The role of neutrophils in mycobacterial infection in zebrafish

The University Of Sheffield.

Piotr Tymon Szkuta

Department of Infection, Immunity and Cardiovascular Disease

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Abstract

Tuberculosis (TB) is a chronic granulomatous disease caused by the pathogen *Mycobacterium tuberculosis* (*Mtb*). Antibiotic resistance has led to TB being one of the leading infectious causes of death worldwide. *Mtb* is notable for surviving within leukocytes in which it creates a permissive niche essential for its proliferation and dissemination. Previous research has demonstrated that stabilised hypoxia inducible factor 1-alpha (Hif-1α) reduced bacterial burden due to increased levels of potent antimicrobial nitric oxide (NO), predominantly found in neutrophils. However, the roles of the neutrophil in this protective effect are not fully elucidated.

Here I used a zebrafish model of TB to investigate neutrophils and macrophages during both initial stages of infection and granuloma formation. I tested the hypothesis that Hif-1α stabilisation is protective for the host by maintaining high levels of antimicrobial neutrophil NO, better controlling bacteria. I used a natural fish pathogen, *Mycobacterium marinum* (*Mm*), to investigate the roles of neutrophils and macrophages by altering cell populations *in vivo*. I then modulated Hif signalling to assess the effects on infection outcome when macrophage and neutrophil populations were altered.

Here, I demonstrated that Hif-1α stabilisation is able to help the host immune response control infection, even when macrophages, a normally critical leukocyte in *Mm* control, are depleted. Conversely, genetic depletion of neutrophils in Hif-1α stabilised zebrafish larvae abolished the host-protective effect. Blocking NO in this context abrogated the protective effect, suggesting that neutrophil NO is responsible for the improved infection control. My data also shown that Hif-1α stabilisation maintains high levels of NO and increases neutrophil internalisation of *Mm* within granulomas, even in macrophage-depleted fish. These findings add further mechanistic insights to evidence suggesting that targeting neutrophils and Hif-1α could be used as future therapeutic avenues, potentially bypassing the emerging problem of emerging antibiotic resistance.

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List of abbreviations

AA - arachidonic acid

AECs - airway epithelial cells

AMK - amikacin

AMPs - antimicrobial peptides

AMs - alveolar macrophages

ANOVA - analysis of variance

ANX1 - Annexin A1

Arg1 - arginase 1

ARNT- aryl hydrocarbon nuclear translocator

ASL^1 - airway surface liquid

ASL^2 - argininosuccinate lyase

ASS - argininosuccinate synthase

Ass1 - arginine-succinate synthase

BCG - Bacillus Calmette-Guérin

CAP - capreomycin

Cas9 - CRISPR associated protein 9

CD - cluster of differentiation

cDCs - conventional dendritic cells

C-di-AMP - cyclic-di-adenosine monophosphate

CFU - colony forming unit

CG - cathepsin G

cGAS - cyclic GMP-AMP synthase

CGD - chronic granulomatous disease

CHT - caudal hematopoietic tissue

CIP - ciprofloxacin

clod lipo - clodronate-injected embryos

CLRs - C-type lectin receptors

COX-2 - Cyclo-oxygenase-2

CRISPR - regularly interspaced short palindromic repeats

CSF3 - Colony-stimulating Factor 3

CSP - cytoplasmic surveillance pathway

ctrl lipo - control liposomes

DCs - dendritic cells

DC-SIGN - DC-specific ICAM-grabbing nonintegrin

DMOG - dimethyloxalylglycine

dpf - days post fertilisation

ECM - extracellular matrix

EMB - ethambutol

Erp - exported repetitive protein

FIH - factor inhibiting HIF

FITM - interferon-induced transmembrane

FLD - first line drugs

fMLP - N-formyl-methionine-leucine-phenylalanine

FQs - fluoroquinolones

GCSF - granulocyte colony-stimulating factor

GFP - green fluorescent protein

GSH - glutathione

H2O2 - hydrogen peroxide

HBHA - heparin-binding hemagglutinin

HBV - hindbrain ventricle

HDTs - host-directed therapies

HIF-α - hypoxia inducible factor alpha

HNO - nitroxyl

HNP-1 - human neutrophil peptide 1

hpi - hours post infection

hpf - hours post fertilisation

hpt - hours post treatment

HREs - hypoxia responsive elements

HSPCs - hematopoietic stem and progenitor cells

iDC - inflammatory dendritic cells

IFN - interferon

IFN-β - interferon beta

IFN-γ - interferon gamma

IL - interleukin

ILCs - innate lymphoid cells

INH - isoniazid

INKTs - invariant NK T cells

iNOS - inducible nitric oxide synthase

IRF - interferon regulatory factor

IRGM - immunity-related GTPase family M protein

KAN - kanamycin

LAM - lipoarabinomannan

LAMP 1 - lysosome-associated membrane protein

LC3 - light chain 3

Lcn2 - lipocalin 2

LDG - low density granulocytes

LEV - levofloxacin

LF - lactoferrin

L-NIL - N6-(1-iminoethyl)-L-lysine

LO - lipoxygenase

LPS - lipopolysaccharide

Lta4h - leukotriene A4 hydrolase

LTB4 - leukotriene B4

LXA4 - lipoxin A4

MAITs - mucosal associated invariant T cells

ManLAM - Mtb mannose-capped lipoarabinomannan

MDR-TB - multidrug resistant TB

MDSCs - Myeloid-derived suppressor cells

Met - Metformin

MHC - Major Histocompatibility Complex

MIP^{1} - maximum intensity projection

MIP^{2} - macrophage inflammatory protein

Mm - *Mycobacterium marinum*

MMP-8 - matrix metalloproteinase-8

MMP-9 - matrix metalloproteinase-9

MO - morpholino oligonucleotide

MOX - moxifloxacin

mpeg1 - macrophage expressed gene 1 promoter

MPO - myeloperoxidase

mpx - myeloperoxidase promoter

MR - mannose receptor

mtz - metronidazole

Mtb - *Mycobacterium tuberculosis*

N_2O_3 - dinitrogen trioxide

NCRs - natural cytotoxicity receptors

NE - neutrophil elastase

NETs - neutrophil extracellular traps

NHPs - Non-human primates

NKs - natural killer cells

NO - nitric oxide

NO_2 - nitrogen dioxide

NOD2 - nucleotide-binding oligomerisation domain-containing protein 2

nt - non-treated

NTR - nitroreductase

O_2^- - oxygen radicals

OAT - ornithine aminotransferase

ODC - ornithine decarboxylase

OFX - ofloxacin

ONOO - peroxynitrite

PAMPs - pathogen-associated molecular patterns

pDCs - plasmacytoid dendritic cells

PDIM - phthiocerol dimycoceroserate

PE - proline glutamate

PGE2 - prostaglandin E2

PGLs - phenolic glycolipids

PHDs - prolyl hydroxylases

PIMS - phosphatidylinositol mannosides

PMNs - polymorphonuclear neutrophils

PRR - pattern recognition receptors

pTB – pulmonary TB

PTPA - protein tyrosine phosphatase

pVHL - Von Hippel-Lindau protein

PVP - polyvinylpyrrolidone

PZA - pyrazinamide

Rab5/7/22a - Ras-related protein 5/7/22a

RIF - rifampicin RIF

RNS - reactive nitrogen species

ROI[1] - reactive oxygen intermediates

ROI[2] - region of interest

ROS - reactive oxygen species

SCMO - control morpholino

SDGs - Sustainable Development Goals

sgRNA - single guide RNA

SLD - second line drugs

SM - streptomycin

T7SS - ESX-1 type VII

TACO - tryptophan-asparatate containing coat protein

TB - Tuberculosis

TDM - lipid trehalose dimycolate

TGF-β - transforming growth factor beta

Th1 - T helper 1 cells

Th2 - T helper 2 cells

TLB4 - leukotriene B4

TLRs - Toll-like receptors

TNF - tumour necrosis factor

VEGF - vascular endothelial growth factor

WHO - World Health Organization

XDR-TB - extensively drug-resistant

Ym1 - chitinase-like 3

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Chapter 1: Introduction

1.1 Tuberculosis

Tuberculosis (TB) is a communicable disease caused by the bacillus *Mycobacterium tuberculosis* (Mtb). Despite a decrease in cases in the last century due to the antibiotic revolution, TB is still one of the major health problems worldwide. According to the World Health Organization (WHO) in 2018, more than 10 million new cases were diagnosed and there were nearly 2 million deaths which makes TB the leading cause of death from a single infectious agent (WHO Global Tuberculosis Report 2018, 2018). The Bacillus Calmette-Guérin (BCG) vaccine (an attenuated strain of *Mycobacterium bovis*) is almost 100 years old and has some protective effects against TB infection (Eisenhut *et al.*, 2009; Hawn *et al*., 2014). However, the protection depends on the maintenance of an intact immunological response that can be diminished by immunosuppression caused by drugs, ageing and malnutrition. In addition, BCG efficacy still does not provide comprehensive and adequate control of TB in the case of immune competent adults from developing pulmonary TB and HIV-infected patients, who are 20-30 times more likely to develop an active form of TB (Ottenhoff and Kaufmann, 2012).

Although over the last 15 years early diagnostic methods and the widespread availability of standard treatment regimes have saved nearly 50 million lives, the proportion of new patients is significantly higher than hoped for in the framework of the project "End TB Strategy". The key goal of this programme was to eradicate the global TB epidemic from 20 countries with the highest burden of TB by 2020 (WHO The End TB Strategy 2014, 2014). Based on information included in the latest Global Tuberculosis Report 2019, the average rate of decline in many high TB burden countries is insufficient to achieve this goal. Globally, between 2000-2018, the average rate of decline was only 1.6% per year and 6.3% over 2015-2018 which is significantly less than 20% reduction approved for the period between 2015-2020. Similarly, the guidelines aiming to decrease the total number of TB deaths of 35% by 2020 proved to be too ambitious and reached only one-third of this rate. In 2019, new milestones and targets for the coming years have been established. By 2030, a 90% reduction in the numbers of TB deaths and 80% reduction in the TB incidence rate is planned compared to 2015. In addition, WHO has set out new global targets such as: to treat 40 million TB patients and reach no less than 30 million people with TB preventive treatment for a latent TB infection by 2022. Yet, irrespective of the promising rate of

decline in some countries, the alarming increasing trend with regard to incident cases per year in places like India and Indonesia, as well as increasing prevalence of drug-resistant Mtb strains highlight the burning need for development of better treatment strategies (WHO Global Tuberculosis Report 2016, 2016).

1.2 Multidrug resistance in mycobacterial infection

A substantial breakthrough in the treatment of TB was made in the 1940s with the development of the first effective antimycobacterial antibiotic - streptomycin. However, this promising drug became ineffective in the wake of resistance development, a well-known feature of many bacterial infections (Goldberg *et al.*, 2012). The current standard treatment in cases of fully sensitive TB is associated with long-term intake of 4 potentially toxic antibiotics (involving 4 drugs for 2 months followed by 2 drugs for a further 4 months) where lack of compliance may contribute to drug resistance (Wallis *et al.*, 2016; Worthington and Melander, 2013). The first line drugs (FLD) such as isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) ethambutol (EMB) and streptomycin (SM) are used to treat TB. However, genetic resistance (Yang resistance), which refers to mutations in bacteria chromosomal genes, has led to the emergence of multidrug resistant (MDR-TB) and extensively drug-resistant (XDR-TB) strains. The new antitubercular agents such as bedaqualine and delamanid are the first drugs of new classes registered in 40 years and have become more commonly used to target MDR-TB (Dooley *et al.,* 2021). Recent studies evaluating clinical efficacy and effectiveness of these drugs against MDR-TB have shown a high culture conversion rate (65-100%) and satisfactory treatment outcomes (Li *et al.,* 2019). However, the safety and tolerability of bedaqualine and delamanid have not been yet fully evaluated in paediatric patients which will be prerequisite to treat MDR- and XDR-TB in children (D'Ambrosio *et al.,* 2017).

A phenotypic resistance (Yin resistance), encompasses numerous mechanisms, such as: drug target alternation, overexpression of drug target, disruption of prodrug activation and the activation of efflux pump resulting in the need for extended treatment and risk of post-treatment relapse. According to data from a WHO report in 2014, 3,5% of newly diagnosed and more than one fifth of previously treated patients had multidrug-resistant TB. MDR-TB data from Eastern Europe and Central Asia are the least optimistic reaching 20% and 50% of total TB cases respectively (Miotto *et al.*, 2018; Zhang and Yew, 2015). MDR-TB is resistant to at least INH and

RIF which necessitates treatment with second-line drugs (SLD) such as fluoroquinolones (FQs): ofloxacin (OFX), levofloxacin (LEV) moxifloxacin (MOX), ciprofloxacin (CIP) and aminoglycosides - injectable antituberculosis drugs including kanamycin (KAN), amikacin (AMK) and capreomycin (CAP) (Jnawali *et al*., 2013). According to forecasts a more worrying increasing problem is the rise in cases of XDR Mtb strains (Jnawali and Ryoo, 2013), which in 2014 were confirmed in almost every tenth of MDR-TB patient (Zhang and Yew, 2015). XDR-TB is resistant to either INH or RIF any fluoroquinolone and at least one of three SLD antituberculosis injectable drugs (Jnawali and Ryoo, 2013).

1.3 Host-directed therapies in Tuberculosis

Drug resistance compounds the TB problem, increasing the number of agents required and the treatment duration. In 2016, as a part of Sustainable Development Goals (SDGs) blueprint, new strategies (next-generation diagnostic tests and platforms, novel anti-TB drugs and vaccine candidates) were proposed with the aim to achieve a substantial reduction of TB deaths and new cases by 2030 (WHO Global Tuberculosis Report 2018, 2018). Both the global reach of the TB problem and the high treatment costs clearly underline a need for effective new medications. In recent years there been a number of studies suggesting that host-directed therapies (HDTs) against TB are plausible. There are many avenues to explore with HDT, with multiple potential drug mechanisms and targets, some of which have yet to be uncovered. For example, a direct solution for TB HDT would be to improve bacterial killing of TB by the immune system. In both *in vitro* and *in vivo* studies, tyrosine kinase (TK) inhibitors such as imatinib have been demonstrated to promote bacterial killing, reduce granulomatous lesions and decrease bacterial loads, even against rifampicin-resistant strains. Both Mtb and Mm use Abl and related TKs for entry and intracellular survival in host macrophages. A study using murine macrophages deprived of Abl-family TKs showed impaired regulation of phagolysosomal trafficking of mycobacteria. In addition, imatinib has been showed to reduce liver pathology and tail lesions in mice infected with both antibiotic-susceptible and rifampicin-resistant strains of Mm. Histopathological lung examination also confirmed reduced bacterial loads of Mtb-infected mice proving its effectiveness against mycobacteria in acutely infected animals. Therefore, targeting of Abl1, Abl2 and imatinib-sensitive kinases offers untapped therapeutic approach which might be used in concert with anti-TB drugs rifampicin and rifabutin (Napier *et al.*, 2011).

The containment of Mtb is complex and requires pro-/-anti-inflammatory control and immune cell death. In *in vivo* experiments, treatment with anti-inflammatory drugs triggers expression of anti-inflammatory eicosanoids which have been shown effective in people with TB, reducing mortality (Critchley *et al.*, 2013). Recently, Mayer-Barber et al demonstrated that interleukin-1 alpha/beta (IL-1 α/β) confers host resistance in mouse and humans through the induction of eicosanoids that restrict the excess of type I interferon (IFN) and promote bacterial containment (Mayer-Barber *et al.*, 2014). Based on the treatment strategies against cancer in humans and chronic hepatitis C virus infection in chimpanzees (Lanford *et al.*, 2010; Takeshita *et al.*, 2010), a therapeutic silencing of micro RNA such as miR-145 and miR-20a-5p may promote bacteria eradication (Tahamtan *et al.,* 2018). One of the most promising HDTs targets killing of extracellular bacteria. Defensins are able to permeabilise bacterial membranes, target extracellular bacteria, serve as chemoattractant for phagocytic cells. Importantly, defensins can be exploited to avert the development of resistance which highlight their therapeutic potential during TB (Dong *et al.*, 2016). Recently, suppressor cell-depleting immunotherapy using denileukin diftitox has been shown to be host-beneficial during Mtb infection in mice, enhancing standard TB treatment (Gupta *et al*., 2017). Mtb is known to disseminate and form lesions in numerous organs such as spleen, lymph nodes and brain and targeting infection dissemination is another potential HDT avenue. Extra-pulmonary dissemination is facilitated by heparin-binding hemagglutinin (HBHA) adhesin which promotes attachment to lung epithelial cells. Reduced recruitment of these cells using heparin or impairing HBHA function with antibodies has been shown effective strategy to evade bacteria spreading in mice (Kohama *et al.*, 2008). Targeting HBHA virulence factor with BCG vaccine and glucocorticoids have also been shown to be important in preventing from Mtb dissemination and meningitis in children (Simmons, 2005).

Macrophages are an important innate immune cell type involved in the control of Mtb and targeting this cell type with HDTs is an important area of study. Macrophage phenotype and behaviours during TB pathogenesis could be manipulated to the host's benefit if more was understood about macrophage polarisation during infection. In mice, TLR2 activation with an agonist limits chronically stimulated T-helper type 1 cells from undergoing exhaustion, resulting in lower Mtb infection (Chodisetti *et al.*, 2015). Khan et al demonstrated that NOD-2 and TLR-4 signalling can activate dendritic cells (DCs) resulting in extensive release of cytokine and nitric

oxide (NO) and improved autophagic cell capacity, impairing Mtb survival in mice (Khan *et al.,* 2016). In humans, vitamin D3 increases cathelicidin-dependent phagosome maturation and phagolysosome assembly with the pivotal role of microRNA hsa-mir-21 (Liu *et al.,* 2012; Selvaraj *et al.,* 2009). Metformin (MET), an antidiabetic drug reduced intracellular drug-resistant Mtb growth, increased levels of mitochondrial reactive oxygen species (ROS), promotes phagosome-lysosome fusion and ameliorates lung pathology in TB patients (Restrepo and Subramanian, 2016; Singhal *et al.*, 2014). The above examples highlight that emerging HDTs represent promising alternatives for existing TB treatment.

1.4 TB pathogenesis

Mtb infection is initiated in the lungs following inhalation of aerosolised bacteria-containing droplets and passage through the host's airways. After accessing the alveolar space, bacteria encounter alveolar epithelium (type I and II epithelial cells) containing antibacterial pneumocytes (Scordo *et al.*, 2016) and alveolar macrophages (AMs) which constitute the first line of defence against invading pathogens. In mice, Mtb is recognised and phagocytosed by the patrolling AMs which are the major cells containing intracellular bacteria during the early stages of infection (Rothchild *et al.,* 2019). Whereas type I epithelial cells form the walls of the alveolus and are mainly responsible for gas exchange, type II are extensively infected with Mtb (Lerner *et al.*, 2015). In addition, type II epithelial cells produce bactericidal molecules and secrete pulmonary surfactant, hydrolytic enzymes and hydrolases (Rivas-Santiago *et al.,* 2005). Surfactant proteins adhere to Mtb resulting in agglutination which increases Mtb phagocytosis by human macrophages (Gaynor *et al.*, 1995). Mtb-human lung interactions depend upon surfactant hydrolases which have capacity to modify Mtb cell envelope leading to decreased intracellular growth within macrophages and enhanced proinflammatory response to infection (Arcos *et al.*, 2011).

Once phagocytosed, Mtb replicates inside host macrophages which leads to two primary outcomes. Either Mtb survives and primary progressive disease is established, or bacterial growth is inhibited and mycobacteria is eliminated. Mtb is able to adapt to survive within host cells due to its virulence factors and can remain in a non-replicating state within granulomas for decades in a latent infection. In order to subvert host defence Mtb use specialised type VII (T7S) secretion system to transport crucial virulence factors across their cell envelope into infected

host cells (Daleke *et al.,* 2012). Mtb uses other virulence factors such as exported repetitive protein (Erp) to grow both in cultured macrophages and *in vivo*. Erp-deprived zebrafish pathogen *Mycobacterium marinum* (Mm) is compromised during the early stages of infection and has affected survival ability following phagocytosis by host macrophages (Cosma *et al.,* 2006). Mtb has evolved to circumvent host defences and survive inside macrophages by blocking phagosome acidification and acquisition of hydrolytic enzymes and antibacterial peptides. In order to avoid phagosomal maturation Mtb uses two main virulence factors: the glycolipid lipoarabinomannan (LAM) and the secreted tyrosine phosphatase (PtpA) (Lerner *et al.,* 2015). Another Mtb virulence factor: phthiocerol dimycocerosates (PDIM) present on the outer surface of virulent Mtb profoundly contribute to the pathogenesis. PDIM promotes access to the cytosol and intracellular burden of Mtb in macrophages, dendritic cells and lymphatic endothelial cells (Lerner *et al.,* 2018). In addition, Mtb is able to escape from human macrophages by expressing miR-30A which inhibits autophagy (Chen *et al.,* 2015). Virulent Mtb is also able to survive inside host neutrophils despite prompt activation of these cells' antimicrobial effectors. The bacterial survival is accompanied with necrotic cell death of infected neutrophils and depends on radical oxygen species production (Corleis *et al.,* 2012) (Mtb virulence factors and host' cell-targeted strategies will be discussed in detail in section 1.9). After many years of latent infection, Mtb may undergo reactivation and, especially in immunocompromised patients (HIV infection), become active disease. Therefore, taking into account Mtb's ability to survive within granulomas there is a burning need for better understanding of Mtb virulence and intracellular survival strategies (Guirado *et al.*, 2013) (Figure 1.1).

