning the time point of 100 mpi may be too early to observe an effect of CXCR4 knockdown. The unresolved questions outlined in this paragraph can be addressed in future work by adapting the experimental procedures, which is discussed below. This work also shows the versatile application of the somite infection model and how it can be used to study neutrophil swarming.

How does the zebrafish somite infection model compare to the more conventional mouse model? Investigation into the neutrophil swarming response has been spear-headed and primarily conducted in mice (Lämmermann et al., 2013), but use of the zebrafish has become recognised as a useful adjunct in studying this neutrophil behaviour (Song et al., 2023). For example, zebrafish studies have shown involvement of LTB4 signalling (Isles et al., 2021), calcium signalling (Poplimont et al., 2020), and CXCR1 and CXCR2 signalling (Coombs et al., 2019). The model described in this thesis utilised a localised infection and imaging-based quantification to characterise the neutrophil swarming response in zebrafish. Similar visualisation of the swarming response in other models, like the mouse, require difficult imaging methods and likely the use of endogenous neutrophil injections (Stackowicz et al., 2020), which may have additional, unwanted effects on the swarming response to, for example, a laser-induced wound by introducing further damage signals created by injections. Additionally, the number of injected neutrophils does not necessarily reflect a natural neutrophil response. These injected neutrophils may be more plentiful than the numbers seen in a natural response, thereby inflating the observed swarming response compared to realistic scenarios. The zebrafish avoids this problem with the use of transgenic lines, in this case particularly the *Tg(mpx:GFP)i144* line (Renshaw et al., 2006), which allow for visualisation of endogenous neutrophils. However, the number of circulating neutrophils in zebrafish is considerable lower than in mice and humans, with up to 115 cells per larva at 2 dpf in zebrafish (Isles et al., 2019), and billions in mice (Strydom & Rankin, 2013) and humans (Mayadas et

al., 2014). The well-known study by Lämmermann and colleagues injected upwards of 300 neutrophils for the swarming experiments (Lämmermann et al., 2013), further exacerbating the neutrophil count difference between the two animal models. The zebrafish may therefore be more representative of a naturally occurring swarming response compared to the mouse. The difference is well-illustrated by the number of neutrophils found within injected zebrafish somites, that averaged below 20, compared to a markedly larger number of injected swarming neutrophils in response to a mouse ear dermis wound (Lämmermann et al., 2013). The relatively low number of neutrophils in the zebrafish results in smaller swarms and increases the possibility of false positives, but this issue was tackled by introducing a minimum time for co-localising neutrophils to be designated as swarming. Work in the zebrafish has also well-described a significant host-protective effect of Hif-1α stabilisation during early *Mm* infection (Elks et al., 2013), which has not been established in the mouse. Furthermore, none of the work performed in this thesis used animals protected by the ASPA, whereas similar work in mice would be classified under the use of protected animals (Home Office, n.d.). This also means that confirmation of Hif-1α stabilisation in the zebrafish using the *Tg(phd3:GFP)i144* line (Santhakumar et al., 2012) for individual experiments is permitted. In contrast, such confirmation in mice, which are protected from birth (Home Office, n.d.), would result in use of protected animals, even though methods of confirmation, like transgenic lines (Safran et al., 2006) or injection of chimeric reporter neutrophils (Moroz et al., 2009), are available. In summary, the zebrafish model is a valuable addition to mice models for the studying of neutrophil swarming, and the somite infection model is a reliable instigator of neutrophil swarming that can be altered to study swarming dynamics *in vivo*.