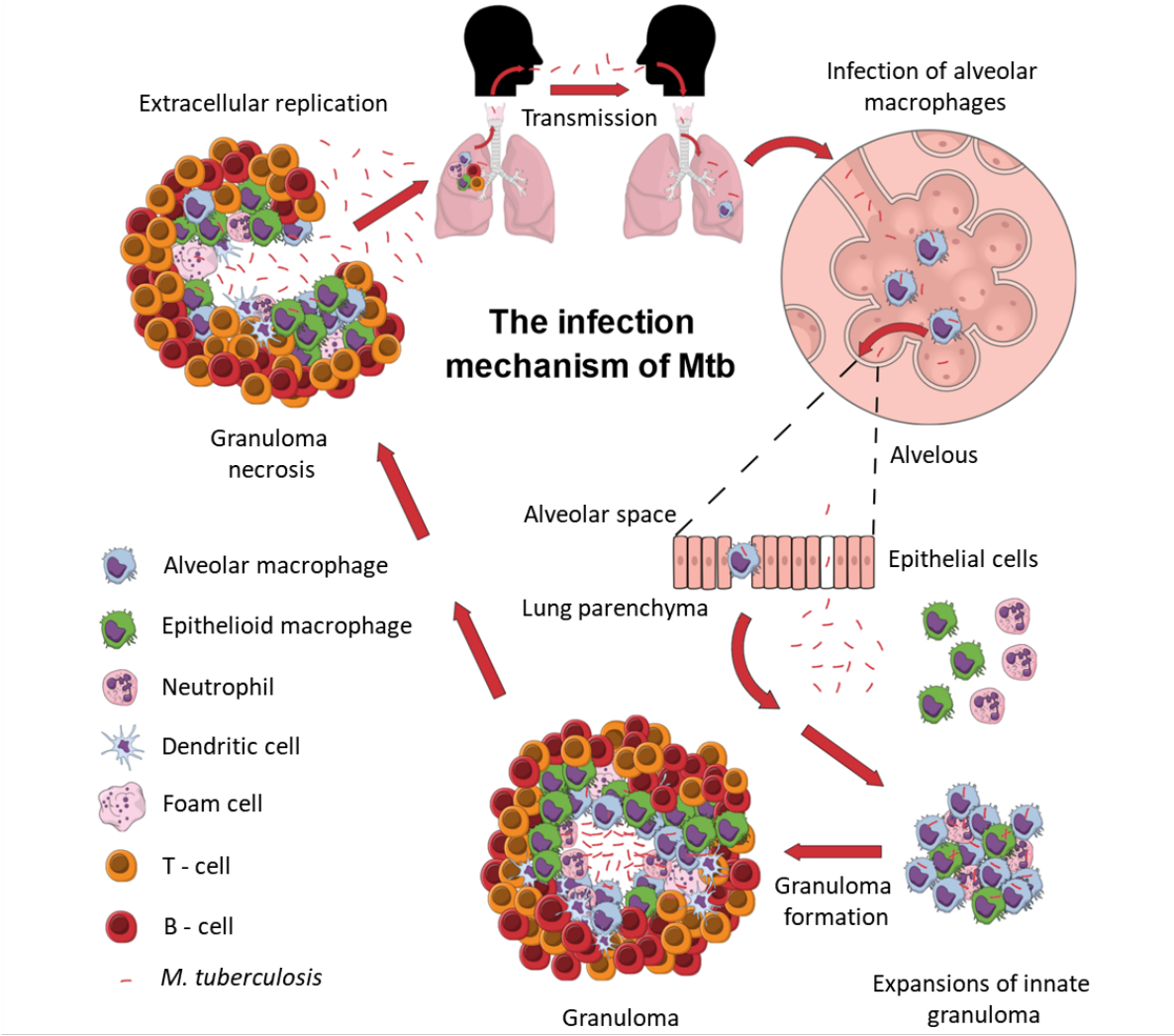


Figure 1.1 *Mycobacterium tuberculosis* infection

Mycobacterium tuberculosis (Mtb) infection initiates when fine aerosol particles containing the bacteria enters the lungs via inhalation, reaches the alveolar space and encounters the resident alveolar macrophages. If these cells fail to kill the bacteria, Mtb invades the lung interstitial tissue, either by the bacteria directly infecting the alveolar epithelium or the infected alveolar macrophages migrating to the lung parenchyma. A new round of macrophage and neutrophil recruitment to the original infected macrophage is initiated resulting in granuloma formation. This compact structure begins to grow and allow bacteria to spread to the newly arriving macrophages. The advent of adaptive immune response leads to the recruitment of immune cells, including T cells and B cells and restriction of bacterial growth. However, if the bacterial load becomes excessive, the granuloma will fail to contain the infection and bacteria will disseminate eventually to other organs. Infected macrophages can undergo necrosis, forming a necrotic core that promotes Mtb growth and transmission to the next host. Based on: C. Cambier, S. Falkow and L. Ramakrishnan (2014) Host Evasion and Exploitation Schemes of *Mycobacterium tuberculosis* and M. Pai, M. Behr, D. Dowdy, K. Dheda, M. Divangahi, C. Boehme, A. Ginsberg, S. Swaminathan, M. Spigelman, H. Getahun, D. Menzies and M. Raviglione (2016) Tuberculosis (Cambier *et al.,* 2014; Pai *et al.,* 2016).

1.4.1 The TB Granuloma

Macrophages are the primary innate immune defense against invading mycobacteria, mainly due to their ability to internalise and eliminate Mtb. However, macrophages are the primary target of Mtb virulence factors and are exploited as the main cellular niche in hallmark TB granulomas (Weiss and Schaible, 2015). Following Mtb uptake by macrophages, both bacteria and host trigger the early inflammatory response which initiates recruitment of monocytes - (precursors capable of differentiation into macrophages) - from the circulation to the infection site. As well as tissue macrophages, neutrophils and dendritic cells (DCs), adaptive immune cells such as T-cells and B-cells contribute to the complex granuloma structure. As infection progresses, granulomas undergo caseation which may contribute to lesion formation and subsequent spreading to other organs (Russell *et al.,* 2010). The word "caseating" usually, but not exclusively, refers to active human tuberculosis and is related to a necrotic process (Adams, 1976). Identification of necrotising granulomas is important from a diagnostic point of view as this process contributes to transmission and morbidity of TB (Cosma *et al.,* 2003; Mukhopadhyay *et al.,* 2012). The heterogeneity of granuloma compositions was illustrated by distinct degrees of immune activation, macrophage immune-phenotypes and capacity to restrict Mtb growth (Gideon *et al*., 2015; Kim *et al*., 2010; Lin *et al*., 2014). This variability in pulmonary TB patients applies to diversity of lesion types, complexity of local immune responses in the lungs and processes leading to independent differentiation (Subbian *et al.,* 2015).

1.4.2 Active TB disease

Granuloma stability is maintained due to local production of T_H1 type cytokines by antigen-specific T lymphocytes. Mtb antigens produced by bacteria are present within granulomas which constantly activate phagocytic cells and trigger adequate proinflammatory response and bactericidal activity (Egen *et al.,* 2011). Efficient host immune responses ensure bacterial control and a gradual reduction of granuloma size (Ehlers and Schaible, 2013). However, when the host's response to granuloma becomes imbalanced due to, for example immunosuppression due to HIV, mycobacteria begin uncontrolled growth resulting in an active form of disease (Lawn *et al.*, 2002). In consequence, lung granulomas start expanding resulting in development of cavitary pulmonary disease. This process depends not only on a variety of interactions which occur between bacteria and host macrophages but also on the virulence of Mtb strains (Matty *et al.,*

2015). Histopathological examination of cavitating lesions revealed considerable changes in lung composition and host phagocytes ringed with caseous necrotic lesions. In addition, the active form of disease is characterised by a totally acellular necrotic centre and granulomatous-fibrotic tissue with Langerhans-type giant cells, epithelioid macrophages, foam cells and numerous dispersed lymphocytes (Kaplan *et al.,* 2003). In consequence, imbalanced cellular integrity and a loss of colocalisation of host immune cells leads to impaired macrophage functions and unrestricted Mtb growth (Guirado *et al.,* 2013).

1.5 Innate immune response to TB

The outcome of TB depends, to a large extent, on the complex interplay between host immune cells during pathogenesis. During Mtb infection, numerous host immune cells such as macrophages, neutrophils, conventional/-plasmacytoid dendritic cells (cDCs/-pDCs), natural killer (NK) cells, B cells, T cells and innate lymphoid cells (ILCs) contribute to mounting an adequate early immune response against bacteria (Lloyd and Marsland, 2017). DCs are potent phagocytes and after Mtb internalisation utilise Major Histocompatibility Complex (MHC) to present antigens to T cells (Marino *et al.,* 2004). In addition, numerous innate-like cells including non-conventional T cells such as mucosal associated invariant T (MAIT) cells, CD1-restricted lymphocytes and NKT cells, airway epithelial cells and mast cells have been shown to participate in limiting Mtb growth (Liu *et al.,* 2017).

1.5.1 Macrophages

Macrophages are important phagocytic cells which play a key role in the host response to invading bacteria. Tissue homeostasis is maintained by a balance between pro- and anti-inflammatory macrophage functions. Their versatility has been studied in the context of immunity, intracellular communication, inflammation, protective and pathogenic functions during antimicrobial defence (Murray and Wynn, 2011). Classically, two extreme macrophage polarisation states have been described in the literature, based on iNOS (M1)/arginase (M2) expression and stimulation by Th1/Th2 cells (Mills *et al.,* 2000). The inflammatory and anti-inflammatory types of macrophages are distinguishable depending on their response to lipopolysaccharide (LPS) and different T cell-derived cytokines. Macrophages polarised by cytokines such as IFN-γ secreted by T helper 1 (Th1) cells are classified as M1 classically-activated

macrophages (Atri *et al.,* 2018). The second type, M2-polarised macrophages are defined as alternatively activated by interleukin 4 (IL-4) and interleukin 13 (IL-13) secreted by T helper 2 (Th2) cells (Bonecini-Almeida *et al.*, 1998; Mantovani *et al.,* 2004; Mosser and Edwards, 2008). However, this simplified classification system does not mirror the full diversity of macrophages *in vivo*. Recent studies have substantially enriched our understanding of macrophage biology and highlighted the need for standardised terminology referring to their activation states (Murray *et al.,* 2014; Xue *et al.,* 2014). The polarised immune response has recently been considered from a 'macrophage first' point of view (Mills and Ley, 2014). This concept assumes that the first M1/M2 differentiation occurs in response to microbial or parasite stimuli sensed by macrophages via TLRs, PRR and cluster of differentiation 14 (CD14), before interactions with adaptive immunity. For instance, LPS triggers innate-activated M1 macrophages, ROS, NO and pro-inflammatory cytokines formation (Tan *et al.,* 2016). As a consequence, IFN-γ stimulates T cells to form Th1 cells which then promote classically-activated macrophages by a positive feedback loop. Similarly, parasitic stimuli trigger M2 polarisation resulting in anti-inflammatory macrophage formation and high arginase activity. IL-4 and IL-13 stimulate T cells to form Th2 cells which enhances differentiation of alternatively-activated macrophages through a positive feedback loop (Wiegertjes *et al.,* 2016).

The important roles of macrophages in controlling TB infection have been extensively studied (Weiss and Schaible, 2015). M1 macrophages are essential for effective immune responses during the early stages of TB (Chacón-Salinas *et al.,* 2005). M1 cells are involved in bacterial killing and this process is regulated by reactive oxygen species (ROS) and nitric oxide (NO) (Benoit *et al.,* 2008). Arginase competes with NOS for a common substrate and hence inhibits NO formation, which partially explains why M2 macrophages do not control Mtb (Rath *et al.,* 2014). These data indicate that, M1 macrophages play a protective role during Mtb infection, whereas M2 macrophages act in the opposite way by competitively blocking potent antimicrobial responses leading to an attenuated immune response. The outcome of macrophage intracellular processing of Mtb is a crucial step in TB pathogenesis and will be discussed in detail in section 1.8.1.

1.5.2 Neutrophils

Neutrophils are remarkably short-lived white blood cells and are produced in high numbers, with numbers produced per day, estimated to be between 5×10^{10}-10×10^{10} (Summers *et al.,* 2010). They are produced and stored in bone marrow from where are deployed into bloodstream and circulatory system (Yamashiro *et al.*, 2001). Neutrophils are phagocytic cells and first responders during the early stages of infection (Riedel and Kaufmann, 1997). The roles of neutrophils in human TB are not as well-characterised as the roles of macrophages, in part because neutrophils have a very short lifespan *in vitro* and are not easy to study. Human neutrophils have been shown to act against Mtb through reactive oxygen species (ROS) and secretion of a range of antimicrobial proteases and proteins (May and Spagnuolo, 1987). Additionally, neutrophils are able to extrude structures know as neutrophil extracellular traps (NETs), comprised of decondensed chromatin fibres coated with antimicrobial granular and cytoplasmic proteins, such as myeloperoxidase (MPO), neutrophil elastase (NE), and α- defensins (Porto and Stein, 2016). In TB, neutrophils release NETs coated with NE and histones in response to Mtb genotypes (H37Rv and *Mycobacterium canetti*). NETs immobilise bacteria but are unable to eliminate Mtb which can have a damaging effect on human lungs in active TB (Ramos-Kichik *et al.,* 2009). The Mtb-induced activation of circulating polymorphonuclear neutrophils (PMN) present in peripheral blood from patients with active TB results in accelerated apoptosis (Alemán *et al.,* 2002). Further investigation of this process revealed that apoptosis of PMNs is mediated through interaction of Mtb with TLR2 leading to activation of the p38MAPK pathway (Alemán *et al.,* 2004). A number of cytokines such as TNF-α, IL-8 and IL-18 have been proposed to promote pro-inflammatory neutrophil behaviour during infection, whereas transforming growth factor beta (TGF-β) and IL-10 regulate anti-inflammatory cell activity (Kany *et al.,* 2019). Interestingly, human neutrophils can affect dendritic cells (DCs) stimulated by Mtb, disrupting their interactions with T cells and inhibiting their antimicrobial activity (Alemán *et al.*, 2007). In zebrafish, neutrophils migrate to sites of inflammation and contribute to innate immune response against *Mycobacterium marinum* (Mm) including bacteria-induced granuloma formation (Meijer *et al.,* 2008). In addition, impaired recruitment of zebrafish neutrophils leads to increased number of bacteria (Yang *et al.,* 2012). Moreover, there is a growing body of evidence that neutrophils can defend the host during mycobacterial infection (Lowe *et al.,* 2012) and control Mtb more effectively via a Hif-dependent pathway (Elks *et al.,* 2013).

1.5.3 Dendritic cells

Dendritic cells (DC) represent a subset of important antigen presenting cells indispensable for both initiation and maintenance of T-cell-dependent immunity. They are involved in the production of anti-mycobacterial cytokines such as TNF-α, IFN-γ and interleukin (IL)-12 (Prendergast and Kirman, 2013). Early *in vitro* studies have shown that DCs are able to phagocytose and restrict Mtb growth in a nitric oxide synthase-dependent fashion however, lack capacity to eliminate Mtb (Bodnar *et al.,* 2001). In mice, Mtb uptake by DCs has been confirmed for both intranasal and intravenous infections with exclusive role of DCs in antigen presentation (Jiao *et al.,* 2002). DCs interact with apoptotic macrophages (Winau *et al.,* 2004) and neutrophils which leads to elevated IL-12 levels, enhanced antigen presentation and migration (Blomgran and Ernst, 2011; Hedlund *et al.,* 2010; Morel *et al.,* 2008). The recognition of Mtb ligands such as LprG and mannosylated phosphatidylinositol mannosides (PIMs), is mainly due to DC-specific ICAM-grabbing nonintegrin (DC-SIGN) expressed by DCs (Carroll *et al.,* 2010). The adaptive immune response during human TB has been shown to dampen as a result of reduced integrin expression, adhesion and migration to chemokines in Mtb-infected DCs (Roberts and Robinson, 2014). Recently, *in vivo* studies suggest that Mtb proline-glutamate (PE)27 is a promising vaccine candidate due to DC-dependent activation of Th1-polarized memory immune response in mice (Kim *et al.,* 2016). During Mtb infection human DCs have elevated expression of CD13 which augments T-cell activation indicating that targeting receptor of this cell surface marker may enhance T-cell-dependent immune response (Kuo *et al.,* 2016).

1.5.4 Natural killer cells

Natural killer (NK) cells contribute to the early protection during Mtb infection due to their cytolytic capacity. NK cells are granular innate lymphocytes able to amplify the antimicrobial defence and recognise Mtb-infected macrophages via natural cytotoxicity receptors (NCRs) such as NKp44, NKp46 and NKG2D (Vankayalapati *et al.,* 2005). NK cells are not MHC-restricted (Allen *et al.,* 2015) and in humans have been shown to lyse infected monocytes and restrict Mtb growth (Esin *et al.,* 2013). NKs activate human macrophages via IFN-γ production and their cytokine secretion leads to induction of regulatory T cells (Tregs) (Vankayalapati and Barnes, 2009). NK cell-derived IFN-γ is responsible for regulation of innate resistance and neutrophil response in T cell-deficient mice which might be important for AIDS patients and other individuals with

compromised CD4+ T cell function (Feng *et al.,* 2006). In addition, human NKs increase γδ T-cell population - a T-cell subset involved in recognition of Mtb phosphoantigens and elimination of Mtb-infected macrophages through production of CD54, TNFα, GM-CSF and IL-12 (Ismaili *et al.,* 2002). In TB patients Mtb induces enhanced expression of cytolytic proteins: perforin and granulysin, via activation of the NKG2D/NCR cell-surface receptor and intracellular signalling pathway involving ERK, JNK and p38 MAPKs. Lu et al demonstrated that in mice NK cells use the same contact dependent mechanism to eradicate Mtb which may be useful in developing new strategies during TB (Lu *et al.,* 2013).

1.6. Recognition of mycobacteria

Immune cells are equipped with a wide range of receptors facilitating appropriate reactions to virulence factors during infections. After Mtb invasion of human lungs pathogen-associated molecular patterns (PAMPs) present on bacteria surface are recognized by pattern recognition receptors (PRR) such as Toll-like receptors, Dectin-1, C-type lectin receptors (CLRs), nucleotide-binding oligomerisation domain-containing protein 2 (NOD2), dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin and mannose receptor expressed in airway epithelial cells (AECs) (Elomaa *et al.,* 2005; Li *et al.,* 2012). The recognition of mycobacteria by Toll-like receptors (TLRs), in particular TLR4, is a crucial step to trigger the innate immune response (Tjärnlund *et al.,* 2006). In response to Mtb advent, human AECs conduct antigen presentation to mucosal-associated invariant T cells (MAITs) which trigger interferon (IFN)-γ, tumour necrosis factor (TNF)-α and granzyme production and induce IFN-γ-dependent macrophage activation (Gold and Gebhart, 2010). In addition to antigen presentation human AECs are indirectly involved in regulation of airway surface liquid (ASL) components such as β-defensin 2, cathelicidin (LL-37), hepcidin and a wide range of AECs-secreted cytokines responsible for phagocyte recruitment and activation (Lerner *et al.,* 2015). Besides being professional phagocytes, macrophages and neutrophils produce common effector molecules including granular proteins, oxidants, chemokines and cytokines indispensable to mount an adequate immune response (Kumar *et al.,* 2018). Despite overlapping antigens and PRRs expression, neutrophils are able regulate macrophage functions providing bacterial antigens and facilitate their presentation to T cells (Megiovanni *et al.,* 2006; Morel *et al.,* 2008).

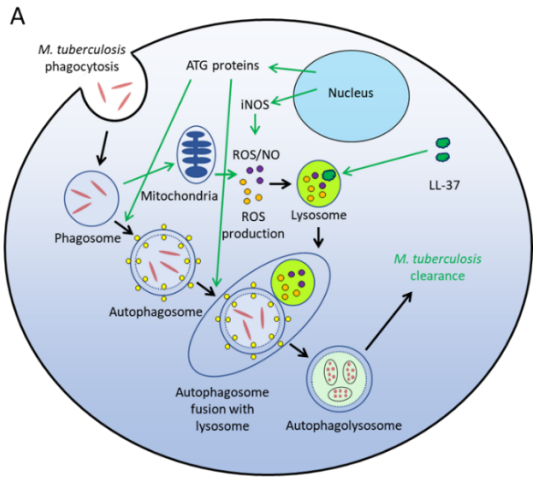
1.7 Phagocytic cell recruitment

Once Mtb reaches the alveoli it encounters alveolar macrophages (AMs), neutrophils, dendritic cells (DCs) and type I and II epithelial cells. Host innate immune cells are equipped with a large variety of antimicrobial strategies targeting bacteria which is evolutionary adapted to evade these mechanisms. As a result, host cells either eliminate bacteria or, in some cases, bacteria survive and infection progresses toward active disease (Lerner *et al.,* 2015). After the first two weeks of Mtb infection in mice, macrophages and neutrophils are the most infected phagocytic cells (Wolf *et al.,* 2007). Mtb-infected macrophages provide an early Mtb niche facilitating bacteria replication or may serve as a vehicle and promote dissemination to other organs (Cohen *et al.,* 2018; Frieden *et al.,* 2003). However, macrophages play a pivotal role in protection and bacteria elimination and together with MAITs, ILCs, natural killer cells (NK), invariant NK T cells (INKTs) and T cells initiate myeloid cells activation (Dhiman *et al.,* 2009; Rothchild *et al.,* 2014; Steinbach *et al.,* 2016). In consequence, neutrophils, DCs and multiple macrophage subsets migrate and infiltrate lungs (Repasy *et al.,* 2013). Neutrophil influx is important for trafficking of DCs, initiation of adaptive immune response by activation of naïve antigen-specific CD+ T cells and granuloma formation (Blomgran and Ernst, 2011). Nonetheless, the excessive numbers of infected neutrophils contribute to disease progression and an efficient removal of these cells is necessary for protection of surrounding tissues from damaging impact of neutrophil bioactive molecules (Dallenga *et al.,* 2017). The diversity of recruited immune cells is crucial for the infection outcome. If the immune response is unbalanced then it limits host's ability to restrict Mtb and further activation of adaptive immune cells (Norris and Ernst, 2018; Wolf *et al.,* 2007).

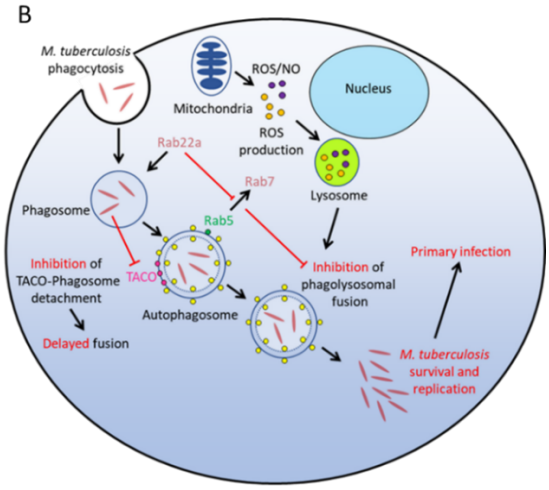
1.8 Phagocytosis and intracellular effector mechanisms against mycobacteria

In response to Mtb infection, a variety of innate immune cells such as alveolar macrophages, dendritic cells (DCs), neutrophils, NK cells epithelial cells and other cells are activated to counteract pathogen invasion (Lyadova, 2017). In accordance with human and animal studies of TB, both macrophages and neutrophils are the primary innate immune cells against Mtb. Macrophages are main phagocytic cells during mycobacterial infection accountable for mounting adequate antimicrobial response but also serving as a protective niche for Mtb (Kumar *et al.,* 2019). Mtb has been found to be phagocytosed by mice macrophages (Ehrt *et al.,* 2001), which play also an important role in efferocytosis - a process of removing bacteria encapsulated in the

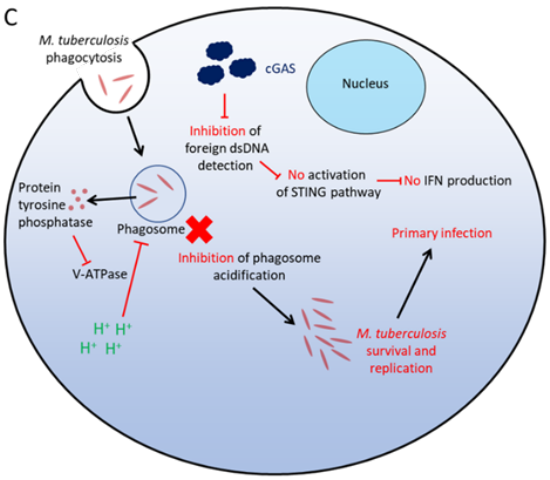
necrotic or apoptotic cells (Hosseini *et al.,* 2016; Weiss and Schaible, 2015). Efferocytosis of apoptotic neutrophils by macrophages has been show to limit bacteria growth (Greenlee-Wacker, 2016). Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB, promoting rapid bacteria replication and spreading (Eum *et al.,* 2010). Mycobacteria elimination occurs via defence strategies including phagocytosis, apoptosis, autophagy, phagosome acidification, ROS and RNS production, NET formation and septin caging, amongst others (Deretic *et al.,* 2013; Mostowy *et al.,* 2010). Both macrophages and neutrophils share an essential role in elimination of bacteria using granular proteins and oxidants (McCracken and Allen, 2014). Tan et al shown that granule contents of human neutrophils can be delivered to macrophage endosome and used against Mtb (Tan *et al.,* 2006) (Figure 1.2).



***M. tuberculosis* killing**



Inhibition of phagolysosomal formation



Inhibition of phagosome maturation and interferon production

Figure 1.2 Intracellular fate of *Mycobacterium tuberculosis* and bacterial clearance via the autophagic pathway

(A) *Mycobacterium tuberculosis* (Mtb) killing. Following phagocytosis Mtb is encapsulated in phagosome which fuses with lysosome resulting in phagolysosome formation. Inside phagolysosome bacterial components are degraded by enzymes and released outside by endocytosis. ROS production is increased upon Mtb infection. Upregulation of LL-37, iNOS and ATG proteins in macrophage activate the autophagy process and contribute to the killing of Mtb. (B) Inhibition of phagolysosome formation. Mtb prevents the formation of phagolysosome by blocking the fusion of autophagosome with lysosome. Phagolysosome fusion occurs after exchange of phagosome surface protein Rab5 to Rab7. Phagosome encapsulating bacteria recruits Rab22a protein which prevents Rab5/Rab7 exchange and precludes phagolysosome formation. Mtb-induced inhibition of TACO-phagosome detachment leads to delayed phagolysosome fusion. As a result, bacteria survive, replicate and establish primary infection. (C) Inhibition of phagosome maturation and interferon production. Under normal conditions V-ATPase pumps the protons into the phagosome to increase its acidity. Mtb release PTPA protein which binds to subunit of V-ATPase pump and inhibits the acidification of the phagosome, enabling the intracellular survival of bacteria. cGAS proteins are responsible for detection of foreign DNA. Without degradation and lysis there is no detection of foreign DNA which results in precluded activation of STING pathway and inhibited interferon production. Green arrows indicate stimulation of the process and the red lines indicates inhibition of the process. NO – nitric oxide, ROS – reactive oxygen species, iNOS - nitric oxide synthase, PTPA – protein tyrosine phosphatase, cGAS – cyclic GMP-AMP synthase, Rab5/7/22a - Ras-related protein 5/7/22a, TACO – tryptophan-asparatate containing coat protein. Adapted from: S. Ahmed, R. Raqib, G. Guðmundsson, P. Bergman, B. Agerberth, R. Rekha (2020) Host-Directed Therapy as a Novel Treatment Strategy to Overcome Tuberculosis: Targeting Immune Modulation (Ahmed *et al.,* 2020).

1.8.1 Phagosome maturation and acidification

After recognition and engulfment, pathogens are enclosed in the phagosome, a vesicle structure which fuses with lysosomes to form a phagolysosome. However, once phagocytosed by host cells Mtb can prevent phagosome maturation to avoid unfavourable conditions such as low pH, hosts hydroxylases and other antibacterial lysosomal components (Liu *et al.,* 2017). Mtb use phosphatidylinositol mannosides (PIMs), to specifically stimulate homotypic fusion of early endosomes to modify the intracellular niche to its advantage (Vergne *et al.,* 2004) in a process which requires small GTPase Rab14 (Kyei *et al.,* 2006). In humans, phagosome maturation can also be blocked by Mtb mannose-capped lipoarabinomannan (ManLAM) which binds to the pattern recognition mannose receptor (MR) and limit phagosome-lysosome (P-S) fusion (Kang *et al.,* 2005). Walburger et al demonstrated that Mtb secrete eukaryotic-like serine/threonine protein kinase within human macrophages to arrest phagosome-lysosome fusion and promote bacteria survival (Walburger *et al.,* 2004). In addition, Mtb has been shown to use protein

phosphatase (PtpA) which block H+-ATPase prerequisite to luminal acidification (Wong *et al.,* 2011). Another Mtb strategy to survive within host macrophage is via the acid and phagosome regulated (*aprABC*) locus which is used by bacteria to withstand the acidic pH of the macrophage phagolysosome (Abramovitch *et al.,* 2011). More recently, interferon-induced transmembrane (IFITM) 3 protein has been shown to bind to endosomal membranes and enhance its maturation in mice (Wee *et al.,* 2012). Regardless of numerous strategies within the merged phagolysosome, bacteria are subjected to antimicrobial mechanisms such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) activity, acidification and exposure to a range of proteases (Torraca *et al.,* 2014).

1.8.2 ROS and RNS production

In response to infection, Mtb is exposed to a wide array of host-derived antimicrobials including reactive oxygen and nitrogen species (ROS and RNS) (Voskuil *et al.,* 2011). Activity of two crucial phagocytic antimicrobial pathways, NADPH phagocyte oxidase (phox) and inducible nitric oxide synthase (iNOS), leads to generation of superoxide (O_2^{-*}) (ROS) and nitric oxide (NO^*) (RNS) radicals, respectively (Fang, 2004) ROS and NOS are highly antimicrobial. Following IFN-γ activation, macrophages generate NO from L-arginine and oxygen through NOS2 enzyme activity (Bogdan, 2001), and reactive oxygen intermediates (ROI) through NOX2 (Feng *et al.,* 2004). Both are regarded as important mechanisms against TB (MacMicking *et al.,* 2003). In *in vitro* IFN-γ induces apoptosis in Mtb-infected macrophages in an NO-dependent manner which results in killing mycobacteria (Herbst *et al.,* 2011). NO determines the availability of nitrogen sources required for T-cell activation and proliferation, therefore is important for an effective immune response and host's ability to eliminate Mtb. Production of this antimicrobial depends on iNOS activity which compete with arginase for its common substrate L-arginine. Arginase-mediated conversion of arginine to ornithine and urea augments mycobacterial growth (Das *et al.,* 2010). Mice deprived of arginine hydrolytic enzyme arginase 1 (Arg1) have lower bacterial counts following Mtb infection (El Kasmi *et al.,* 2008). However, in hypoxic conditions of necrotic granulomas NOS2-dependent mycobacterial killing facilitated by NO is ineffective due to high oxygen requirements. In contrast, Arg1 does not require oxygen and contribute to T-cell regulation and tissue repair (Duque-Correa *et al.,* 2014). Mice macrophages with abrogated Arg1 expression have augmented lung granuloma pathology and increased bacterial burden (Duque-

Correa *et al.,* 2014). These findings indicate an important role of Arg1 in Mtb control in the low-oxygen environment of granuloma, where NOS2 has been shown ineffective. NO plays important role in the regulation of Mtb-triggered tissue damage by inhibiting the assembly of the NLRP3 inflammasome via thiol nitrosylation. NO mediates lymphocyte-derived IFN-γ suppression of both IL-1β and infection induced immunopathology (Mishra *et al.,* 2013). In mice and human macrophages, NOX-2-driven enhancement of ROI formation also contribute to cathelicidin-dependent intracellular killing of Mtb (Sonawane *et al.,* 2011). ROI can be also generated by mitochondria following TNF-α stimulation however, abundance of this cytokine leads to increased macrophage necrosis and apoptosis and a consequent augmentation of Mtb infection (Roca and Ramakrishnan, 2013). Inside phagosomes, NO and ROI generate nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), nitrosothiols, nitroxyl (HNO) and dinitrosyl-iron complexes, which damage bacterial membrane lipids, DNA, thiol and tyrosine residues (Fang *et al.,* 2004). In mice, NOS2, which is indispensable for NO production, plays pivotal roles in controlling Mtb infection and disrupting *NOS2* allele leads to progression of chronic tuberculosis (MacMicking *et al.,* 1997). In the process of generation NO from arginine by macrophages citrulline is generated. This by-product is required for consecutive arginine synthesis, a process facilitated by arginine-succinate synthase (Ass1), and mice macrophages lacking this enzyme have impaired ability to restrict Mtb (Qualls *et al.,* 2012) (Figure 1.3).

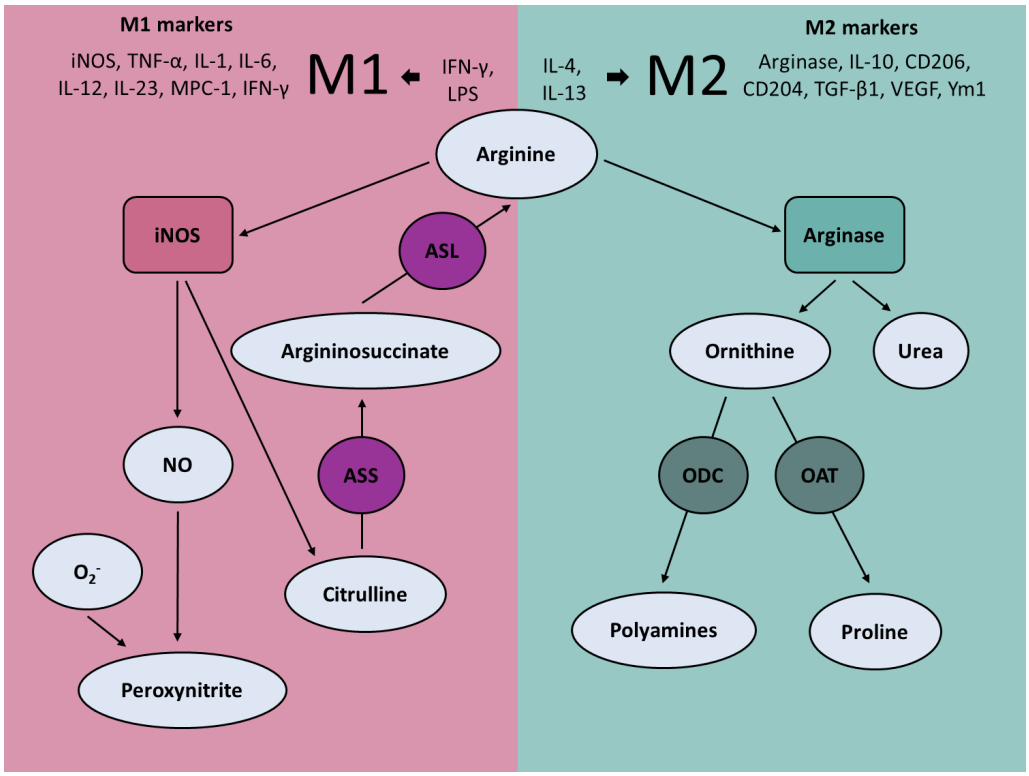


Figure 1.3 Arginine metabolism via iNOS or arginase and M1/M2 macrophage polarisation

Pro-inflammatory M1 and anti-inflammatory M2 macrophages are characterised by the metabolism of arginine via two competing enzymes: iNOS or arginase. IFN-γ and (LPS are the main stimulators of M1 polarization whilst IL-4 and IL-13 are inducers of M2 polarisation. The diagram focuses on relevant enzymes M1 (magenta-shaded circles), M2 (green-shaded circles), metabolites (grey ellipses) and the position of iNOS (pink rectangular) and arginase (turquoise rectangular) is highlighted. Abbreviations: ASS - argininosuccinate synthase, ASL - argininosuccinate lyase, OAT - ornithine aminotransferase; ODC - ornithine decarboxylase, IFN - interferon, LPS - lipopolysaccharide, IL - interleukin, iNOS - inducible nitric oxide synthase, TNF - tumour necrosis factor, CD - cluster of differentiation, Ym1 - chitinase-like 3, TGF - transforming growth factor, VEGF - vascular endothelial growth factor. Based on: K. Lee (2019) M1 and M2 polarisation of macrophages: a mini review and M. Rath, I. Müller, P. Kropf, E. Closs and M. Munder (2014) Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages (Lee, 2019; Rath *et al.,* 2014).

Neutrophils alongside macrophages contribute to host immune defense against Mtb and are able to kill bacteria through degranulation, reactive oxygen intermediates (ROI) production and neutrophil extracellular traps (NETs) (section 1.8.3). ROI are produced by NADPH-dependent oxidase and superoxide dismutase. In zebrafish, following macrophage apoptosis and Mtb release neutrophils arrive to nascent granuloma and kill internalised bacteria through NADPH oxidase-dependent mechanisms (Yang *et al.,* 2012). Activated neutrophils produce nitric oxide (NO) and peroxynitrate, which is generated from NO oxidation. Neutrophil phagosome

maturation and anti-microbial granule release are rapid, practically overlapping with bacterial uptake (Nordenfelt and Tapper, 2011). Neutrophil granules can be divided into three different types: primary azurophilic, secondary specific and tertiary gelatinase granules. First two types mainly contain AMP, lactoferrin (LF), lysozyme, myeloperoxidase (MPO), elastase, cathepsins and proteisase-3. The latter group is equipped with anti-microbials targeting extracellular matrix such as matrix metalloproteinase 9 (MMP-9), collagenase and gelatinase. The iron-sequestrating proteins, lipocalin 2 (Lcn2) and LF retrieve iron from loaded mycobacterial siderophores or directly bind Fe^{3+}. Oxygen radicals are produced via activity of vesicular NADPH-oxidase and H_2O_2 reacting with oxygen radicals (O_2^-), hydroxyl radicals and singlet oxygen. H_2O_2 is subsequently metabolised by MPO to hypochlorous acid and chloramines. Cathelicidins, β-defensins and the human neutrophil peptide 1 (HNP-1) constitute a non-oxidative effectors (Weiss and Schaible, 2015). Mtb are able to avoid MPO/NOX2-dependent oxidative killing and escape, initiating RD1-dependent ROI-mediated necrosis of human neutrophils (Corleis *et al.,* 2012). In humans, following neutrophil apoptosis and subsequent phagocytosis by macrophages, bactericidal peptides contained with neutrophil granules target Mtb, which demonstrate a cooperative defense strategy between these two important phagocytic cells (Tan *et al.,* 2006) (Figure 1.4).

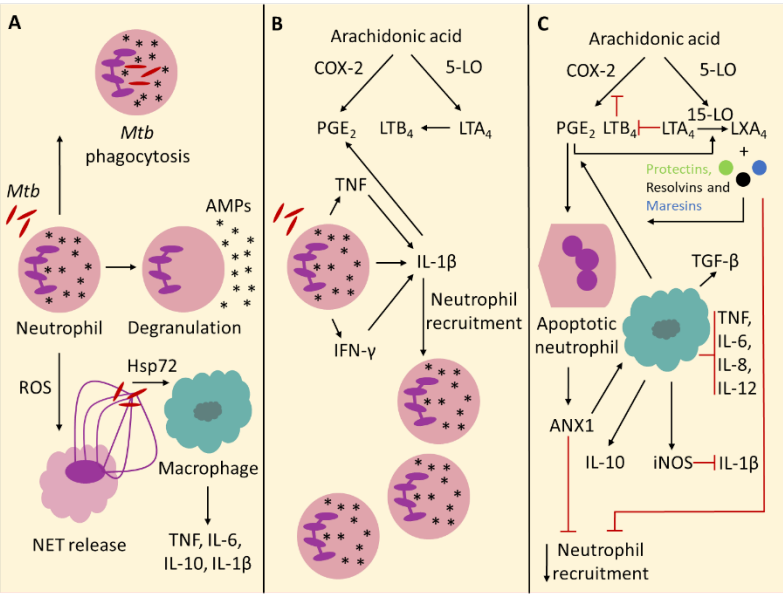


Figure 1.4 Neutrophils in *Mycobacterium tuberculosis* infection and disease

(A) Neutrophils use a wide range of mechanisms to mediate *Mycobacterium tuberculosis* (Mtb) infection. These included phagocytosis, degranulation, ROS formation and NET release. Neutrophil granules can fuse with the phagolysosome, degranulate and release antimicrobial peptides (AMPs). Mtb-induced NETs transfer the danger signal heat shock protein 72 (Hsp72) to adjacent macrophages. This interaction induces a pro-inflammatory response in macrophages leading to the release of IL-6, TNF, IL-1β, IL-10. (B) Interaction of recruited neutrophils with Mtb mediates the activation of several pathways which contribute to inflammation and clearance of Mtb infection. Interleukin-1β (IL-1β) release is mostly mediated in an inflammasome dependent manner. Tumour necrosis factor (TNF) induces NF-κB which mediates the induction of gene expression of IL-1β in neutrophils. Interferon-γ (IFN-γ) may also regulate the release of IL-1β. IL-1β is a key player in mediating the release of eicosanoid prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) both of which contribute to inflammation and the recruitment of neutrophils. Eicosanoids are important lipid mediators derived from arachidonic acid (AA) and are rapidly synthesised by phagocytes after acute challenge with Mtb. (C) Cyclo-oxygenase-2 (COX-2) competes with 5-Lipoxygenase (5-LO) or 15-lipoxygenase (15-LO) for the generation of each of the different eicosanoids. PGE2 eventually becomes a stop signal and has a negative feedback on COX-2 and 5-LO. The production of lipoxin A4 (LXA4) is favoured. In addition, annexin A1 (ANX1) stimulates release of the anti-inflammatory cytokine, IL-10, by macrophages, inhibits neutrophil migration, and promotes efferocytosis of apoptotic cells. Neutrophils express inducible nitric oxide synthase (iNOS) which has a further negative feedback on IL-1β release. The net effect is an increase in neutrophil apoptosis and clearance by tissue macrophages. A hallmark of the anti-inflammatory response is the production of TGF-β and PGE2, and the inhibition of IL-6, IL-8, IL-12, and TNF release by the phagocytosing macrophages. In addition to the release of endogenous anti-inflammatory mediators, pro-resolution action is also required. Lipoxins, protectins, resolvins and macrophage mediator in resolving inflammation (maresins) are unique mediators fulfilling this duality. More macrophages are recruited and further neutrophil recruitment is inhibited and inflammation is resolved. Adapted from E. Kroon, A. Coussens, C. Kinnear, M. Orlova, M. Möller, A. Seeger, R. Wilkinson, E. Hoal and E. Schurr (2018) Neutrophils: Innate Effectors of TB Resistance? (Kroon *et al.,* 2018).

1.8.3 NET formation

Neutrophils have a variety of killing mechanisms and following bacteria engulfment, degranulation and generation of ROI they can form specialised structures called neutrophil extracellular traps (NETs) (Lyadova, 2017). These structures are comprised of decondensed chromatin, contents from azurophilic granules and cytoplasmic proteins such as myeloperoxidase, neutrophil elastase (NE), and α-defensins. NETs are formed by activated neutrophil granulocytes also known as polymorphonuclear leukocytes (PMNs) as a consequence of an active cell death program called NETosis. This process is activated in response to microbes however, it can also lead to inflammatory injury indicating that NETosis participate in both acute lung injury and in autoimmunity (Almyroudis *et al.,* 2013). NETs are formed in response to pathogens, activated platelets or pathogen components. Following stimulation of PMNs the Raf/MEK/ERK pathway is activated resulting in the assembly of the multimeric NADPH oxidase complex and ROS production. After disintegration of membranes neutrophil elastase is released into the nucleus resulting in hypercitrullination of histones, decondensation and chromatin mobilisation (Brinkmann *et al.,* 2012). NETs are regarded as a form of innate immune response that binds pathogens, limit their dissemination, and providing a high local concentration of antimicrobial agents to degrade virulence factors and eliminate bacteria (Brinkmann *et al.,* 2004). *In vitro*, Mtb induces NETs formation which trap bacteria but are unable to kill them (Ramos-Kichik *et al.,* 2009). However, their relevance in TB remains unclear, as recent findings suggest that NETs can enhance Mtb growth and promote bacteria expansion (Cardona and Prats, 2016; Ehlers and Schaible, 2013).

1.8.4 Autophagy

Autophagy (macroautophagy) is an important innate defence mechanism in which cells target cytoplasmic contents for lysosomal degradation. Autophagy is a dynamic process initiated by autophagic vacuole formation and leads to elimination of damaged organelles, protein aggregates and intracellular pathogen (Gutierrez *et al.,* 2004). Whereas autophagy is a broad term, xenophagy strictly refers to targeting intracellular pathogens to lysosomes and limiting their growth. This process encompasses bacteria ubiquitination, recognition of the ubiquitin by autophagy receptors that interact with microtubule-associated protein 1 light chain 3 (LC3), recruitment the bacteria to autophagosomes, fusion with lysosomes (resulting in

phagolysosome) and cargo degradation (Mizushima and Komatsu, 2011). Mtb has been the first intracellular pathogen used to elucidate the multifaced mechanism of xenophagy and explain how host cells limit bacteria replication (Kimmey and Stallings, 2016). IFN-γ is well-established autophagy activator, prerequisite to overcoming the phagosome maturation block triggered by Mtb. Dutta et al demonstrated that in human macrophages anti-inflammatory IL-6 hampers both IFN-γ and starvation-induced autophagy in Mtb-infected cells and using anti-IL-6 antibody rescued IFN-γ-dependent mycobacterial killing (Dutta *et al.,* 2012). Numerous knockdown studies specified autophagy genes such as *Ulk1* (Jayaswal *et al.,* 2010), *Becilin1* and *Atg5* (Kim *et al.,* 2012), *Atg7* (Wang *et al.,* 2013) and *p62* (Watson *et al.,* 2012) which limit Mtb survival and promote effectiveness of antimycobacterial first-line drugs such as isoniazid and pyrazinamide (Kim *et al.,* 2012). Autophagy modulation can be achieved by TLR3 and TLR4 stimulation with LPS or poly (I:C) which induce autophagy-mediated elimination of mycobacteria in macrophages (Xu *et al.,* 2013).