6.1. Potential avenues for treatment of TB through mediation of neutrophil swarming

With the rise of drug resistance in TB (Dartois & Rubin, 2022), targeting the host immune response may present new treatment avenues for TB. More specifically, targeting neutrophil swarming and its mediators may improve disease outcomes. However, it will be important to tailor such advances to the stage of TB disease in the patient, as neutrophils play markedly different roles in early and late stage TB (Gaffney et al., 2022). In early infection, neutrophils have been described to aid in *Mtb* (Hu et al., 2017; Sugawara et al., 2004) and *Mm* bacterial killing (Elks et al., 2013). Hif-1α stabilisation increased swarming prevalence in the somite infection model, and may therefore exert its protective effect by potentiating the swarming response. HIF-1α has been shown to be crucial to *Mtb* infection control in mice, as HIF-1α knockout mice succumbed to infection months before wildtype mice (Braverman et al., 2016). A common clinical approach to HIF stabilisation is through the inhibition of prolyl hydroxylases,

which target HIF for proteasomal degradation by hydroxylation of HIF-α (Miao et al., 2022). One of these PHD inhibitors is roxadustat, which was approved for treatment of anaemia patients receiving dialysis in 2018, and for treatment of anaemia during chronic kidney disease in 2019 (Zhu et al., 2022). Since then, other drugs targeting PHD enzymes have either passed clinical trials or are currently undergoing clinical trials, including daprodustat, vadadustat, molidustat, and enarodustat (Miao et al., 2022). Molidustat has been used in a mouse study, showing that treatment with the drug reduced *Mtb* proliferation within human macrophages (Zenk et al., 2021). However, this exemplifies that HIF stabilisation may not exert a protective effect through neutrophil swarming. HIF regulates many target genes that are involved in many different processes, and may therefore have significant off-target effects (Miao et al., 2022). Fortunately, roxadustat treatment has thus far not seen severely dangerous off-target effects (Miao et al., 2022), but it is unclear if this would also be the case in TB patients.

Another potential target for treatment is LTB4. As discussed above, inhibition of LTB4 signalling has been shown to both positively (Peres et al., 2007) and negatively (Bafica et al., 2005; Sorgi et al., 2020; Tobin et al., 2013) affect TB disease outcome. In the somite infection model, LTB4 inhibition resulted in a significant increase in somite bacterial burden, suggesting that LTB4 may be protective during early infection. However, there are no known stimulants of LTB4 production in literature. One way to stimulate LTB4-mediated neutrophil recruitment is through inhibition of LXA4 (Papayianni et al., 1996), but there again do not appear to be any LXA4 inhibitors on the market. Inhibition of NOX2 could also increase neutrophil swarm size through increased production of LTB4 and other pro-inflammatory cytokines (Song et al., 2023). While there are inhibitors for NOX1/NOX4 undergoing clinical trials, this is not the case for NOX2 (Sylvester et al., 2022). Even if stimulants of LTB4 signalling were available, their application in TB might be difficult if their host-protective effects were time-dependent. For example, neutrophilia induced by LPS was host-protective against *Mtb* infection immediately after infection, but this effect was lost when neutrophilia was induced at 10 dpi (Sugawara et al., 2004). Such early detection of TB infection will be difficult in humans. The earliest time of diagnosis is during LTBI, in which granulomas with contained *Mtb* will already have developed (Muñoz et al., 2015). This may already be too late to repel the bacterium with a host-derived approach.

In late stage TB neutrophils are often complicit in disease progression by damaging host tissues and contributing to chronic inflammation (Gaffney et al., 2022). Inhibition, rather than stimulation, of neutrophil swarming may therefore be the desired approach for host-derived therapies. As discussed above, inhibition of LTB4 signalling has been shown to be host-protective in *Mtb* infection (Bafica et al., 2005; Sugawara et al., 2004; Tobin et al., 2013). Only