In vivo, autophagy has been shown to play both antibacterial and anti-inflammatory role during Mtb infection. Castillo et al demonstrated that Arg5 (flox/flox) LysMCre mice are severely susceptible to Mtb infection highlighting autophagy as a mechanism which supresses Mtb growth and damage inflammation (Castillo *et al.,* 2012). Recent studies in a Chinese population revealed that immunity-related GTPase family M protein (IRGM) polymorphism differences between latent and active TB (Lu *et al.,* 2016). The host resistance to tuberculosis also depends on cytoplasmic surveillance pathway (CSP). Its functionality is essential to sense cyclic-di-adenosine monophosphate (C-di-AMP), as levels of this bacterial second messenger determine IRF/IFN-β-dependent response and eventually the infection outcome (Dey *et al.,* 2015). In addition, other factors such as GTPase Rab5 and Rab7, lysosome-associated membrane protein (LAMP1) and EEA1 have been shown essential for phagosome-lysosome fusion (Roberts *et al.,* 2006; Via *et al.,* 1997) and phagosomal maturation (Fratti *et al.,* 2003). Recently, a new line of evidence corroborated that P27 (also referred as LprG) and well-established Mtb phthiocerol dimycocerosate (PDIM) favour Mtb-induced phagosome-lysosome blockage, bacteria escape from phagosome and host cell exit (Quigley *et al.,* 2017; Vázquez *et al.,* 2017)

1.8.5 Degranulation

The abundance of antimicrobial molecules produced during granulopoiesis and stored in granules are critical for host defence (Cowland and Borregaard, 1997). Neutrophils use surface receptors to bind opsonised pathogens and internalise them into the phagosome. The fate of phagocytosed bacteria depends on activity of numerous granule proteins predominantly present in neutrophils such as defensins, cathelicidins and lactoferrin (Levay and Viljoen, 1995; Selsted and Ouellette, 2005; Zanetti, 2004). Inside the phagosome, neutrophils produce ROS as a consequence of oxygen burst, which together with cytoplasmic granules are responsible for killing pathogens. Neutrophils are also equipped with myeloperoxidase (MPO), an important enzyme present in phagosome after degranulation and NADPH which oxidases chloride ions of hydrogen peroxide (H2O2) and chloride. As a result, hypochlorous acid is produced alongside other bactericidal oxidants which target internalised pathogen or can be released outside of neutrophils (Klebanoff, 2005). The importance of NADPH oxidase is evident amongst people carrying mutations in the NADPH oxidase gene who are diagnosed with chronic granulomatous disease (CGD) who suffer from perennial infections (Segal *et al.,* 2008). Neutrophils not only shape an appropriate immune response, but can also contribute to damage of host cells and tissues due to lack of granule specificity (Nathan, 2006). High cytotoxicity of neutrophil granules may drive tissue damage which explains why neutrophil number and deployment must be precisely controlled in an appropriate immune response.

In TB, neutrophils can undergo priming or be activated by Mtb and proinflammatory cytokines which induce degranulation and respiratory burst (Kroon *et al.,* 2018). Host's activity against Mtb is enhanced due to phagocytosis of apoptotic neutrophils by macrophages which acquire their granules inhibiting bacterial growth (Tan *et al.,* 2006). Neutrophil elastase (NE) and cathepsin G (CG) are a key hematopoietic serine proteases present in azurophilic granules inhibiting bacterial replication and their deficiency results in attenuated control of mycobacterial infection in mice (Steinwede *et al.,* 2012). Impaired activity of cathepsin G within regions of lung granulomas of Nos2-/- mice causes distorted granuloma structure, increased hypoxia and augmented bacterial growth (Reece *et al.,* 2010). These finding were in contradiction with previous observations that down-regulation of cathepsin G in THP-1 monocytes following infection is associated with increased Mtb intracellular survival (Rivera-Marrero *et al.,* 2004). Recent studies demonstrated a correlation between low density granulocytes (LDG) and more

severe form of TB (Deng *et al.,* 2016). However, despite undoubted importance of neutrophil granules in TB, their detailed roles in mycobacterial infection need to be fully elucidated.

1.9 The intracellular fate of *Mycobacterium tuberculosis*

Phagocytes such as macrophages and neutrophils are the principal phagocytic cells to Mtb. However, Mtb have developed a number of counter-strategies to act against the host defensive mechanisms. For instance, pathogens use different secretion systems to distribute virulence factors. Moreover, bacteria are able to interfere with host signalling pathways and force phagocytes to undergo cell-death (Daleke *et al.,* 2012; Hilbi *et al.,* 1998; Masaki *et al.,* 2013). Virulent Mtb has been shown to escape from oxidative killing by neutrophils. Mtb is able to survive within human neutrophils despite prompt activation of microbicidal effectors and induce a reactive oxygen species (ROS)-dependent necrotic cell death of infected neutrophils (Corleis *et al.,* 2012). During Mtb infection, engulfed bacteria are able to survive in macrophages and successfully proliferate. Subsequently, uninfected macrophages and other immune cells migrate towards the infection site resulting in formation of compact structures called granulomas - a distinctive feature of TB (Cosma *et al.,* 2003).

1.9.1 How *Mycobacterium tuberculosis* escape innate immune cells – Mtb virulence factors

In the course of evolution Mtb has developed numerous strategies such as blocking phagosome acidification or acquisition of hydrolytic enzymes and bactericidal peptides which increase its survival inside host cells. In humans, there are two major Mtb virulence factors which prevent from phagosomal maturation: the glycolipid lipoarabinomannan (LAM) and the secreted tyrosine phosphatase (PtpA) (Lerner *et al.,* 2015). In mice, surface lipid trehalose dimycolate (TDM) has been shown to preclude phagosome maturation and recent findings in humans corroborated that a polymorphism in the CLR for TDM CLECSF8 (MCL) is associated with an increased susceptibility to pulmonary tuberculosis (Wilson *et al.,* 2015). In addition, Mtb has the ability to escape from macrophage phagolysosome to cytosol using ESX-1 type VII (T7SS) secretion system which impaired function has been used in BCG vaccine strain development (Abdallah *et al.,* 2007). The ESX-1 T7SS substrate ESAT-6 protein has been shown to promote necrotic death in THP-1 human macrophages in NLRP3-dependent fashion (Wong and Jacobs, 2011). In addition, ESX-1

T7SS secretes EsxA/ESAT-6/EsxB/CFP-10 heterodimer with EsxA being responsible for phagosome perforation and membrane lytic activity (Upadhyay *et al.,* 2018). Multiple lines of evidence indicate that EsxA, together with phthiocerol dimycocerosate (PDIM), determines the bacteria's ability to rapture phagosome and promote cell apoptosis (Augenstreich *et al.,* 2017; Quigley *et al.,* 2017). Mtb is able to inhibit autophagy by expressing miR-30A in THP-1 macrophages (Chen *et al.,* 2015) and block IFN-γ production via ESAT-6 in $CD3^+$ T cells (Wang *et al*., 2009). Mtb impairs autophagy at the step of autophagosome-lysosome fusion through ESX-1 system in DCs (Romagnoli *et al.,* 2012).

Apoptosis is a form of death that maintains an intact plasma membrane and is associated with diminished pathogen viability. Apoptosis is an innate defense function of macrophages against Mtb and occurs following TNF-dependent activation of the extrinsic death domain pathway resulting in caspase-8 activation. In order to activate caspase-3 and trigger apoptosis, mitochondrial outer membrane permeabilisation and activation of the intrinsic apoptotic pathway is needed (Behar *et al.,* 2011). Mtb aims to avoid apoptosis using serine/threonine kinase PknE (Jayakumar *et al.,* 2008) and the Rv3364c proteins (Danelishvili *et al.,* 2012). Another two Mtb proteins: SecA2 and NuoG have been suggested to participate in the suppression of apoptosis in THP-1 macrophages (Miller *et al.,* 2010; Velmurugan *et al.,* 2007). Mtb can manipulate host cells by triggering necrosis which creates more beneficial conditions for bacteria due to cell lysis. In primary human macrophages, necrosis is achieved by mitochondrial inner membrane disruption (Chen *et al.,* 2006) and inhibition of plasma membrane repair (Divangahi *et al.,* 2009). The importance of necrotic cell death for Mtb has also been shown in human macrophages infected with a high burden of ESAT-6-expressing Mtb which undergo caspase-1-dependent necrosis (Welin *et al.,* 2011).

1.9.2 Mycobacterial escape from granulomas

Macrophages and neutrophils are the first responders in TB and their key functions in the phagocytosis of Mtb has been the subject of extensive research (Srivastava *et al.,* 2014). However, mycobacteria has the ability to evade leukocyte defence mechanisms, surviving within these cells and proliferating (Eum *et al.,* 2010; Repasy *et al.,* 2013). Apart from macrophages and neutrophils other cells of both the innate and adaptive immune system, such as T-cells, B-cells and dendritic cells are recruited to the site of infection forming the hallmark structure of TB - the

caseating granuloma (Flynn *et al.,* 2011; Ulrichs and Kaufmann, 2006). According to the classical model, granulomas were considered to play a protective role for the host by limiting bacterial dissemination (Ulrichs and Kaufmann, 2006). These assumptions were supported by results from animal models where inhibited growth of bacteria correlated with activation of adaptive immunity (Saunders and Cooper, 2000; Swaim *et al.,* 2006). In fact, zebrafish granulomas have been shown to form far earlier than secondary adaptive immune responses emerged (Davis *et al.,* 2002).

The tuberculosis lesion is dynamic and formed by both immune response and pathogen contributions (Ehlers and Schaible, 2013). Currently, the predominant view of the field is that TB granulomas act to shelter the bacteria from host immunity to allow them to survive until an immune compromise situation when they can escape and cause a systemic infection. Early stages of this process were comprehensively studied using fluorescent strains of Mm in a zebrafish model. Macrophages serve as effective niches utilised by mycobacteria to enhance the recruitment of uninfected macrophages in ESX-1/RD1-dependent manner. In consequence, arriving macrophages promote secondary granuloma formation and the kinetics of this process has been found to be crucial for successful bacteria dissemination (Davis and Ramakrishnan, 2009). The mycobacterial ESAT-6 protein induces matrix metalloproteinase-9 (MMP9) in neighbouring epithelial cells which augment recruitment of new macrophages resulting in nascent granuloma maturation and bacterial growth (Volkman *et al.,* 2010). In mice, macrophages infected with BCG differentiate by a TLR2-dependent mechanism (D'Avila *et al.,* 2006) into foamy macrophages which constitute a reservoir for long-term persistence (Peyron *et al.,* 2008). Recently, based on previously established zebrafish genetic screen, Berg and colleagues demonstrated that mutant deficient in lysosomal cysteine cathepsins are hypersusceptible to Mm infection. Zebrafish mutant macrophages due to accumulated undigested lysosomal material exhibit impaired migration and engulfment of dying cells. As a result, apoptotic macrophages within granuloma undergo secondary necrosis leading to granuloma breakdown and enhanced mycobacterial growth (Berg *et al.,* 2016).

Myeloid dendritic cells are a major cell population infected with Mtb in the lungs and lymph nodes. Wolf et al showed that Mtb targets DC migration inhibit MHC class II Ag presentation to promote persistent infection in mice (Wolf *et al.,* 2007). Granulomas can recruit inflammatory dendritic cells (iDCs) which control local T-cell responses and transfer mycobacteria into the

lymph nodes. Mycobacterium-infected iDCs are associated with bacteria-specific T-cell in infected tissue, outside granuloma, which leads to formation new multi focal lesions and dissemination of granulomatous inflammation (Harding *et al.,* 2015). In mice, Mtb is able to utilise DCs to migrate from lungs to the local lymph node and impair antigen presentation to delay adaptive response due to late initial activation of CD4(+) T cells (Wolf *et al.,* 2008).

In mice and non-human primates (NHP), polymorphonuclear neutrophils (PMNs) represent a cell subset recruited early during Mtb infection (Dorhoi *et al.,* 2014; Nouailles *et al.,* 2014; Repasy *et al.,* 2013). In murine TB models it was demonstrated that neutrophils can play a host-protective role by activation of naive antigen-specific CD4+ T cells and granuloma formation (Blomgran and Ernst, 2011; Seiler *et al.,* 2003), or play the role of a "Trojan horse" and promote mycobacterial infection (Eruslanov *et al.,* 2004). Recently, a detrimental role of neutrophils in human TB has been shown, where neutrophil-derived NF-κB-dependent matrix metalloproteinase-8 (MMP-8) drives immunopathology (Ong *et al.,* 2015). Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature and progenitor myeloid cells with strong immunosuppressive ability for both natural and adaptive immunity (Yang *et al.,* 2014). They provide a niche for Mtb survival in mice (Knaul *et al.,* 2014), diminish protective T-cell response and may participate in the human's impaired ability to eradicate the infection (Du Plessis *et al.,* 2013).

MMPs are a superfamily of zinc- and calcium-dependent proteolytic enzymes which have diverse physiological functions and are responsible for both tissue homeostasis and pathologic conditions (Rohlwink *et al.,* 2019). However, MMPs are not limited to immune-protection and tissue destruction but also determine cell signalling and leukocyte migration by altering gradients of cytokines and chemokines (Green and Friedland, 2007). They play an important role in pulmonary TB (pTB), contributing to breakdown of lung extracellular matrix (ECM) and Mtb spreading from the lung parenchyma into the airways (Elkington *et al.,* 2011). Clinical studies confirmed that in bronchoalveolar lavage from TB patients, concentrations of MMP-1, -2, -3, -7, -8 and -9 were significantly elevated when compared with patients with other pulmonary conditions. Additionally, Singh et al demonstrated a positive correlation between MMP-3, MMP-7 and MMP-8 and a chest radiographs of cavitation and parenchymal damage (Singh *et al.,* 2014). Bronchoalveolar lavage of patients with active pulmonary TB showed that release of MMP-9 and upregulation of gene expression of MMP-1 and MMP-9 is stimulated by lipoarabinomannan

(LAM) - a major Mtb antigenic cell wall component (Chang *et al.,* 1996). MMP-9 is regulated by TNF-α and associated with tissue destruction within granuloma (Price *et al.,* 2003). Elevated levels of MMP-9 have been also shown to correlate with disease severity in TB patients (Hrabec *et al.,* 2002). Elkington et al demonstrated that both host- and pathogen-derived factors act synergistically to drive MAPK-dependent MMP-9 secretion from respiratory epithelial cells during Mtb infection (Elkington *et al.,* 2007). TB-dependent secretion of MMP-1 and MMP-9 has been shown to decline in primary human macrophages and epithelial cells as a result of inhibition of promoter activation with doxycycline (Walker *et al.,* 2012).

Macrophages present in the areas of tissue destruction of patients with active TB have elevated levels of MMP-1 and MMP-7 expression (Elkington *et al.,* 2005), with p38 phosphorylation as a pivotal regulatory point in the generation of a matrix-degrading phenotype (Elkington *et al.,* 2005). In addition to p38 and NF-κB, STAT 3 has been shown to play a critical role regulation of fibroblast-dependent unopposed MMP-1 production with potential impact on tissue destruction in TB patients (O'Kane *et al.,* 2010). MMP-1 activity can be decreased by targeting p38 MAPK-PG signalling pathway with p-aminosalicylic acid - a antimycobacterial drug which may be used to reduce inflammatory tissue destruction (Rand *et al.,* 2009). Mtb infection of transgenic mice expressing human MMP-1 also led to elevated MMP-1 expression and subsequent alveolar destruction in lung granulomas and increased collagen breakdown (Elkington *et al.,* 2011). Recent epigenetic studies demonstrated that modification of histone acetylation via histone deacetylases (HDACs) and histone acetyltransferases (HAT) activity has a pivotal regulatory role in secretion of MMP-1 and MMP-3 (Moores *et al.,* 2017). IL-17, which is instrumental in host defence against Mtb has been shown to promote airway stromal cell-derived MMP-3 by p38 MAP kinase and PI3K pathways (Singh *et al.,* 2018). MMP-10 is an important activator of MMP-1 and it has been shown that Mtb is able to activate inflammatory and stromal cells to secrete MMP-10 - a TB-induced process which depends on Early Secretory Antigenic Target-6 (ESAT-6) virulence factor (Brilha *et al.,* 2016).

In human tuberculous granulomas, the appearance of T and B cells occurs in the final stage of granuloma formation which induces peripheral lymphoid follicle-like structures in the peripheral rim of granulomas. These structures are considered essential for the orchestration of the enduring host response in pulmonary tuberculosis (Ulrichs *et al.,* 2003). Mtb drive T cell immunity until necrotising granulomas leak into bronchial cavities to be transmitted to a new

host (Ehlers and Schaible, 2013). This phenomenon explains why granuloma can fail to limit Mtb infection even after adaptive immunity develops (Ramakrishnan, 2012). However, tumour necrosis factor alpha (TNF-α), a key effector in controlling tuberculosis, mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death, but is not required to granuloma formation. Loss of TNF signalling leads to the progression of tuberculosis in humans, increased death rate of Mtb-infected mice associated with disorganised necrotic granulomas (Clay *et al.,* 2008) and impaired reactive nitrogen production by macrophages early in infection (Flynn *et al.,* 1995). The tuberculostatic macrophage functions also depends on interferon-gamma (IFN-γ) as well as the B cell-stimulatory factors interleukin-4 (IL-4) and IL-6 (Flesch and Kaufmann, 1993). IFN-γ regulates neutrophils limiting lung inflammation (Nandi and Behar, 2011) and nonhematopoietic cells to shape immune response to Mtb (Desvignes and Ernst, 2009). As Mtb infection progresses the granuloma architecture changes with development of a caseous granuloma that cavitates and release infectious Mtb. Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism (Kim *et al.,* 2010). Mtb infection *in vitro* triggers lipid droplet formation in murine and human macrophages. In *in vivo* Mtb cell wall lipid, trehalose dimycolate, induces a strong granulomatous response in mice which was accompanied by foam cell formation (Kim *et al.,* 2010).

1.10 Hypoxia signalling in TB

Hypoxia is defined as a physiological condition in which oxygen levels are not sufficient for the needs of the tissue. Low oxygen tension is characteristic feature of tumour microenvironment, infection and inflammation (Kennedy *et al.,* 1997). Hypoxia is an important wide-range modulator and has influence on various cells, organs and physiological systems. The links between mycobacterial diseases and hypoxia have been discussed in the literature from the beginning of the 20th century (Imboden and Schoolnik, 1998; Wayne, 1994). Indeed, tuberculous granulomas in human and non-human models have been shown to be extensively hypoxic (Belton *et al.,* 2016; Via *et al.,* 2008). Research into TB has revealed that the hypoxic environment of granulomas is correlated with higher resistance of Mtb and its response to host and pharmacological treatment (Boshoff and Barry, 2005; Via *et al.,* 2008).

1.10.1 Cellular response to hypoxia

The cellular response to the low level of oxygen is controlled through stabilisation of hypoxia inducible factor alpha (HIF-α). This transcription factor is predominantly regulated at a post-translational level and its protein stability depends on oxygen sensitive enzymes: prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIH) (Epstein *et al.,* 2001; Lando *et al.,* 2002). Both enzymes are highly reactive to changes in cellular oxygen concentration which determines hypoxia responses (Hirsilä *et al.,* 2003). Low oxygen leads to the silencing of FIH and PHD enzyme activity and a hypoxic genetic response.

In normoxia, HIF-α is hydroxylated by PHDs, undergoes ubiquitination in the cytoplasm and is subsequently degraded (Huang *et al.,* 1998) (Figure 1.5). The process of degradation is facilitated by Von Hippel-Lindau (pVHL) E3 ligase complex (Huang *et al.,* 1998; Robinson and Ohh, 2014). The role of tumour suppressor protein pVHL in the degradation of HIF-α was demonstrated by constitutive activation of HIF signalling in the setting of VHL disease (in which inactivating mutations of VHL are associated with a range of benign and malignant tumours) (Iliopoulos *et al.,* 1996; Maxwell *et al.,* 1999). HIF-α is highly conserved across vertebrates (and in some invertebrates) and there are three mammalian isoforms: HIF-1α, HIF-2α and HIF-3α (Lisy and Peet, 2008). HIF-1α and HIF-2α require their stable binding counterpart aryl hydrocarbon nuclear translocator (ARNT), also called HIF-1β, to facilitate transcriptional activation of HIF target genes (Wang *et al.,* 1995). As a consequence, the emerging (α-β) complex binds to elements in target gene promoters - hypoxia responsive elements (HRE), upregulating target gene expression. The second oxygen sensitive enzyme FIH plays an important role in preventing the fusion between HIF transcriptional cofactors CREB-binding protein/p300 and HIF, an interaction which mutes its activation capability (Freedman *et al.,* 2002; Sang *et al.,* 2002).

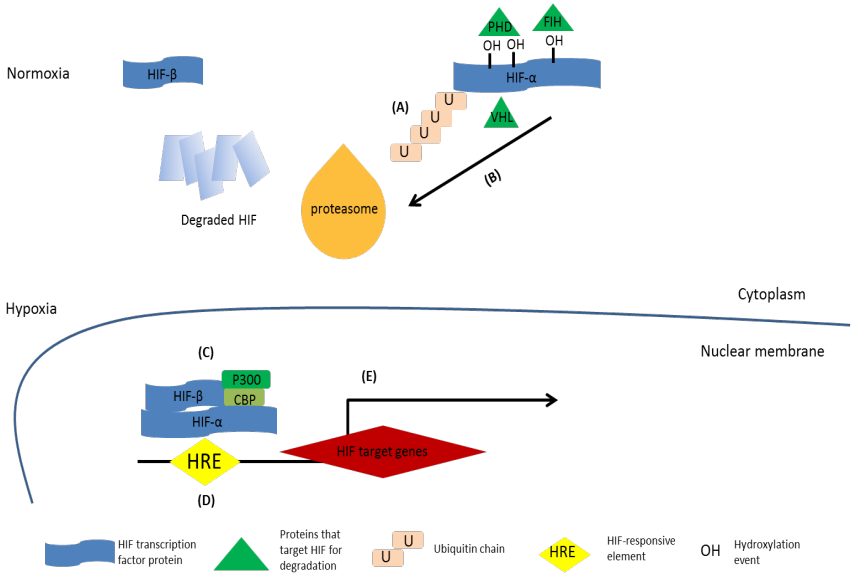


Figure 1.5 The HIF signalling system

In normoxia HIF-α is hydroxylated by PHD enzyme and undergoes ubiquitination (A) in cytoplasm which is facilitated by VHL protein. Afterwards, ubiquitinated HIF-α is directed for degradation (B) in the proteasome. The C-terminal end is hydroxylated by FIH enzyme to supress the transactivation of HIF. In hypoxia conditions both PHD and FIH enzyme are not active, HIF-α binds to its counterpart HIF-β and cofactors (C) in nucleus. Subsequently, HIF-α binds to HRE (D) and upregulates target gene expression (E). Adapted from: Elks, P.M., Renshaw, S.A., Meijer, A.H., Walmsley, S.R., and van Eeden, F.J. (2015). Exploring the HIFs, buts and maybes of hypoxia signalling in disease: lessons from zebrafish models. Dis. Model. Mech. *8*, 1349–1360 (Elks *et al.,* 2015).

Alongside HIF-1α, a second family member, HIF-2α also plays an essential role in hypoxia signalling (Prabhakar and Semenza, 2012; Zhang *et al.,* 2015). Extensive transcriptional analysis has allowed identification of individual HIF-1α and HIF-2α targets as well as genes common for both isoforms. For instance, *Oct-4* is proposed to be an exclusive HIF-2α target gene, HIF-1α induces uniquely *Pgk1* and *Alda* genes, whereas *MT1-MMP and PAI-1* expression is linked with both isoforms (Covello *et al.,* 2006; Petrella *et al.,* 2005; Sato *et al.,* 2004; Semenza, 2003). Moreover, HIF-2α has been shown to be less active than HIF-1α in a number of cells expressing HIF-1α and HIF-2α family members (Hu *et al.,* 2006). A different role of HIF-α isoforms in hypoxia has been also inferred based on extended research on erythropoietin (EPO), a glycoprotein hormone regulated by HIF. Rankin et al generated mice with conditional inactivation of Hif-1α and/or Hif-2α in hepatocytes and demonstrated that conditional inactivation of Hif-2α in pVHL-deficient mice supressed hepatic *Epo* and the development of polycythemia. These findings indicate that HIF-1 and HIF-2 have distinct roles in the regulation of hypoxia-inducible genes (Rankin *et al.,* 2007). In contrast to the widely expressed HIF-1α, the HIF-2α expression is limited

to certain types of cells and genetically correlates with evolution of vertebrates. A study on mice and humans confirmed that HIF-2α is vital for processes related to regulation of EPO gene expression and red blood cells production (Gruber *et al.,* 2007; Percy *et al.,* 2008). A HIF-3α isoform potentially acts as a negative regulator of hypoxia-inducible gene expression (Makino *et al.,* 2001), but less is known about this lowly expressed isoform and in this thesis I will focus on HIF-1α and HIF-2α.

1.10.2 Hypoxia and myeloid cells in mycobacterial disease

Hypoxia and myeloid cells are intimately related. Recruited myeloid cells are abundant in sites of inflammation and require ATP to maintain their activity and functions (Kawaguchi *et al.,* 2001). This product is obtained under high and low oxygen conditions from glycolysis (Sbarra and Karnovsky, 1959), a process chiefly governed by HIF-1α (Seagroves *et al.,* 2001). Additionally, macrophages lacking in HIF-1α showed abolished TNF-α response to LPS during hypoxia. Loss of HIF-1α impaired myeloid cells capacity to eliminate bacteria, diminishing their aggregation and motility (Cramer *et al.,* 2003). Studies on human neutrophils incubated in hypoxic conditions or treated with hypoxia mimetics (which stabilise HIF-1α) show that this leads to NF-κB activation and autocrine secretion of the neutrophil survival factor MIP-1β; both of these processes contribute to prolonged neutrophil survival (Walmsley *et al.,* 2005). Additionally, study on human blood showed that gain-of function HIF-2α mutation reduces apoptosis in neutrophils without altering their essential phagocytic features and respiratory burst. Moreover, over-activation of *hif-1αb* and *hif-2αa* (HIF-α zebrafish orthologues) hampers resolution of neutrophilic inflammation and disrupts tissue regeneration (Elks *et al.,* 2013; Thompson *et al.,* 2014). Hif-1α/Hif-2α have opposing effects on nitric oxide production and hence RNS and mycobacterial burden in a zebrafish model of *Mycobacterium marinum* (Mm) infection, with Hif-1α stabilisation increasing RNS and decreasing bacterial burden whilst Hif-2α led to reduced RNS and increased infection (Elks *et al.,* 2013; Hall *et al.,* 2012). Myeloid-derived mouse neutrophils deprived of *Hif2a* show unchanged level of apoptosis in normal oxygen conditions. In hypoxia, apoptosis rates of Hif-2α-deficient neutrophils are suppressed and comparable to unmodified cells (Renshaw *et al.,* 2006; Thompson *et al.,* 2014). Together, these data show that HIF-1α and HIF-2α isoforms play important roles in governing macrophage and neutrophil activity (Lewis and Elks, 2019; Ogryzko *et al.,* 2019; Schild *et al.,* 2020).

1.11 *In vivo* models of TB

Our understanding of the processes associated with Mtb infection is primarily due to extensive research *in vitro* supplemented by knowledge and broad experience gained through the development of *in vivo* models (Dharmadhikari and Nardell, 2008; Gupta and Katoch, 2005). Animal models have provided invaluable information about the details of disease progression and have been utilised to verify the effectiveness of numerous drugs and prospective vaccines (Orme and Ordway, 2016). Due to some similarities to human TB, animals such as mice, guinea pigs, rabbits, zebrafish and nonhuman primates are used in the experiments. However, there are a number of limitations of some of these models, the main obstacles being the lack of reagents for immunological analysis, expensive housing and ethical controversy (Flynn, 2006).

The history of the use of mice in experiments with TB is long. Early research on TB conducted by Koch showed a similarity between human and mice lesions after inoculation with Mtb (Gupta and Katoch, 2009). Murine models have been used to illuminate the mechanisms of pathogenesis, examine the virulence factors and determine the susceptibility to TB infection (Browning and Gulbransen, 1921; Keller *et al.,* 2006; Repasy *et al.,* 2013). The mouse is the most predominately used TB model, mainly due to well-defined immunology, cost-effectiveness, ease of handling, little space requirements and wide-spread access to transgenic/mutant lines (Vandamme, 2014). Mice are commonly used in pre-clinical trials and the current TB treatment regimen has been developed, to some extent, on the basis of data from murine models (Gupta *et al.,* 2016; Kerantzas and Jacobs, 2017; Kumar *et al.,* 2016). The mouse model, despite its many advantages, has some issues as a model of human TB. Mice do not fully reproduce many of the key features present in the human granuloma microenvironment (Barry *et al.,* 2009), including lung cavitation and organised granulomas (Ordonez *et al.,* 2016). The structure of mice granulomas is less complex than human, with key pathological features such as necrosis, fibrosis and hypoxia missing. However, the C3HeB/FeJ mice has been shown to produce necrotic granulomas, recapitulating some features observed in human disease (Irwin *et al.,* 2015). Additionally, during murine disease the number of bacteria stays high and progressive infection is lethal to mice (Rhoades *et al.,* 1997). Mouse susceptibility to Mtb infection vary and some strains such as C57BL/6 exhibit decreases in bacteria counts after adaptive immunity is activated (Franzblau *et al.,* 2012). Nevertheless, irrespective of aforementioned variability murine models

of TB have driven the field forwards and have proven particularly useful as a pre-clinical model for vaccine development (Cardona and Williams, 2017).

The guinea pig is a well-used TB model, in part due to high susceptibility to Mtb infection and recapitulation of necrotic primary granulomas (Sakamoto, 2012). They have been particularly useful models to test novel drug development (Dutta *et al.,* 2013; Dutta *et al.,* 2012) and, in vaccine development (Dascher *et al.,* 2003; Lenaerts *et al.,* 2007). However, the guinea pig model is more expensive than mouse model and has some important limitations such as limited availability of genetic/immunological reagents and is still missing some features of human TB (Gupta and Katoch, 2009).

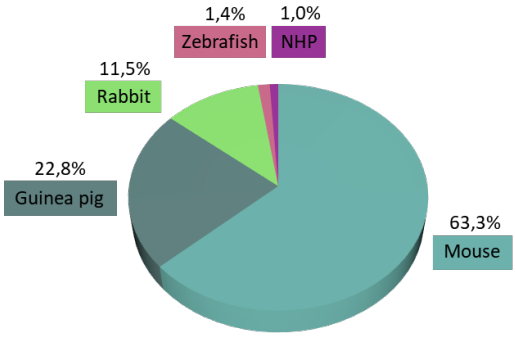
The rabbit model is another informative model of TB, having diversified susceptibility to Mtb infection and recapitulating important features of human TB, such as formation of cavitary disease (Manabe *et al.,* 2003). Some strains, such as HN878, reproduce characteristics of human granulomas and have cavity production (Subbian *et al.,* 2011). TB-infected rabbits have been used to elucidate the role of anti-vascular endothelial growth factor in normalisation of tuberculosis granuloma vasculature to improve treatment strategies (Datta *et al.,* 2015; Kjellsson *et al.,* 2012). However, similarly to other models, using rabbits have some important limitations such as body size and shortage of immunological reagents (Yang *et al.,* 2017).

Non-human primates (NHPs) models most accurately recapitulate human TB conditions and are extensively used in HIV/TB coinfection studies (Lin *et al.,* 2006). Despite ethical controversy and high expenses associated with housing, NHPs are indispensable in testing novel treatment strategies (Kaushal *et al.,* 2012; Via *et al.,* 2008). Due to similarities to humans there is an overlap with immunological reagents and anti-TB drug testing efficacy (McLeay *et al.,* 2012). However, NHPs vary in their susceptibility to Mtb infection (Lin *et al.,* 2009) which has some disadvantages in novel vaccine development (Cardona and Williams, 2017).

The zebrafish model due to its optical transparency and genetic tractability has become an extensively used model in mycobacterial research. Fish have their own host adapted TB bacterial infection by its natural pathogen *Mycobacterium marinum* (Mm). Zebrafish has comparable innate immune cells to humans including macrophages and neutrophils, which allow for in-depth studies on pathogenesis, development and drug candidate testing (Renshaw and Trede, 2012; Traver *et al.,* 2003). The zebrafish pathogen Mm has high similarity to Mtb (85%) and causes

systemic infection recapitulating some of important features of human TB such as granuloma formation, latency and reactivation (Myllymäki *et al.,* 2016; Stinear *et al.,* 2008). They are multiple reasons for using zebrafish in TB studies including ease of infection (Benard *et al.,* 2012), genetic manipulation, little space requirements, relatively low cost, high-throughput screening and well-established platform for drug development (Kaufman *et al.,* 2009; Renshaw and Trede, 2012; Takaki *et al.,* 2013). It was particularly informative in terms of inhibitory effect of verapamil on Mtb-mediated drug tolerance, cytokine expression, mechanisms of host-pathogen interactions and host-directed therapies (Roca and Ramakrishnan, 2013; Tobin *et al.,* 2012). However, the zebrafish TB model has some important limitations due to anatomical differences (Singh and Gupta, 2018) (Figure 1.6).

A



B

Model	Lung lesion			Susceptibility to TB	Laboratory space requirements and cost	Cell mediated immunity	Application
	Necrosis	Caseation	Cavitation				
Mouse	Do not form necrotic lessions in granuloma	Usually not	No	Low	Less	Good	TB immunology, rapid evolution of drug and vaccines. Research on mechanism of latency and relapse
Guinea pig	Yes	Yes	Sporadic	High	Moderate	Poor	Vaccine safety and efficacy studies. Drug evaluation
Rabbit	Yes	Yes	Yes	Varies with *Mycobacterium* species	Relatively large	Good	Good model for TB transmission research, studying bone TB, rarer form of TB such as meningeal and cutaneous TB
Zebrafish	-	-	-	Susceptible to *Mycobacterium marinum*	Small	Poor	Research on dynamics of granuloma formation, evaluation of drug efficacy and safety
Non-human primates (NHPs)	Yes	Yes	Yes	High	Large	Poor	Evaluation of drug, vaccine efficacy and treatment strategy, study of pathological and immunological response of the host against *Mycobacterium tuberculosis*

Figure 1.6 Proportion, characteristic and utilisation of different animal models in TB research

(A) Pie chart showing the percentage of different animal models in TB research. The results from a Pubmed search performed on 11 November 2020 using the following search terms: “mouse AND tuberculosis”, “guinea pig AND tuberculosis”, “rabbit AND tuberculosis”, “zebrafish AND tuberculosis” and “non-human primate AND tuberculosis”. Percentages were calculated based on the total number of publications for all animal models. (B) Characteristic and application for each of the most commonly used tuberculosis (TB) animal model. Based on: K. Fonseca, P. Rodriques, I. Olsson, M. Saraiva (2017) Experimental study of tuberculosis: From animal models to complex cell system and organoids and A. Singh and U. Gupta (2018) Animal models of tuberculosis: Lesson learnt (Fonseca *et al.,* 2017; Singh and Gupta, 2018).

1.11.1 The zebrafish

Zebrafish (*Danio rerio*) is a well-established model organism with a fully sequenced genome and optical transparency during the embryonic and larval stages. The high homology to numerous human genes, similarity to human innate and adaptive immunity, and conserved hypoxia inducible factor (HIF - a key regulator of hypoxic signalling) pathway components make zebrafish an enlightening model for studying host-pathogen interactions (Renshaw and Trede, 2012; van der Vaart *et al.,* 2012). Zebrafish are highly amenable to genetic manipulation using techniques such as morpholino oligonucleotide knockdown and, clustered regularly interspaced short palindromic repeats and CRISPR associated protein 9 (CRISPR/Cas9) (Bedell *et al.,* 2011; Hruscha *et al.,* 2013; Hwang *et al.,* 2013). The effectiveness of MO knockdowns allows study of gene functions during early development of embryos. MOs, in contrast to gene knockouts, do not lead to total knockdown of the gene functions (Timme-Laragy *et al.,* 2012). MOs have been widely used in high-throughput gene screening, modification of gene functions and validating zebrafish mutants (Bill *et al.,* 2009).

1.11.2 Zebrafish phagocytic cells

The zebrafish, due to a highly conserved hematopoietic system and optical transparency during early stages of development is an excellent model to investigate fate of blood cells (Stachura and Traver, 2014). Additionally, the activity of macrophages and neutrophils in zebrafish larvae can be investigated without the impact of adaptive immune response which develops later (2 to 3 weeks post fertilisation) (Lam *et al.,* 2004). The emergence of novel zebrafish lines and advances in live imaging techniques have profoundly improved our comprehension of phagocyte biology (Barros-Becker *et al.,* 2017; Cambier *et al.,* 2014; Cronan and Tobin, 2014; Davis and Ramakrishnan, 2009; Hall *et al.,* 2013; Tobin *et al.,* 2010; Volkman *et al.,* 2010). For example, neutrophils and macrophages can be effectively visualised using transgenic lines expressing fluorescent proteins driven by myeloperoxidase (*mpx*) and macrophage expressed gene 1 (*mpeg1*) promoters (Renshaw *et al.,* 2006; Ellett *et al.,* 2011). In combination with real-time live imaging techniques, zebrafish are a useful model to study the lag phase of TB infection (Davis *et al.,* 2002).

1.11.3 The *Mycobacterium marinum* infection model in zebrafish

Mycobacterium marinum (Mm) is the closest relative of *Mycobacterium tuberculosis* (Mtb) widely used to investigate host-pathogen interactions as a model of TB (Davis *et al.,* 2002; Lesley and Ramakrishnan, 2008; van der Vaart *et al.,* 2012). Mm is an intracellular pathogen which, like Mtb, infect and exploit host macrophages to survive and replicate. Importantly, Mm is a natural fish pathogen which produces systemic granuloma-like lesions with a strong histological resemblance to these observed in human disease (Prouty *et al.,* 2003).

The development of the Mm infection model allowed for a better understanding of the processes related to the heterogeneity of pathogenesis observed during Mtb infection and gave a valuable insight into potential anti-TB treatment strategies (Torraca and Mostowy, 2018). Mm and Mtb share numerous features such as conservation of genetic programmes and virulence factors which enable to determine their common infection and survival strategies. Zebrafish infected with Mm have proven a useful model to understand Mm latency development (Parikka *et al.,* 2012) and granuloma formation (Prouty *et al.,* 2003; Swaim *et al.,* 2006) revealing that the virulence locus ESX-1/RD1 promotes mycobacterial infection by enhanced recruitment and motility of granuloma macrophages (Davis and Ramakrishnan, 2009). Mm infection in fish cause nascent granulomas which contain fewer lymphocytes than Mm-induced infection in mammals (Swaim *et al.,* 2006). Furthermore, the zebrafish Mm model has been employed in developing new treatment strategies to combat the drug resistance problem. For example, drug tolerance can be inhibited by targeting bacterial efflux pumps in both zebrafish and human macrophages (Adams *et al.,* 2011). Studies in zebrafish have also shed light on the correlation between reduced inflammation during mycobacterial infection and absence of Leukotriene A4 hydrolase (Lta4h) - an enzyme involved in the induction of TNF biosynthesis (Tobin *et al.,* 2010). The zebrafish model has elucidated the role of mycobacterial-surface-associated phthiocerol dimycocerosate (PDIM) lipids in pathogen strategy to avoid microbicidal macrophage recruitment. It has been demonstrated that PDIM block PAMP-TLR signalling and phenolic glycolipids (PGLs) promote macrophages with lower RNS response (Cambier *et al.,* 2014). In addition, studies in zebrafish have shown that neutrophils are able to kill bacteria in forming granulomas through oxidative mechanisms (Yang *et al.,* 2012).

However, despite the similarities, Mm has several distinct characteristics that distinguish it from Mtb, such as growth temperature (25-35°C vs 37°C for Mtb) and generation time (4h vs more than 20h for Mtb). Mm unlike Mtb relies on actin-based motility which enable fish pathogen to pass through epithelium during the early stages of mycobacterial infection (Stamm and Brown, 2004). Mm also differs from Mtb in terms of transmission route. In humans, it occurs almost exclusively between lungs of individuals, whereas in fish it is extended to aquatic niches such as water reservoirs. These environmental differences partially determine distinct characteristic of Mm genome which is 1.5 times the size of the Mtb. Mm genome differ from Mtb orthologous region in 15% with coding amino acid identity average of 85% (Stinear *et al.,* 2008). A transposon screen identified several Mm infection mutants defective for growth and macrophage infection sharing 70% similarity in gene orthologous to Mtb (Mehta *et al.,* 2006). Further genome analysis identified a multigene locus accountable for production of Sulfolipid-1 - a Mtb-exclusive lipid responsible for enhanced bacterial virulence (Kumar *et al.,* 2007). A majority of macrophage-activated and granuloma-activated bacterial promoters (*maps* and *gaps*) have common orthologues, however differences in pathogenesis between these two mycobacterial species likely depend on divergent niches (Ramakrishnan *et al.,* 2000; Banaiee *et al.,* 2006). Mutation in *erp* locus - a mycobacterial virulence factor which encodes a secreted cell surface protein, shows impaired bacterial survival *in vitro* and *in vivo* and increased susceptibility to lipophilic antibiotics for both Mm and Mtb (Cosma *et al.,* 2006). Mtb also share other virulence determinant with Mm such as PDIM - a glycolipid present on the cell wall which profoundly contributes to mycobacterial pathogenesis by promoting access to the cytosol, intracellular burden and phagosomal escape (Onwueme *et al.,* 2005; Quigley *et al.,* 2017; Lerner *et al.,* 2018). Both Mm and Mtb pathogenesis depends upon ESX-1 secretion system and RD1 deletion result in impaired bacteria capacity to survive within host cells (Tan *et al.,* 2006). The mycobacterial ESX-1 contains ESAT-6 and CFP-10 - two secreted proteins indispensable for phagosome maturation arrest by Mtb and Mm but not BCG strain (MacGurn and Cox, 2007). Secretion of another ESX-1 substrate EspB - required for growth in host macrophage, in Mm depends on secretion of ESAT-6 and ESAT-10, whereas in Mtb is secreted irrespective of CFP-10 presence (McLaughlin *et al.,* 2007). The comparative studies have shown exceptionally insightful unrevealing conserved pathogenesis between mycobacteria species.

1.11.4 Hif signalling in zebrafish

Zebrafish along with other cyprinids as a result of genome duplication have six *hif-α* gene copies: *hif-1A/B, hif-2A/B and hif-3A/B*. Hif-1αb plays a key role in the early response to hypoxia (Elks *et al.,* 2011; Köblitz *et al.,* 2015; Kopp *et al.,* 2011), whereas Hif-2αb homologue has been shown to delay resolution of inflammation and reduce neutrophil apoptosis (Thompson *et al.,* 2014). In zebrafish, the Phd enzyme family consists of Phd1 (Egln2), Phd-2a (Egln1a), Phd-2b (Egln1b) and Phd-3 (Egln3) - used as reporter for hypoxia in *Tg(phd3:EGFP)*^{i144} embryos (Santhakumar *et al.,* 2012). Importantly, zebrafish show genetic similarity in terms of the other Hif-signalling compounds such as FIH (Fih), Arnt (Arnt1a and Arnt1b) and HRE. The hydroxylase, FIH-1, has been proposed to be an important anti-angiogenic factor and act as vascular endothelial growth factor a (VEGFa) modulator in zebrafish (Mahon *et al.,* 2001). In addition, arnt-1 morpholino in a zebrafish model confirmed that Arnt-1 counterpart is necessary to form a heterodimer with Hif-α and delay inflammation resolution (Elks *et al.,* 2011; Prasch *et al.,* 2006). Thus HIF signalling is well-conserved between human and zebrafish, including upstream-regulation and downstream signalling, supporting the use of zebrafish to study hypoxia signalling (Egg *et al.,* 2013; Greenald *et al.,* 2015; Kajimura *et al.,* 2006; Kulkarni *et al.,* 2010). Studies of HIF signalling in zebrafish are particularly attractive due to the access to a large number of genetic tools enabling its modification and comprehensive analysis.