one drug that targets the LTB4 pathway has reached the market and is called ubenimex, also known as bestatin, and is used in chemotherapy in acute non-lymphocytic leukemia (Bhatt et al., 2017). Other drugs in clinical trials include etalocib, which was terminated due to a lack of benefit after phase 2 trials, amelubant, which was terminated due to a lack of benefit and due to side-effects after phase 2 trials, and moxilubant, which was terminated due to lack of effect after a phase 1 clinical trial (Bhatt et al., 2017). Bestatin has been found to significantly decrease bacterial burden and lesion in *Mtb* infected mice (Correa et al., 2017), but has not been used in any published TB clinical studies. The inhibition of another swarming mediator, CXCR2, was shown to partially reverse exacerbation of neutrophil-mediated lethal inflammation in TB in mice with a deletion of a noncoding RNA that increased susceptibility to TB (Dorhoi et al., 2013). There is a wide range of CXCR2 inhibitors that completed clinical trials or are currently in clinical trials for diseases like diabetes mellitus type 1 and a variety of cancers (Sitaru et al., 2023). However, none of these drugs have been approved for clinical use (Sitaru et al., 2023). Specific neutrophil phenotypes have been found in patients with severe TB (L. Wang et al., 2024), indicating phenotype-specific targeting of neutrophils may improve infection outcomes. In conclusion, treatment of TB by targeting host neutrophils may be a valid strategy, but requires further investigation into their involvement in different stages of the disease, as well as the approval of drugs that target swarming mediators for clinical use.

6.2. Future prospects

The initiation of neutrophil swarming in response to injury has been well-described (Lämmermann et al., 2013), but what drives swarm formation in response to TB is unclear. In injury, migration of a few individual neutrophils towards the site of challenge is sufficient to induce a large swarming response (Lämmermann et al., 2013). In zebrafish, a single neutrophil was shown to initiate swarming and to become the centre of this swarm after tailfin injury (Isles et al., 2021). However, studying of the early swarming response in the somite infection model proved difficult. A majority of the swarms initiated within an hour of starting *Mm* infections, therefore making it difficult to examine swarming onset of a single experimental group, and near impossible if more experimental groups were involved. Other locations for injection could be of interest for future swarming investigations if they generally result in later times of swarming onset. One such possibility is injection of the hindbrain ventricle, which is rarely occupied by neutrophils without any influence of injury or infection (Powell et al., 2018). Injection with *Pseudomonas aeruginosa* into the hindbrain was shown to have recruited neutrophils towards the somite by 90 mpi, with neutrophil numbers peaking at 180 mpi (Phennicie et al., 2010). Infection with *Salmonella enterica* recruited neutrophils towards the hindbrain within 1 hpi and peaking at 3 hpi (Du et al., 2017). At 6 hpi, recruitment of neutrophils

to both *Cryptococcus neoformans* yeast and spores showed neutrophil numbers comparable to those found in response to *Mm* infection in this thesis (Davis et al., 2016). No information on neutrophil recruitment towards an *Mm* hindbrain infection was found, but the studies mentioned above might indicate the suitability of the hindbrain as a place of infection for neutrophil swarming assays in an *Mm* context. Similarly, the otic vesicle has been used for *Staphylococcus aureus* infection, which recruited neutrophils to the site of infection and initiated swarming at around 6 hours post infection (Isles et al., 2021). While variable between infections, the observed neutrophil numbers suggest the peak of recruitment in these alternative models occurs later than in the *Mm* somite infection model, which could provide enough additional time to investigate the formation of swarms in response to *Mm* without sacrificing sample size.

The involvement of LTB4 in neutrophil swarming is well-established, but how other mediators affect this neutrophil response remains unclear (Lämmermann et al., 2013). Therefore, in Chapter 4, a number of potential swarming mediators were investigated for their effects on neutrophil swarming in response to *Mm*. Inhibition of CXCR2 significantly reduced neutrophil numbers in the infected somite. It was theorised that this was due to decreased neutrophil recruitment towards the somite, and CXCR2 inhibition was therefore dropped from further investigation in favour of other targets. However, CXCR2 has been shown to be involved in neutrophil aggregation (Jackson-Jones et al., 2020), which could potentially be studied further by applying CXCR2 drug inhibition at around 100 mpi, when neutrophil swarming is likely to already have commenced. Another mediator that may be involved in neutrophil aggregation, as well as recruitment, is GRK2, which inhibition increased neutrophil recruitment and aggregation of mouse neutrophils (Kienle et al., 2021). The homolog of mammalian GRK2 in zebrafish is GRK3, and has recently been described to be successfully inhibited by CRISPR-mediated targeting of *grk3* (Casey et al., 2024). CXCR4 was also examined for its effect on neutrophil swarming in response to *Mm*, as it has been shown to be important for neutrophil retention during inflammation in zebrafish (Isles et al., 2019). Pharmacological inhibition of CXCR4 did not affect clustering prevalence in response to *Mm* infection, but it was hypothesised that the time of imaging at 100 mpi was too early to assess an effect on neutrophil retention during swarming. Repeating these experiments with a later time of imaging may therefore yield different results. A preliminary time lapse experiment may provide the necessary temporal resolution to select an appropriate time point to assess neutrophil clustering. These experiments can also be done with other potential swarming resolution mediators. For example, LXA4 was identified as a swarming resolution signal for swarming human neutrophils in response to zymosan (Reátegui et al., 2017). Exogenous LXA4 can be injected into the zebrafish (Loynes et al., 2018) after 100 minutes post *Mm* infection to assess