1.11.5 HIF modulation in the zebrafish model

The zebrafish is a highly informative model, commonly used to understand features of human diseases and validate novel treatment strategies (Alvarez-Delfin *et al.,* 2009; Cao *et al.,* 2008; Rouhi *et al.,* 2010). Importantly, zebrafish have conserved HIF pathway components (van Rooijen *et al.,* 2009) and variations in HIF lead to differing oxygen tolerance (Rytkönen *et al.,* 2007). Hif signalling can be triggered in zebrafish by physical hypoxia using closed hypoxia chambers and pre-equilibrated media (Manchenkov *et al.,* 2015). Another well-established method to stabilise Hif-α in zebrafish embryos is by adding chemical compounds directly to the embryo media. This method is extensively used mainly due to ease of use and low cost of available inhibitors. *In vitro* studies showed that HIF pathway could be modified using dimethyloxalylglycine (DMOG), a synthetic analogue of α-ketoglutarate which inhibits the PHD enzymes (Jaakkola *et al.,* 2001). Indeed, Phd/Fih activity can be blocked using DMOG in zebrafish

(Elks *et al.,* 2011), with stabilised Hif-1α leading to reduced neutrophil apoptosis and increased retention at site of inflammation. However, none of the pan-hydroxylase inhibitors are able to selectively and exclusively stabilise Hif-α without affecting other hydroxylases in different pathways, hence there may be off-target effects (Elks *et al.,* 2013; Elks *et al.,* 2011; Kajimura *et al.,* 2006). Modulation of Hif signalling can be accomplished by genetic manipulation using morpholino, RNA and DNA injections into one cell-stage zebrafish embryos. Morpholino injections have been effectively exploited to block Arnt (Prasch *et al.,* 2006) and Hif-1α, confirmed by inhibition of Hif-1α targets: *vegf* and *aldo* genes *(Barriga et al.,* 2013). Moreover, the use of dominant-active (Hif-1αa) and dominant-negative (Hif-1αb) constructs have demonstrated that hydroxylation of Hif-1αb regulates the resolution of neutrophilic inflammation. Dominant negative variants lack in N-terminal and C-terminal transactivation domains (N-TAD and C-TAD), resulting in downregulated Hif signalling, whereas dominant active variants are innately stable due to mutation of proline targets to non-hydroxylatible amino acids (Elks *et al.,* 2011). The dominant variants were created based upon well-established human cell model and provided a powerful tool to study Hif signalling pathway (Chan *et al.,* 2005). In addition, Hif signalling has been investigated in zebrafish line with mutations in *vhl* gene resulting in Hif-α stabilisation and up-regulation of hypoxia-induced genes (Rooijen *et al.,* 2009). Finally, Hif modulation can be visualised using zebrafish fluorescent transgenic lines such as *Tg(phd3:EGFP)*^{i114} which has given a profound insight into the role of Vhl in supressing tumours. This reporter line has enabled to track the expression of EGFP being under the *phd3* promoter in *vhl*$^{-/-}$ zebrafish and provided an important tool to test novel anticancer therapeutics (Santhakumar *et al.,* 2012). High genetic homology of HIF-signalling between human and zebrafish enables dissection of crucial events taking place in the early stages of infection.

1.12 Thesis aims

The ultimate goal of this project is to understand the interaction between innate immune cell populations and mycobacteria with the aim to identify potential therapeutic strategies against TB. According to the WHO's Post Global Tuberculosis Strategy report it is imperative to discover new treatments to combat the growing scourge of tuberculosis. Augmenting host anti-mycobacterial immunity is one such route. However, for such host-derived strategies to be developed and implemented, we need better understanding of the interactions of innate immune cells with the pathogen and with each other. My overall aim is to further the understanding of innate immune interactions *in vivo* in a disease relevant model of tuberculosis allowing future translation into mammalian and ultimately human disease.

I hypothesise that neutrophils, and their nitric oxide production, aid the host to control mycobacterial infection.

The aims of this thesis are:

- Delineate the kinetics of neutrophil recruitment in zebrafish model of mycobacterial infection

- Explore the relative contribution of neutrophils and macrophages in early infection control

- Manipulate HIF signalling and determine the impact on mycobacterial infection in zebrafish

- Determine the key neutrophil anti-mycobacterial mechanisms in early mycobacterial infection in zebrafish

Chapter 2: Material and Methods

2.1 Common procedures

2.1.1 RNA preparation protocol

RNA for single-cell stage injection was prepared using Ambion mMessage mMachine SP6 kit (AM1340). The reaction was prepared at room temperature by adding components in the following order:

1) 10µl 2x NTP/CAP
2) Diethyl pyrocarbonate (DEPC) - treated water to 18µl
3) 1µg linearised DNA template
4) 2µl 10x reaction buffer

Next, the components were mixed and centrifuged (1min/14000g) at room temperature. Instantly after this spinning step 2µl SP6 enzyme (kept in a -20°C freezer box) was added and the sample was transferred to 37°C hot block and incubated for ~2-3hrs. Subsequently, 1µl Turbo DNase was added in order to remove any remaining template DNA and the sample was incubated at 37°C for further 30mins. The sample was transferred to -20°C for at least 20min to allow more efficient DNA precipitation. After the DNA precipitation step, 480ul DEPC-treated water, and 500µl acid:phenol:chloroform (ChCl3) (Ambion AM9720) were added, and the sample was vortex for 2mins. The sample was centrifuged (10mins/10000g) at 4°C, 400ul of upper phase was placed in an Amicon Ultracel 100k filter (Millipore UFC510024) within a collection tube and centrifuged (10mins/14000g) at room temperature. The filter was transferred to fresh collection tube, placed upside-down and centrifuged (2mins/1000g) at room temperature. The RNA concentration was quantified using a Nanodrop™ spectrophotometer (ND1000) (expected RNA concentration = 4000µg/µl) and stored in -80°C until required.

2.1.2 *Mycobacterium marinum* culture preparation protocol

The day before a planned *Mycobacterium marinum* (Mm) injection (approximately 2pm), a bacterial culture was prepared by inoculation of small plastic loop filled with bacteria from the Mm culture plate (stored at 28°C) into a T25 tissue culture flask (Greiner Bio-One 690175)

containing 10ml 7H9 media with hygromycin antibiotic and ADC (see section 2.4). When the bacteria were well suspended in the 7H9 medium, 100ul of the resulting suspension was transferred to spectrophotometer cuvettes containing 900ul 7H9 medium. The spectrophotometer was calibrated using 900ul 7H9 medium alone and the optical density at 600nm (OD_{600}) was determined (expected OD_{600} ~0.2). The culture was incubated for 24hrs at 28°C and optical density was measured again when bacteria reached appropriate growth phase (expected OD_{600} between 0.6 and 1.0). The bacterial pellet from the whole 10ml Mm culture was re-suspended in 1ml PBS, washed and centrifuged three times. The spectrophotometer was calibrated with PBS alone and depending on the final readout from OD_{600} the pellet was diluted in 2% polyvinylpyrrolidone (PVP) (Sigma Aldrich, US) in PBS to a target of 200 colony forming units per nl (200CFU/nl).

2.1.3 *Mycobacterium marinum* glycerol stock preparation protocol

The bacteria to prepare glycerol stock were cultured as described (see section 2.1.2). In the final step the pellet was diluted in 20% glycerol in PBS to approximately 500 colony forming units per ml (500CFU/nl) and frozen in -80°C. Before zebrafish injection a 10ul aliquot was defrosted at room temperature and diluted 1:1 in 2% PVP in PBS (500CFU/nl).

2.1.4 Needles for microinjections

Each experiment involving microinjection into zebrafish tissue, circulation and single-stage cell was performed using borosilicate glass capillaries (Kwik-Fil World Precision Instruments, WPI, TW100-4). Needles were prepared using a Flaming Brown micropipette puller (Sutter Instrument Co., Novato, USA) located in the Fly Facility (Bateson Centre, Firth Court). Before microinjection under a dissecting microscope attached to a microinjection rig (WPI, PV820), pulled needle was loaded with adequate suspension of injection material. The end of the needle was broken using forceps and final droplet size (volume 1nl) was determined using a glass graticule with mineral oil to inject into.

2.2 Ethics and group II organisms

According to UK Animals Act 1986, zebrafish larvae used in experiments were not considered as protected animals (up to 5.2dpf/kept in 28°C) and were culled before reaching age of protection. Zebrafish were maintained in accordance with UK Home Office Licence (Project licence number P1A4A7A5E). According to Health and Safety Executive and approved list of biological agents, experiments including procedures with pathogen *Mycobacterium marinum* (Mm) (http://www.hse.gov.uk/pubns/misc208.pdf, page 13) were performed in the accordingly appointed and equipped Category II laboratory at the Royal Hallamshire Hospital, Department of Infection & Immunity and Cardiovascular Disease, Sheffield, UK.

2.3 Zebrafish work

2.3.1 Husbandry

Zebrafish were maintained in designated aquarium facilities under approved animal care protocols. Adult in-crossed (the same transgenic line) and outcrossed (different transgenic lines) fish were kept in the aquarium at 28°C in tanks (up to 40 fish per tank) illuminated by 14 hours of light/10 hours of dark per day. Zebrafish were bred both by the marbling technique as well as by pairing individual females and males in tanks with dividers. Marbling is a method of breeding zebrafish which involves using marble tank. It is placed into a fish tank and fish eggs are collected to plastic bottom container of the marble tank. The pairing method provides tight control of the number of recently fertilised single-cell stage eggs at a specified time. Fish embryos were incubated in petri dishes (Menzel-Gläser, Germany) (up to 60 embryos per plate) containing 1xembryo medium (E3) with methylene blue to prevent the development of fungal infections and all procedures were carried out on fish for up to 5dpf. Fish subjected to experiments at or before 2dpf were prior dechorionated using two pairs of forceps. Zebrafish were anaesthetised using 1.5ml of 4.2% tricaine MS322 (Sigma, MO, US) added to 25 ml of 1xE3.

2.3.2 Zebrafish anaesthesia

In all experiments with zebrafish tricaine was used as an anaesthetic. Zebrafish larvae were anaesthetised by immersion in 0.168mg/ml tricaine in E3. Before imaging zebrafish larvae

were kept in 0.168mg/ml tricaine in 0.8-1.0% low point melting (LPM) agarose. Zebrafish larvae recovery was performed by replacing medium containing anaesthetic with fresh E3.

2.3.3 Zebrafish injections

Zebrafish injections were performed at single cell stage (morpholino (MO) and RNA injections), 1dpf (Mm systemic infection and clodronate liposomes injections), 2dpf (Mm muscle somite infection) or 3dpf (Mm tail fin infection) using non-filament glass capillary needles (Kwik-Fil™ Borosilicate Glass Capillaries, World Precision Instruments (WPI), Herts , UK) (Figure 2.1).

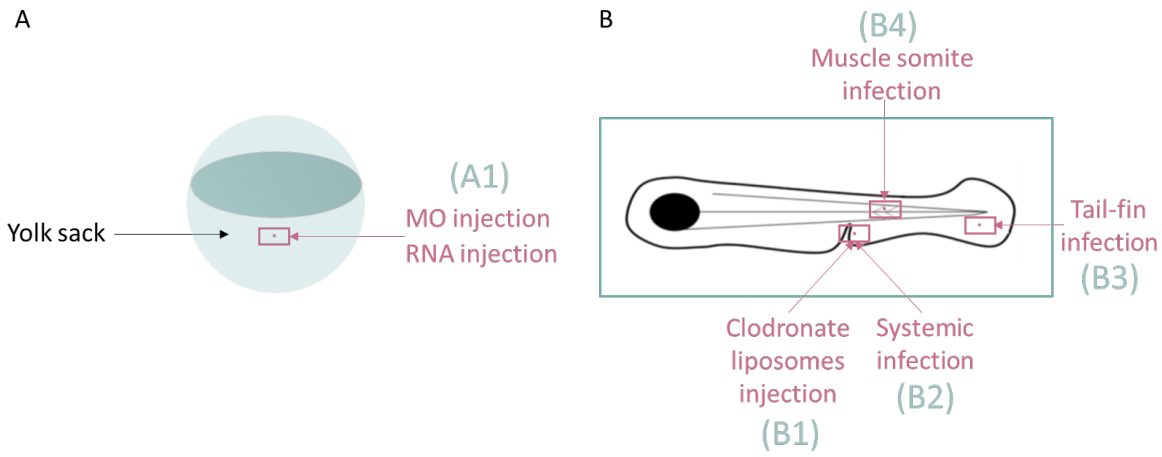


Figure 2.1 Different sites of zebrafish injections/infections

(A) Single cell stage injections (MO and RNA injections (A1)). (B) Caudal vein injections at the posterior blood island (Mm systemic infection (B2) and clodronate liposomes injection (B1)); Injection into the muscle somite midway between the tail fin and yolk sac extension (B4) (Mm local infection) and injection into the tail fin (B3) (Mm local infection).

2.3.3.1 Morpholino oligonucleotides injections

Morpholino (Gene Tools, US) against *pu.1* (Rhodes *et al.,* 2005), *csf3r* (Stachura *et al.,* 2013) and *irf8* (MO^{atg}) (Li *et al.,* 2011) were injected at the single-cell stage as described (Prajsnar *et al.,* 2012). The needle was loaded with ~3µl of each morpholino oligonucleotide (1mM) and calibrated as previously described (see section 2.1.4). Fish were collected from single paired male/female tanks and approximately 60 single-stage eggs (<20mpf) were mounted on glass microscope slide adhesive to petri dish plate. After microinjection into the centre of the yolk sack, eggs were transferred to a fresh petri dish plate with E3 with methylene blue and kept in a 28°C incubator. Additionally, standard control morpholino (Gene Tools, US) was injected into single-

cell stage eggs as a negative control. Both dead and damaged embryos were removed from each experimental group approximately 5hrs after injection (Figure 2.1A1).

2.3.3.2 RNA injections

RNA was injected at the single-cell stage as described (Elks *et al.,* 2011). From RNA stock (~4000 ng/ul) a 1:40 dilution was prepared using 1xphenol red. The needle was loaded with ~3µl of RNA and calibrated as previously described (see section 2.1.4). Fish were collected from single male/female individual tanks and approximately 60 single-stage eggs per group (<20mpf) were mounted on a glass microscope slide adherent to a petri dish. After microinjection into the centre of the yolk sack, eggs were transferred to fresh E3 petri dish plate with methylene blue and kept at 28°C. Additionally, phenol red was injected into the single-cell stage embryos as a vehicle control. Both dead and damaged embryos were removed from each experimental group approximately 5hrs after injection (Figure 2.1A1).

2.3.3.3 Clodronate liposomes injections

Liposome-encapsulated clodronate (clod lipo) and liposome-encapsulated PBS (ctrl lipo) (http://www.clodronateliposomes.org/) were prepared to concentration 5mg/ml as previously described (Van Rooijen and Sanders, 1994) and injected (Bernut *et al.,* 2015). Before microinjection 1dpf *Tg(mpeg:mCherry)* fish were dechorionated, anaesthetised with tricaine (see section 2.3.2) and lined up on 1% agarose injection pad. The needle was loaded with ~3µl of clod lipo or ctrl lipo and calibrated as previously described (see section 2.1.4). Zebrafish embryos were microinjected with 2-3nl of clod lipo or ctrl lipo into the caudal vein at the posterior blood island as described (Benard *et al.,* 2012) at 24hpf. To assess the effectiveness of macrophage depletion in *Tg(mpeg:mCherry)* zebrafish the numbers of macrophages present between yolk sac extension and tail fin were manually counted at 6hpt, 24hpt and 4dpt (Figure 2.1B1).

2.3.3.4 Systemic injections

Before microinjection 1dpf fish were dechorionated, anaesthetised with tricaine (see section 2.3.2) and lined up on 1% agarose injection pad. The needle was loaded with ~3µl 200CFU/nl of Mm suspension and calibrated (see section 2.1.4). Fish were injected into the caudal vein at the

posterior blood island as described (Benard *et al.,* 2012). After microinjection fish were transferred to fresh E3 petri dish plates kept at 28°C (Figure 2.1B2). At day 5 anaesthetised fish were, placed on the tissue culture dish (FluoroDish) and widefield microscopy (FITC, TRITC filters; Leica DMi8 SPE-TCS) were used to image whole body of infected fish. After the imaging fish were culled and recorded images were analysed as described (Figure 2.1B2).

2.3.3.5 Tail fin injections

At 2dpf fish were dechorionated and left in E3 petri dish for subsequent procedures. Before microinjection 3dpf fish were anaesthetised with tricaine (see section 2.3.2) and lined up on 1% agarose injection pad. The needle was loaded with ~3µl 500CFU/nl of Mm suspension and calibrated as described (see section 2.1.4). Fish were injected into the tail fin as described (Hosseini *et al.,* 2016). After microinjection fish were transferred to fresh E3 petri dish plate without methylene blue and kept at 28°C. Additionally, 2% PVP in PBS was injected into the tail fin as a control (Figure 2.1B3).

To image neutrophil recruitment 2hpi, anaesthetised fish were placed on the 1% agarose injection pad and imaged using a fluorescent stereomicroscope (Leica MZ10F) with attached GX Camera. After the imaging fish were transferred to 24-well (VWR) and incubated at 28°C for further analysis. The same imaging procedure was repeated 24hpi and 48hpi. After the imaging at day 5 fish were culled and recorded images were analysed.

To image granuloma 1dpi *Tg(mpx:Gal4)*^{i222}*(UAS:Kaede)*^{s1999t} referred later as *mpx:*Kaede, larvae were screened for tail fin granuloma, imaged and green neutrophils within granuloma were photoconverted. At 5dpf (2dpi) previously photoconverted *mpx*:Kaede fish were reimaged and neutrophil counts present within tail fin granuloma were compared.

2.3.3.6 Muscle somite injection with *Mycobacterium marinum*

Before microinjection 2dpf fish were dechorionated, anaesthetised with tricaine (see section 2.3.2) and lined up on a 1% agarose injection pad. The needle was loaded with ~3µl 200CFU/nl of Mm suspension and calibrated (see section 2.1.4). Fish were injected into the muscle somite

midway between the tail fin and yolk sac extension as described (Oehlers *et al.,* 2015). After microinjection fish were transferred to fresh E3 petri dish plate without methylene blue and kept at 28°C. Additionally, 2% PVP in PBS was injected into the muscle somite as a control (Figure 2.1B4).

Depends on experiment (2dpi or 3dpi) fish were placed on the 1% agarose injection pad and any neutrophils present in the muscle somite were counted using a fluorescent stereomicroscope (Leica MZ10F). After counting, fish were transferred to 24-well (VWR) and incubated at 28°C for further analysis. The same counting procedure was repeated. After the imaging fish were culled and results were analysed.

3dpi *Tg(mpx:GFP)*^{i114} larvae were imaged and acquired images were used to asses neutrophil number within granuloma or proportion of neutrophils with internalised Mm within granuloma.

2.3.3.7 Muscle somite injection with Zymosan

Before microinjection 2dpf fish were dechorionated, anaesthetised with tricaine (see section 2.3.2) and lined up on 1% agarose injection pad. From frozen aliquot (working stock concentration 400ug/ml) was prepared in 200ul fresh eppendorf tube by adding purified milli-Q water. The needle was loaded with ~3µl of 1mM Zymosan (Sigma Aldrich, UK) dilution and calibrated as previously described (see section 2.1.4). Fish were injected into the muscle somite midway between the tail fin and yolk sac extension as described (Oehlers *et al.,* 2015). After microinjection fish were transferred to fresh E3 petri dish plate without methylene blue and kept at 28°C. Additionally, purified milli-Q water was injected into the muscle somite as a control (Figure 2.1B4).

2.3.4 Metronidazole treatment

NTR/Mtz system has been previously used to ablate cardiomyocytes, hepatocytes, pancreatic β-cells and macrophages in zebrafish and larvae (Curado *et al.,* 2007; Gray *et al.,* 2011; Pisharath *et al.,* 2007). Fresh 10mM metronidazole stock was prepared by adding metronidazole powder to E3 and mixing until dissolved. To achieve neutrophil ablation 1dpf *Tg(lyzC:NTRmCherry)*

zebrafish were kept in 5mM metronidazole overnight (about 15hrs) and fresh media with metronidazole were added. To maintain cell ablation fish kept in metronidazole and plate was wrapped in foil or placed in dark incubator. After 36 hours zebrafish were imaged using confocal microscopy.

2.3.5 Pharmacological inhibition of iNOS

To determine the role of NO in host protection 4 hours before infection L-NIL (N6-(1-iminoethyl)-L-lysine, dihydrochloride, Tocris Bioscience) was added to the embryo water at a 200µM concentration as previously described (Elks *et al.,* 2013). 24 hours post Mm infection the inhibitor was washed off and bacterial burden levels in 4dpi infected were assessed using dedicated pixel counting software. DMSO solvent controls were used at corresponding concentrations for each treatment.

2.3.6 Generation of CRISPant nos2ab knockdown

CRISPR/Cas system has been shown to be an effective gene knockout tool in zebrafish (Cade *et al., 2012;* Hwang *et al.,* 2013) and has been used to knockdown genes in F0 injected larvae (termed CRISPants) (Isles *et al.,* 2019). Two single guides RNA (sgRNA) per gene (*nos2a* and *nos2b*) were designed for both ATG and exon 1 guides sites using ChopChop gRNA design tool https://chopchop.rc.fas.harvard.edu/

Target ID/Clone Name	Target Sequence: 5'-3' (does not include PAM)
nos2a_ATG	**CAAAATATTTTTAGCAAGAT**
nos2a_ex1	**TTTCTCATTTTCAATGATAG**
nos2b_ATG	**GCAGTATTTCAGAAACACAT**
nos2b_ex1	**GTTCGCTCTTGTGAGTGACC**

Following injections with single guide RNA (sgRNA) targeting ATG at single cell stage 5dpf *nacre* larvae were fixed and nitrotyrosine levels were immune-labelled using a rabbit polyclonal anti-nitrotyrosine antibody (Elks *et al.,* 2013). After staining fish were imaged in caudal hematopoietic tissue (CHT), the site of embryonic hematopoietic stem and progenitor cells (HSPC) expansion in zebrafish (Wolf *et al.,* 2017).