its effect on swarm resolution. The extensive zebrafish toolbox will allow for further investigation into other potential swarming mediators that have not been mentioned here. However, it is important to note that neutrophil swarming is a dynamic process with a range of mediators, and that individual knockdown of such mediators only indicates whether or not they uniquely mediate certain neutrophil behaviours during swarming (Lämmermann et al., 2013).

Release of NETs has been implicated in onset of neutrophil swarming in a zebrafish tailfin injury model (Isles et al., 2021), but further research will need to be conducted to solidify NETs as one of the contributors to neutrophil swarming and whether they drive neutrophil swarming in response to *Mm* infection. A transgenic reporter line for NET release, which labels neutrophil histone H2az2a (H2A) (Isles et al., 2021), was used in the somite infection model to examine NET involvement in the neutrophil swarming response to *Mm* infection (data not shown). Unfortunately, a considerable amount of autofluorescence of *Mm* mCherry caused issues with the determination of H2A-derived fluorescent signal, and the reporter line was not used in any further experimentation. Inhibition of NET formation with LDC7559 did not affect neutrophil clustering prevalence in the somite infection model, but did significantly reduce swarming prevalence in a tailfin injury model (Isles et al., 2021). Inhibition of NET-component NE, but not *mpx*, in this model similarly significantly reduced swarming prevalence (Isles et al., 2021). Inhibition of these targets can be applied to the somite infection model to investigate NET involvement in neutrophil swarming in response to *Mm* infection. Furthermore, NETs can be inhibited by injection of DNase I (W. Chen et al., 2021), which could be co-injected with *Mm* to investigate NET involvement during swarming onset. However, treatment through submersion in water containing a range of DNase I concentrations was toxic to live zebrafish (Isles et al., 2021), indicating a potential risk in the injection of DNase I as well. There are numerous other agents that have been shown to inhibit NET formation in various experimental models (R. Liu et al., 2024), which could be tested in the zebrafish model as well. This includes PAD4 inhibitors like BMS-P5 and JBI-589, NE inhibitor sivelestat (R. Liu et al., 2024), and gasdermin D inhibitor disulfiram (Adrover et al., 2022). In short, there are numerous paths to affect NET formation, which can be utilised to examine NET involvement in swarming to *Mm*.

It is unclear if macrophages are involved in neutrophil swarming in response to *Mm* infection. In humans, alveolar macrophages are the first immune cells to phagocytose *Mtb*, and their interaction with the bacteria causes recruitment of additional immune cells, including neutrophils (Chandra et al., 2022b). During *Mtb* infection, macrophages produce cytokines that recruit neutrophils towards the site of infection, including IL-8 and IL-1β (Kroon et al., 2018), and they have been shown to promote neutrophil production through release of IL-1 (Alcantara et al., 2023). Neutrophils are able to phagocytose dying, infected macrophages (C.-