2.3.7 Antibody detection of nitrotyrosine levels

5dpf *nacre* larvae were fixed in 4% formaldehyde (PFA, Fisher) with 0.4% Triton X-100 (Tx – Sigma) overnight at 4°C or alternatively for 3hrs at room temperature (RT). Following fixation step larvae were rinsed with PBS-Tween (PBS-0.1% Tween20) and stored at 4°C in PBS-Tween. At day 1 larvae were washed 4x5mins with 1ml PBS-0.4%Tx on shaker and treated for 30mins at RT with 1ml Proteinase K (10mg/ml Sigma) in PBS-0.4%Tx on shaker. Larvae were washed 4x10mins in 1ml PBS-0.4%Tx on shaker and incubated at 4°C in 100-200µl α-nitrotyrosine antibody (Millipore) diluted 1:250 in 5% SS:PBS-0.4%Tx. At day 2 larvae were washed 4x10mins in 1ml PBS-0.4%Tx and blocked for 1hr at RT in 1ml 5% SS:PBS-0.4%Tx. Larvae were incubated for 2hrs, in the dark, at RT in 200µl GAR secondary antibody diluted 1:500 in 5% SS:PBS-0.4%Tx. Following incubation step fish were washed 4x20mins in 1ml PBS-0.4%Tx, in the dark, kept in 1ml 4%PFA-0.4%Tx for 20mins at RT, rinsed briefly in PBS-0.4%Tx and stored in the dark until imaging (up to 1 week).

To quantify nitrotyrosine levels in zebrafish larvae the caudal vein region of antibody stained zebrafish were assessed. For each labelled cell the corrected total cell fluorescence was calculated using ImageJ software as previously described (Elks *et al.,* 2013). Neutrophils were selected for the expression of GFP from the *Tg(mpx:GFP)*^{i114} zebrafish line. Cell fluorescence was corrected for cell size and background fluorescence of the image.

2.3.8 Time-lapse microscopy

Before time-lapse microscopy fish need to be immobilised. Fish were anaesthetised with tricaine added to E3 plate and then transferred to µ-Slide 4 Well Glass Bottom plate (Ibidi). 0.8% low gelling temperature agarose (Sigma, BioReagent, US) was microwaved until it melted and left to cool down. Residual E3 was removed from 4-well plate and 0.5ml melted agarose was added. In order to facilitate imaging fish were laid as flat as possible and arranged to form 15-18 fish column with heads facing the same way. The 4-well slide with mounted fish was left until the agarose set and its surface was covered with few drops of E3 to prevent drying out.

2.3.9 Software analysis

Images were analysed using ZF4 software which compares the number of pixels from imported images to a 'blank' image. The data from remaining microscopy experiments were quantified manually. The data captured using GX camera were archived and exported using GX Capture-T software. The generated results were exported and analysed within the Prism 7.0 statistics software (GraphPad Software, Inc, US). The comparison between 2 groups with normally distributed data was performed using unpaired 2-tailed t-test. More than 2 groups were analysed using one-way ANOVA test with Bonferroni adjustment. The data from live imaging were generated using LAS X software (Leica Microsystems).

2.4. Antibiotics, Solutions and Reagents

Antibiotic	**Stock concentration**	**Working concentration**
Carbenicillin	50mg/ml	50µg/ml
Hygromycin	50mg/ml	50µg/ml
Kanamycin	50mg/ml	50µg/ml

E3 embryo water (10X) - diluted to 1x using dH_2O (900ml distilled water was added to 100ml E3 10X). To avoid fungal contamination 3 drops of methylene blue/litre were added.

- 50mM NaCl
- 1.7mM KCl
- 3.3mM $CaCl_2$
- 3.3mM $MgSO_4$

PBS Phosphate buffered saline - (Fisher Scientific) - used as a washing solution (NaCl, Na_2HPO_4, KCl and KH_2PO_4 were dissolved in 1l of distilled water).

- NaCl 8g/l
- Na_2HPO_4 1.4g/l
- KCl 0.2g/l

- KH_2PO_4 0.2g/l
- distilled water 1l

2 % PVP Polyvinylpyrrolidone - (Sigma Aldrich, US) - used to re-suspend *Mycobacterium marinum* pellet for microinjections (PVP was dissolved in 100ml PBS and autoclaved).

- 2g PVP
- 100 ml PBS

7H10 growth agar - (Middlebrook BD 262710) - medium used to grow *Mycobacterium marinum* strains (glycerol and agar powder were added to distilled water to final volume 450ml and autoclaved).

- 2.5ml glycerol
- 9.5g 7H10 agar powder
- 450ml mQ water

7H10 agar plates - 7H10 growth agar was microwaved and kept in 50°C water bath. Next, 50ml 10% oleic acid, albumin, dextrose, catalase (OADC) enrichment (Middlebrook, US) and hygromycin antibiotic (Sigma, H0654) were added (see section 2.4). Petri dishes were poured and stored in the 4°C.

- 450ml growth agar
- 50ml OADS
- 50µg/ml hygromycin antibiotic

7H9 bacterial growth media - (Middlebrook BD, 271310) - medium used for culturing *Mycobacterium marinum* strains (mCherry and Crimson) for microinjections (glycerol and agar powder were fulfilled with distilled water up to 450ml and autoclaved. Next, 50ml 10% albumin, dextrose, catalase (ADC) enrichment (Middlebrook, US), hygromycin antibiotic were added (see section 2.4) and stored in 4°C.

- 1ml glycerol
- 2.35g 7H9 broth powder
- 50ml ADC
- 50µg/ml hygromycin antibiotic

Chapter 3: Neutrophils are recruited to *Mycobacterium marinum* infection sites and phagocytose mycobacteria

3.1 Introduction

Mycobacterium tuberculosis (Mtb) is a pulmonary pathogen transmitted by droplet inhalation and upon invading human airways the first immune cells encountered are resident alveolar macrophages (Weiss and Schaible, 2015). These primary phagocytic cells release pro-inflammatory cytokines such as tumour necrosis factor (TNF), IL-6, IL-1α and IL-1β (Law *et al.,* 1996) that attract further phagocyte migration to the site of infection, that amplify cytokine signals and enhance the host immune response against mycobacterium (Giacomini *et al.,* 2001; Medzhitov, 2007). Early evidence that Mtb is phagocytosed by macrophages came from *in vivo* experiments performed in the 1920s where authors showed that rabbit macrophages contained acid-fast debris of phagocytosed tubercle bacilli (Sabin and Doan, 1927). Since then, numerous studies have demonstrated that neutrophils alongside macrophages are able to phagocytose Mtb and play an important role during Mtb infection (Eruslanov *et al.,* 2005; Ganbat *et al.,* 2016; Kisich *et al.,* 2002; Lombard *et al.,* 2016). However, it remains unclear how neutrophil recruitment and phagocytosis at the initial site of infection, determines the outcome of infection.

In humans, neutrophils are the most prolific circulating leukocytes and one of the first immune responders to invading pathogens (Amulic *et al.,* 2012). During Mtb infection neutrophils, alongside macrophages, play a pivotal role in maintaining pro-inflammatory signals required for monocyte recruitment from the bloodstream (Russell *et al.,* 2010). Circulating neutrophils are released from the bone marrow and their recruitment to infected tissues is a tightly orchestrated multistage process (Schmidt *et al.,* 2013). The activation of neutrophils and their migration towards infectious foci is tightly controlled by numerous cytokines and chemokines (Nathan, 2002). On reaching infected tissue, human neutrophils become activated, recognise, phagocytose and eliminate invading Mtb, thus fulfilling essential roles in innate immunity (Kisich *et al.,* 2002). It is widely accepted that neutrophil activity is not limited to bacterial eradication but encompasses modulation of innate and adaptive immune response via interactions with other host cells (Amulic *et al.,* 2012; Kolaczkowska and Kubes, 2013; Mantovani *et al.,* 2011).

Neutrophils can become activated after a process called neutrophil priming. Priming is defined as the response after initial exposure to external stimuli including, bacterial LPS, colony-stimulating factors (GM-CSF and G-CSF) and TNF-α (Nathan, 1989; Swain *et al.,* 2002). Primed neutrophils display enhanced phagocytic capacity and oxidative burst when challenged by a further activating agent (Fossati *et al.,* 1999; Pitrak, 1997). *In vitro* studies have demonstrated that TNF-α stimulation resulted in a 2-fold increase of nonoxidative neutrophil killing of Mtb within 1 hour of phagocytosis when compared with unstimulated controls (Kisich *et al.,* 2002). The outcome of TB is partially determined by neutrophil numbers present during infection. Blood obtained from patients diagnosed with pulmonary TB that was subject to *in vitro* neutrophil depletion showed more than 3-fold reduction in ability to limit Mtb growth (Martineau *et al.,* 2007). Whole-blood augmented with viable neutrophils improved mycobacterial control, whereas addition of necrotic neutrophils had the opposite effect (Lowe *et al.,* 2018). However, mice neutrophils have been shown to be ineffective in limiting Mtb growth and likely facilitate disease progression serving as Trojan horse. The role of neutrophils in rodent and human TB may differ and these conflicting findings highlight the need for a more detailed understanding of neutrophils roles during Mtb infection (Eruslanov *et al.,* 2005).

Early mycobacterial infection is difficult to study in mammalian models. Human peripheral blood neutrophils have a limited life span and their activation status can inadvertently be profoundly altered during experimental procedures. *In vitro*, neutrophil experiments also lack the microenvironmental context of the infection site and interactions with other immune cells. Therefore, non-mammalian animal models have become more widely used to address questions related to neutrophil functions during mycobacterial infection. The zebrafish has emerged as a powerful model to study neutrophil behaviour and a unique platform to explore mechanisms related to infection and immunity (Harvie and Huttenlocher, 2015). Stable transgenic zebrafish lines with fluorescently-tagged phagocytes can be used to study neutrophil-mycobacterial interactions (Hosseini *et al.,* 2014, 2016). A tail fin infection allows study of the recruitment of phagocytes to a localised infection within epithelia which makes it a relevant model to understand conditions present in human TB infection. Neutrophil recruitment to localised mycobacterial infection can also be observed when Mm is injected into the zebrafish muscle somite (Oehlers *et al.,* 2015). The muscle infection model produced a more robust and larger infection site, allowing more detailed study of neutrophil dynamics. The outcome of

mycobacterial infection in zebrafish is dependent on the interplay between macrophages and neutrophils, as well as their individual capacity to phagocytose and contain mycobacteria. A wide array of zebrafish neutrophil functions such as phagocytosis and bacterial killing have been previously studied (Colucci-Guyon *et al.,* 2011; Palić *et al.,* 2007). However, new transgenic zebrafish lines and high resolution confocal microscopy opens up new possibilities for insight into the details of the neutrophil recruitment and phagocytosis of Mm.

3.2 Hypotheses and aims

In this chapter I hypothesise that neutrophils are recruited, alongside macrophages, to local sites of *Mycobacterium marinum* (Mm) infection and phagocytose bacteria in zebrafish larvae.

The aims of experiments presented in this chapter are:

- To optimise bacterial preparation for local infection
- To characterise neutrophil migration towards Mm in different local infection sites
    - Characterisation of neutrophil recruitment to Mm in a muscle infection model
    - Characterisation of neutrophil recruitment to Mm in a tail fin infection model
- To determine whether neutrophils and macrophages are able to phagocytose Mm at local sites of infection

3.3 Results

3.3.1 Local *Mycobacterium marinum* injection of bacteria from glycerol stocks produces higher infection levels than from freshly prepared culture

Before investigating neutrophil recruitment to infection sites, I compared different bacterial preparation methods. The most commonly used model of *Mycobacterium marinum* (Mm) injection used in the lab is systemic infection. For systemic infections the injection stock is usually prepared from agar plates the day before infection and grown overnight in suspension culture to reach the exponential growth phase (van der Sar *et al.,* 2009). This protocol works well to assess bacterial burden 4 days later. Any initial variations in the CFU of freshly prepared bacterial stocks do not make a large difference to outcome days later. For neutrophil recruitment studies, the initial CFU needs to be as reproducible as possible due to the shorter time period of the experiment (a few hours), which is not long enough for Mm to replicate (which has a doubling time of around 10 hours). Therefore, I chose to investigate whether frozen glycerol stocks from a single initial bacterial culture would give a reproducible pathogen burden in the hours following initial infection. An advantage of using glycerol stocks is that a single, large preparation of Mm can be frozen down into aliquots and used over a large number of experiments to ensure better reproducibility.

I hypothesised that preparing mycobacteria from the same frozen stocks would result in a consistent and robust initial bacterial burden and hence be a more reliable method to use in immune cell migration studies. Two groups of 3dpf *nacre* fish were injected into the tail fin with either fluorescent bacteria 500CFU prepared fresh from an overnight culture or from a frozen down glycerol stock (Chapter 2 Materials and Methods) (Figure 3.1). The *nacre* fish are a mutant strain that have no melanophores throughout development meaning that no bacteria is masked by pigment when imaging (Lister *et al.,* 1999).

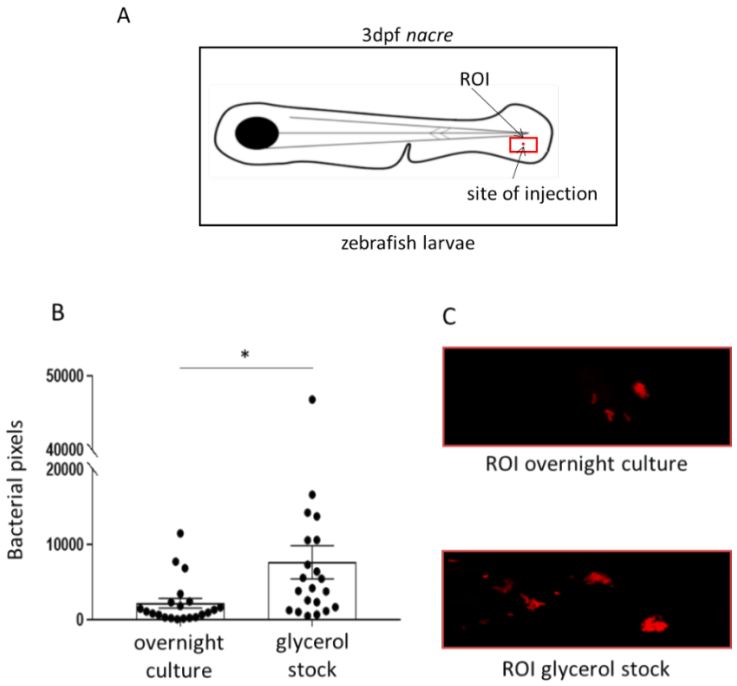


Figure 3.1 Initial *Mycobacterium marinum* bacterial burden is higher using glycerol stock than overnight culture

(A) Schematic of zebrafish larvae with site of tail fin injections (red dot) with either overnight culture or glycerol stock, and marked region of interest (ROI, red frame) following injection. (B) Comparison of the two different mycobacterial preparation methods. Tail fin injection was carried out at 3dpf and injected fish were imaged 2hpi. Images were analysed using dedicated pixel count software and numbers of bacterial pixels in the ROI were quantified. Three experimental repeats (7 fish per group) were performed and each experiment included two groups: overnight culture (n=21) and glycerol stock (n=21). (C) Representative images of zebrafish tail fin taken 2hpi with overnight culture and glycerol stock. Infecting fish with Mm from glycerol stock significantly increased the initial bacterial burden (Unpaired t-test; *-p<.023).

For future experiments I needed an initial infection with high and reproducible pathogen numbers. The glycerol stock was found to give robust and significantly higher infection levels than the overnight culture (the single individual with a very high bacterial burden in the glycerol stock group, likely resulted from a clump of bacteria in the initial inoculum (Figure 3.1). While the freshly prepared Mm produced a more consistent infection in terms of variance, the levels of infection were significantly lower using this method (Figure 3.1B-C). Glycerol stocks have the added advantage of being prepared and aliquoted from a single bacterial stock, and so experiments performed week on week will be performed using the same original stock making infections more consistently high. Therefore, future experiments were performed using glycerol stocks.

3.3.2 Neutrophils are recruited to *Mycobacterium marinum* infection in the muscle infection model

To investigate neutrophil recruitment I adapted a previously established muscle infection model (Oehlers *et al.,* 2015). The muscle infection is technically less challenging than the tail fin infection and it is more proximal to the caudal hematopoietic tissue (CHT), the primary site where hematopoietic stem and progenitor cells (HSPCs) expand in early embryonic development and where macrophages and neutrophils are produced (Wolf *et al.,* 2017).

In the course of experiment, two groups of 2dpf *Tg(mpx:GFP)*^{i114} fish were injected into the muscle somite midway between the tail fin and yolk sac extension with 200CFU Mm glycerol stock. Additionally, a polyvinylpyrrolidone (PVP) - which improves homogeneity of the bacterial suspension - control group was used to determine the number of neutrophils that were recruited to the muscle somite by the injection 'injury' alone. Neutrophils present in the infected somite were quantified by counting at 2 and 4hpi using fluorescence stereo microscopy (Figure 3.2).

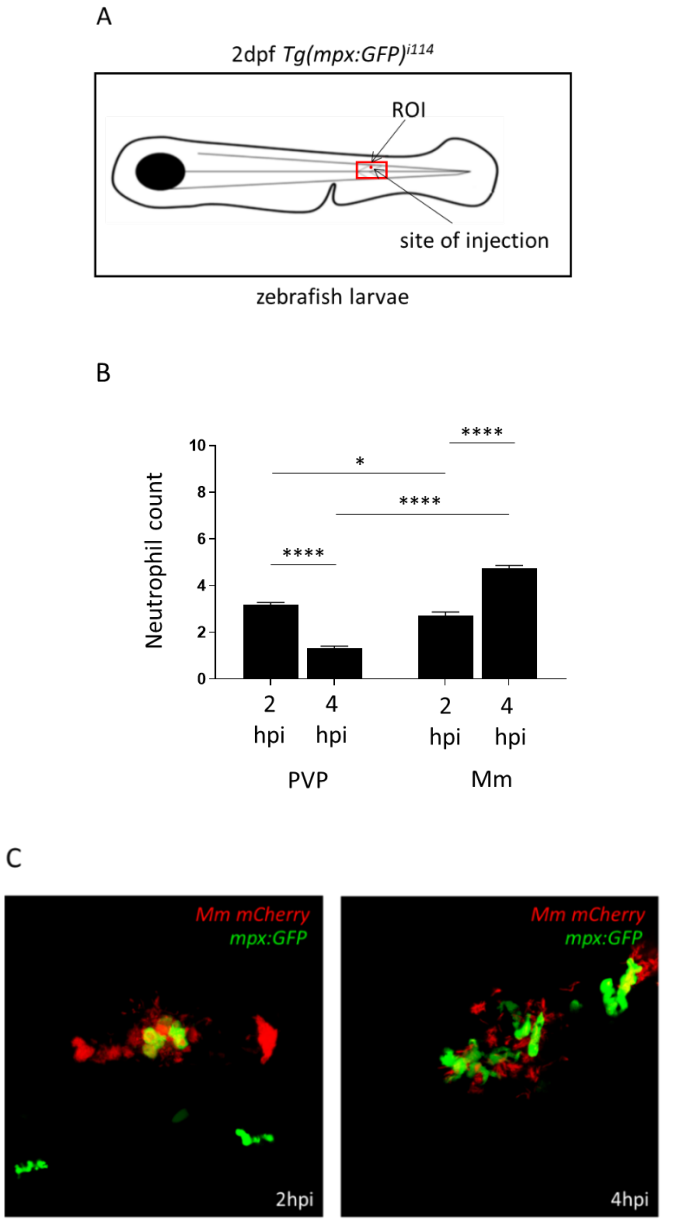


Figure 3.2 *Mycobacterium marinum* infection into the muscle somite recruited neutrophils

(A) Schematic of zebrafish larvae showing site of muscle somite injection (red dot) with either Mm (glycerol stock) or PVP control, and marked region of interest (ROI, red frame). (B) Neutrophil recruitment to muscle somite during the first 4 hours after Mm and PVP control injections. Local injections were performed at 2dpf and neutrophil counts were carried out manually in the ROI 2hpi and 4hpi using a fluorescent stereomicroscope Leica MZ10F. Three experimental repeats were performed with total n=72 fish for both Mm and PVP-injected groups. (C) Representative images of neutrophils (green) recruited to Mm (red) in the muscle somite at 2hpi and 4hpi. The number of neutrophils recruited to the muscle somite after 4hpi was significantly higher in Mm group than in PVP group. After 4hpi the number of neutrophils in Mm increased and in PVP group decreased significantly (Unpaired t-test; *-p<.05, ****-p<0.0001).

The number of neutrophils recruited at the initial stage of infection to the muscle somite was significantly higher in PVP-injected than Mm-injected group at 2hpi, but the biological difference between these groups was less than a single neutrophil difference (p=<.05) (Figure 3.2B). The data from this experiment show that 4 hours after Mm infection the number of neutrophils in Mm-injected group increased significantly, but modestly (by 2+/-SD number of neutrophils), whereas number of neutrophils in PVP-injected significantly decreased (Figure 3.2B-C). I have therefore confirmed that the muscle infection model can be used to study neutrophil recruitment in response to Mm, but that a minimum of 4hpi is required before counting neutrophils to discount for initial recruitment of neutrophils to the injection wound that subsequently resolves.

3.3.3 Recruitment of neutrophils to *Mycobacterium marinum* is lower than to the known neutrophil attractant, Zymosan

The findings from the muscle infection model (shown in figure 3.2) show that neutrophil recruitment in response to Mm at 4hpi is significantly higher than in the PVP control group, however the number of recruited neutrophils is limited (between 4-6). Similarly, low numbers of neutrophils recruited to Mm during systemic infection have been previously reported (Clay *et al.,* 2007; Yang *et al.,* 2012). In order to compare neutrophil recruitment to Mm with a well-characterised neutrophil attractant, I used Zymosan particles (Underhill *et al.,* 1999). Zymosan is an insoluble glucan obtained from the *Saccharomyces cerevisiae* cell wall and ligand for Dectin/TLR2 and is commonly used in zebrafish studies to investigate the recruitment dynamics of neutrophils (Gantner *et al.,* 2003; Taylor *et al.,* 2007).

It is not possible to consistently inject zymosan between the epithelial layers of zebrafish tail fin due to large size of the particles (average diameter 3um). Therefore for this experiment, two groups of 2dpf *Tg(mpx:GFP)*^{i114} fish were injected into the muscle somite with either zymosan (1nl of 1mM) or PVP controls. A PVP control group was used to determine the number of neutrophils recruited to the muscle somite by the injection process alone. At 2hpi neutrophils present in the injected muscle somite were counted. The same counting procedure was repeated 4hpi (Figure 3.3).