T. Yang et al., 2012) and, vice versa, macrophages may facilitate killing of *Mtb* by transporting granules of internalised apoptotic neutrophils to their infected endosomes (Tan et al., 2006). Finally, macrophages contribute to resolution of neutrophilic inflammation by clearance of apoptotic neutrophils (Ortega-Gómez et al., 2013). These studies indicate a high likelihood of macrophage involvement in potentially all of the stages of neutrophil swarming. Such interactions between these immune cell types can be investigated in a transgenic line that combines the transgenic lines *Tg(mpx:EGFP)* and *Tg(mpeg1:mCherry)*, which fluorescently labels both macrophages and neutrophils with different fluorescent proteins (Ellett et al., 2011; Renshaw et al., 2006). Injections can be performed with *Mm* Crimson instead of *Mm* mCherry (Ogryzko et al., 2019) to avoid fluorophore overlap of the bacteria with the macrophages in the transgenic line. Unfortunately, *Mm* is autofluorescent and may introduce difficulty of imaging *mpeg1:mCherry* macrophages which are relatively dim compared to the *mpx:EGPF* neutrophils, which will need to be considered during the experimental procedures. In addition to imaging of neutrophils and macrophages during the swarming response, depletion of macrophages may be used to assess their effect on swarm progression. Lipo-C injected into the caudal vein at 24 hpf leads to significant depletion of macrophages within 24 hours (48 hpf) and does not affect neutrophils (El Omar et al., 2024). These approaches can be combined with drug treatments or CRISPR to investigate if these potential neutrophil-macrophage interactions during swarming are affected by swarming mediators such as LTB4.

The zebrafish model used to examine neutrophil swarming in response to *Mm* in this thesis could be applied to investigate neutrophil swarming in the context of other diseases as well. As mentioned above, neutrophils have been shown to swarm to infections such as *Pseudomonas aeruginosa* in mice (Kienle et al., 2021) and a zebrafish wound/infection comorbidity model (Poplimont et al., 2020), *Candida albicans* on microarrays (Hopke et al., 2020) and in mice (E. K. S. Lee et al., 2018), *Staphylococcus aureus* in mice (Kamenyeva et al., 2015) and zebrafish (Isles et al., 2021), and *Cryptococcus neoformans* in mouse cell culture (D. Sun & Shi, 2016). These studies have investigated roles of, for example, GRK2 (Kienle et al., 2021) and calcium signalling (Coombs et al., 2019) in neutrophil swarming to different infectious agents. The somite infection model can be used to further examine potential drivers of neutrophil swarming, such as NETs and specific chemokines. In zebrafish, neutrophil swarming in response to *Pseudomonas aeruginosa* (Poplimont et al., 2020) and *Staphylococcus aureus* (Isles et al., 2021) has already been described, but further experimentation in the swarming infection model will allow for additional characterisation of neutrophil swarming in response to these infections. Neutrophil swarming in response to *Candida albicans* and *Cryptococcus neoformans* has not been investigated in zebrafish, but the zebrafish is a well-described model for both these diseases (Chalakova & Johnston, 2023;

Chao et al., 2010). Other diseases with a strong neutrophil response, such as *Shigella* infections (Arena et al., 2017), are also candidates for examination in the somite infection model.

6.3. Conclusion

In conclusion, in this thesis I have developed and characterised a model that allows for robust investigation into neutrophil swarming in response to *Mm* infection, which may be applicable to other infections as well. Potential neutrophil swarming mediators were investigated for their involvement in this neutrophil behaviour through modulation with drug treatments and CRISPR-Cas9 gene editing. Knockdown of *lta4h* negatively impacted swarming prevalence to injury and swarm duration to infection, indicating modulation of swarming mediators in this model is an effective tool to investigate their involvement in the swarming process. Additionally, the increase in *Mm* infection following knockdown of *lta4h* indicates that neutrophil swarming may be host protective against early mycobacterial infections. Genetic Hif-1α stabilisation, which is host protective, was found to increase swarming prevalence following *Mm* infection. This could not be linked to clearance of bacteria by neutrophils, but does indicate a potential avenue for host-directed therapies if neutrophil swarming is a host-protective process.

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