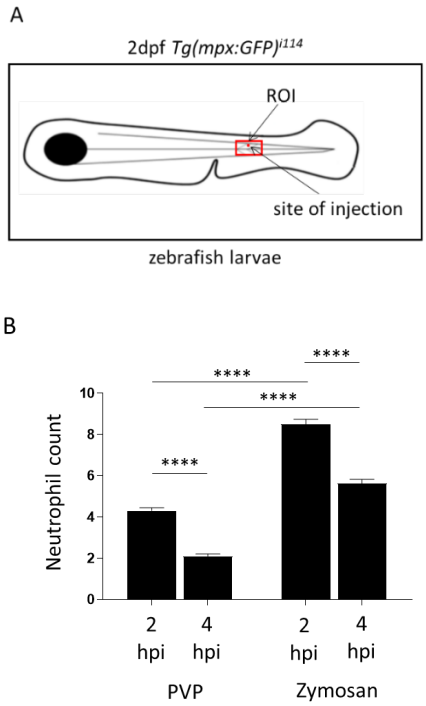


Figure 3.3 Zymosan enhanced the neutrophil recruitment to the muscle somite

(A) Schematic of zebrafish larvae showing site of muscle somite injection (red dot) with either zymosan or PVP controls and marked region of interest (ROI, red frame). (B) Neutrophil recruitment to muscle somite after zymosan and PVP control injections over 4h. Local injections were performed at 2dpf and neutrophil counts were carried out manually in the ROI 2hpi and 4hpi using a fluorescent stereomicroscope Leica MZ10F. Three experimental repeats were performed and two groups were used: zymosan-injected (zymosan; n=48) and PVP-injected (PVP; n=48). The number of neutrophils recruited to the muscle somite after 2hpi and 4hpi was significantly higher in the zymosan group than in the PVP controls. After 4hpi the number of neutrophils in zymosan and PVP groups decreased but remained significantly higher in zymosan group. A statistically significant difference in the neutrophil recruitment was observed between all groups (Unpaired t-test; ****-p<0.0001).

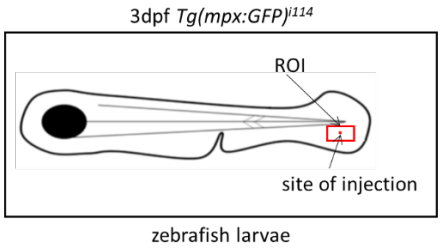
Upon injection with Zymosan neutrophils were recruited to the muscle somite at 2hpi and the number of neutrophils remained high at 4hpi, though decrease (p=<0.0001 for each group) (Figure 3.3). At both the 2 and 4 hour timepoints, the PVP injection recruited the same numbers of neutrophils as the previous Mm experiment (Figure 3.2). However, the Zymosan groups had more neutrophils recruited (5-8) (Figure 3.2) compared with those recruited to Mm (3-5) (Figure 3.3). These data suggest that the neutrophils response to Mm is less than to the well-known neutrophil attractant Zymosan. It is important to note that these experiments were performed on separate batches of embryos and this difference equates to 1-2 neutrophils. As difference is so small the biological relevance of decreased neutrophil recruitment to Mm is not clear from this experiment.

3.3.4 Neutrophils are recruited to *Mycobacterium marinum* infection in the tail fin infection model

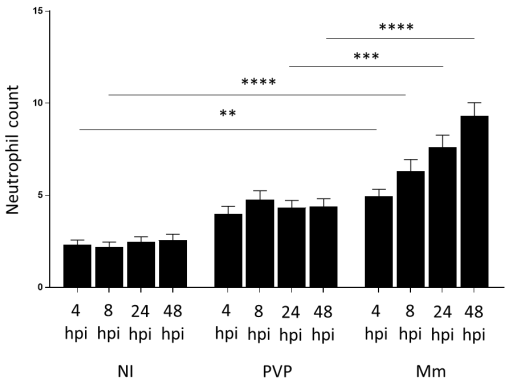
I aimed to use the tail fin infection model to investigate neutrophil recruitment to this very thin tissue as it offers better optical accessibility appropriate for detailed confocal/widefield microscopy. Moreover, the unchallenged zebrafish tail fin is devoid of neutrophils and has been shown to be highly informative to study host-pathogen interactions due to rapid attraction of leukocytes and predictable formation of a single granulomatous lesion following Mm injection (Hosseini *et al.,* 2014). Furthermore, the tail fin model has been used to study neutrophil and macrophage functions such as phagocytosis, and efferocytosis, and the dissemination of mycobacteria (Hosseini *et al.,* 2016).

I have already demonstrated that neutrophils are recruited to Mm in the somite model (Chapters 3.3.2). I hypothesised that initial neutrophil recruitment to the site of infection would significantly increase during the first 48hpi in the tail fin model. I injected the tail fins of 3dpf fish with 500CFU from a glycerol stock, or with PVP as control. The number of neutrophils present in the tail fin was quantified by manual counting using fluorescence microscopy at 4hpi, 8hpi, 24hpi and 48hpi (Figure 3.4).

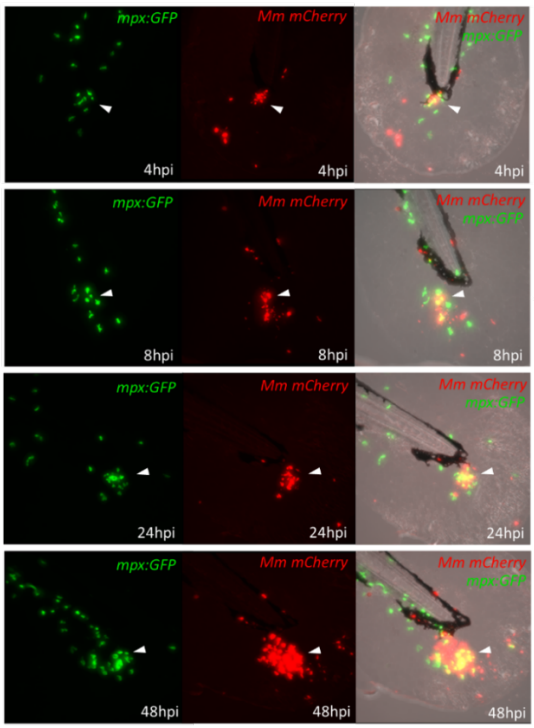
A


3dpf Tg(mpx:GFP)i114
ROI
site of injection
zebrafish larvae

B


Neutrophil count
15
10
5
0
****
***
****
**
4 hpi
8 hpi
24 hpi
48 hpi
NI
PVP
Mm

C


mpx:GFP
Mm mCherry
Mm mCherry
mpx:GFP
4hpi
8hpi
24hpi
48hpi

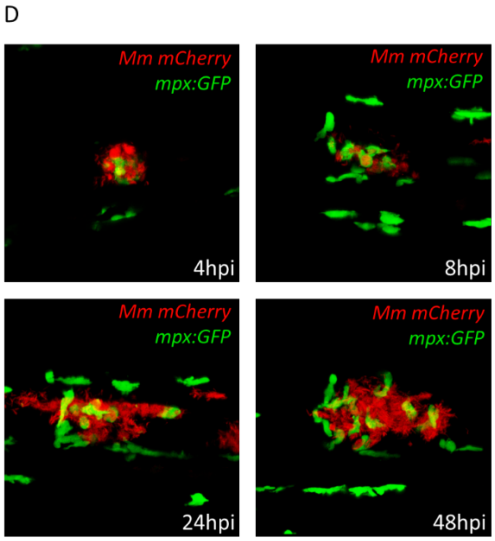


Figure 3.4 *Mycobacterium marinum* recruited neutrophils in a tail fin model

(A) Schematic of zebrafish larvae showing site of tail fin injection (red dot) with either Mm or PVP and marked region of interest (ROI, red frame). (B) Quantification of neutrophil recruitment to tail fin after Mm and PVP control injections during 48 hours post infection. Local injections were performed at 3dpf and neutrophil counts were carried out manually in the ROI 4hpi, 8hpi, 24hpi and 48hpi using a fluorescent stereomicroscope Leica MZ10F. Three experimental repeats were performed and three groups were used: non-injected (NI; n=44); PVP-injected (PVP; n= 42) and Mm-injected (Mm; n=51). (C) Representative images of neutrophils (green) attracted to Mm (red) in the tail fin measured 4hpi, 8hpi, 24hpi and 48hpi. White arrowheads indicate the infection points. Images of zebrafish tail fin taken using widefield microscope Leica DMi8 SPE TCS. (D) Representative images of neutrophils (green) attracted to Mm (red) in the tail fin measured 4hpi, 8hpi, 24hpi and 48hpi. Images of zebrafish tail fin taken using confocal microscope Leica DMi8 SPE TCS. The number of neutrophils recruited to the tail fin after 4hpi and 8hpi was significantly higher in Mm group than in NI group. Neutrophil recruitment in Mm group increased consistently over time and in later stages of infection was significantly higher than in both NI and PVP groups. After 24hpi and 48hpi the number of neutrophils was significantly higher in Mm group than in PVP group (One-way ANOVA with Sidak multiple comparison test; ****-p<0.0001, ***-p<0.001, **-p<0.01).

There was no significant difference between non-injected and PVP-injected fish in neutrophil recruitment at any time point. In Mm infection the number of neutrophils increased progressively with time from around 5 at 4hpi to 9 at 48hpi. The key result from this experiment is that Mm infection recruited significantly greater neutrophil numbers than the PVP controls at all timepoints (8hpi vs 8hpi *-p=0.0461; 24hpi vs 24hpi ***-p=<0.0001; 48hpi vs 48hpi ****-p=<0.0001) (Figure 3.4B).

In combination, the results from the above experiments suggest that the tail fin infection is an informative model in terms of dynamics of interaction between neutrophils and mycobacteria.

The number of neutrophils recruited to site of infection at 4hpi is similar to the number observed in muscle infection model, but more cells arrive during the later stages of infection (24hpi and 48hpi), suggesting these are more suitable to observe neutrophil recruitment. I therefore used the tail fin infection model to investigate how Mm growth changes over this timeframe.

3.3.5 *Mycobacterium marinum* proliferates over time at the local point of infection

Once the neutrophil recruitment in muscle and tail fin models were characterised, I wished to characterise how Mm bacterial burden changes during the course of a localised infection. The localised tail fin infection method allows investigation of granuloma-like structure formation as a result of Mm microinjection into the tissue. In the course of experiment 3dpf fish were injected into the tail fin with 500CFU using bacteria from glycerol stock. Fish were imaged at 2hpi, 24hpi (4dpf) and 48hpi (5dpf) (Figure 3.5) and the acquired images were used to assess mycobacterial density.

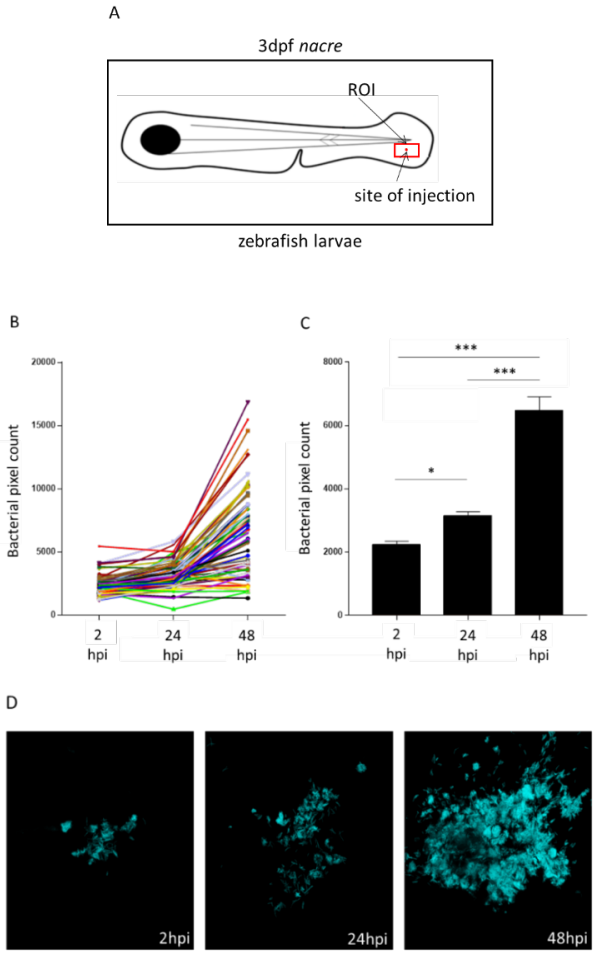


Figure 3.5 *Mycobacterium marinum* bacterial burden increased over time in a tail fin model

(A) Schematic of zebrafish larvae showing site of injection with Mm from glycerol stock (red dot) and marked region of interest (ROI, red frame). (B) Mm bacterial burden in tail fin infection model. Local injections were performed at 3dpf, bacterial burden was imaged using a fluorescent confocal microscope Leica DMi8 SPE TCS and quantified using dedicated pixel count software in the ROI at 2hpi, 24hpi and 48hpi. Three experimental repeats were performed (n=71). Each line corresponds with bacteria growth in an individual fish at the indicated timepoints. (C) Mean bacterial burden was calculated for each time point. A statistically significant difference in bacterial burden was observed between all timepoints (One-way ANOVA with Bonferroni multiple comparison test; ***-$p<0.001$, *-$p<0.05$). (D) Representative images of bacterial burden (cyan) in tail fin model measured 2hpi, 24hpi and 48hpi.

The above results (Figure 3.5) confirmed my hypothesis that mycobacterial burden changes over time in a tail fin infection model. The differences observed between days (Figure 3.5A-C) show that mycobacterial burden changes nonlinearly, with the most marked increase occurring between 24hpi and 48hpi (although a smaller but less significant increase was seen over the first 24hpi). I next wished to investigate phagocytosis of Mm by neutrophils and macrophages in the early phase of infection.

3.3.6 Neutrophils and macrophages phagocytose *Mycobacterium marinum* during early stages of infection

Adapting the previously established tail fin infection model (Hosseini *et al.,* 2014) I wished to verify whether neutrophils and macrophages are able to phagocytose Mm during the early stages of infection. During the experiments two different optical imaging techniques were used. Confocal microscopy was used to provide detailed information about host cells interactions. An advantage of confocal microscopy is reduced out-of-focus light making it potentially a better technique for imaging phagocytosis processes. Widefield microscopy allows for longer time-lapse due to lower power light energy being put into the live sample, and so may be a more useful technique for neutrophil and macrophage recruitment studies.

I hypothesised that Mm would be efficiently phagocytosed by both neutrophils and macrophages. In the course of experiment 3dpf *Tg(mpx:GFP)*^{i114} and *Tg(cfms:GFP)*^{sh377} larvae (with GFP-labelled neutrophils and macrophages respectively, enabling assessment of phagocytosis by each cell type separately) were injected into the tail fin with 500CFU. 2 hours after injections (the earliest timepoint when both neutrophils and macrophages are recruited to tail fin and interact with Mm) fish were imaged using confocal and widefield microscopy (Figure 3.6).

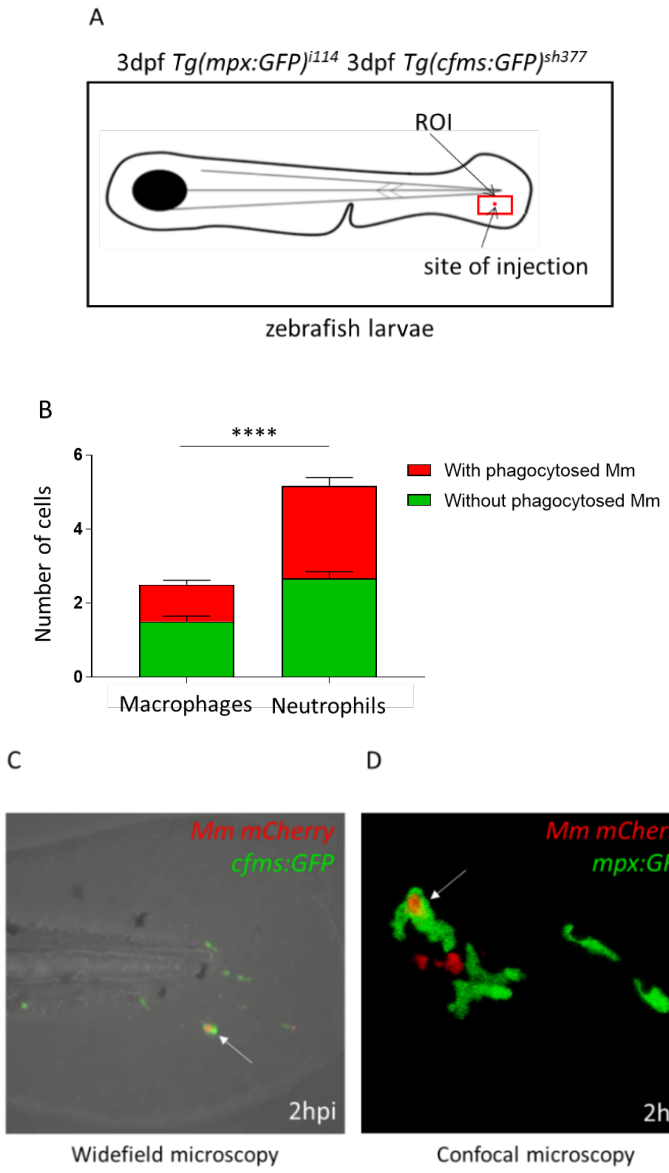


Figure 3.6 Macrophages and neutrophils are able to phagocytose *Mycobacterium marinum* during the early stages of infection

(A) Schematic of zebrafish larvae showing site of injection with Mm from glycerol stock (red dot) and marked region of interest (ROI, red frame). (B) Quantification of macrophages and neutrophils with (red) or without (green) phagocytosed Mm in tail fin at 2hpi (Unpaired t-test; ****-p<0.0001) (C) Early macrophage phagocytosis of Mm (white arrow). Image of zebrafish tail fin taken 2hpi using widefield microscope Leica DMi8 SPE TCS with maximum intensity projection. Macrophages of *Tg(cfms:GFP)*^{sh377}are GPF-labelled (green) and Mm is mCherry-labelled (red). (D) Early neutrophil phagocytosis of Mm (white arrow). Image of zebrafish tail fin taken 2hpi using confocal microscope Leica DMi8 SPE TCS with maximum intensity projection. Neutrophils of *Tg(mpx:GFP)*^{i114}are GPF-labelled (green) and Mm is mCherry-labelled (red). Images are representative of 12 fish (n=12) imaged over three independent experiments. Both phagocytic cells show ability to phagocytose Mm.

Both neutrophils and macrophages phagocytose Mm during the early stages of infection (Figure 3.6). The number of neutrophils recruited to Mm in the tail fin at 2hpi is significantly higher than the number of macrophages (5 neutrophils and 3 macrophages), but both cell types have similar fraction of internalised mycobacterium (about 50%) (Figure 3.6B). Although maximum intensity projections (MIPs) are displayed in figure 3.6, the Z-stacks of confocal and widefield microscopy images revealed that at 2hpi both neutrophils and macrophages contained internalised Mm (Figure 3.6C). These findings suggest that neutrophils and macrophages are equally capable of internalising bacteria in early stages after localised Mm infection.

3.4 Conclusions and chapter discussion

Macrophages are the best characterised innate immune cell type in TB and play pivotal roles in the control of infection. When studying mycobacterial infection, researchers have primarily focused on macrophages whilst neutrophils have been considered to be of lesser importance in this setting (Silva *et al.,* 1989). Crucially, our knowledge of neutrophil biology, on both the functional and molecular levels, has progressed greatly over the past 100 years, in particular with regards to their interactions with pathogens (Lowe *et al.,* 2012). It is now widely accepted that neutrophils are extremely versatile phagocytic cells and may comprise of distinct subpopulations which play key roles in numerous diseases (Cloke *et al.,* 2012; Fine *et al.,* 2016; Massena *et al.,* 2015; Rocha *et al.,* 2015; Tsuda *et al.,* 2004). The first evidence that neutrophils migrate to mycobacterium emerged in 1930s, and was described as a rapid cellular reaction to tubercle bacilli (Gardner, 1930). Recent developments in imaging techniques and advances in *in vivo* models have improved our understanding of neutrophils during the early stages of mycobacterial pathogenesis (Meijer, 2016). However, the exact role and functions of neutrophils in the context of mycobacterial infection are still not completely determined and have yet to be exploited therapeutically.

In this chapter I used zebrafish model to examine neutrophil migration to different stimuli and show that both macrophages and neutrophils are able to internalise Mm during the early stages of mycobacterial infection. In order to investigate neutrophil migration, I adapted new models of localised Mm infection, but first required consistent bacterial inoculum at the localised site. By comparing different mycobacterial preparation methods I confirmed that initial Mm bacterial

burden is higher using glycerol stocks than overnight cultures. Both methods are used widely in the literature, and have proven consistent for systemic infection models (Benard, *et al.,* 2012; Takaki *et al.,* 2012). Glycerol stocks ensure that injections over multiple experiments are derived from the same original stock of liquid culture, thereby decreasing variation that may occur over different cultures (Bernut *et al.,* 2015). Furthermore, the glycerol itself may aid injection at the localised sites, where the physical properties of the glycerol enhance tissue penetration.

In order to characterise neutrophil recruitment I adapted previously established tail fin and muscle infections (Hosseini *et al.,* 2014; Oehlers, *et al.,* 2015) and quantified neutrophil numbers at the infection sites. I demonstrate that local injection of the zebrafish can be successfully used to investigate neutrophil recruitment to Mm during the early stages of infection, utilising the optical transparency of this organism and standard tools such as transgenic lines in which specific cell types express GFP. I noted that in some infections injection in between the epithelial layers led to formation of multiple infection foci. The effect of multiple foci of infection on neutrophil recruitment is not clear and requires further work to elucidate. However, although the tail fin is highly accessible for imaging due to it being only approximately 2µm thick, the thinness of tissue makes it technically very challenging to inject successfully with bacteria (Parichy *et al.,* 2009).

Macrophages are the major phagocytic cells responsible for controlling mycobacterial infection while the roles of neutrophils remain unclear and have been studied less. From my observations in zebrafish both macrophages and neutrophils are equally able to phagocytose the pathogen Mm during the early stages of infection. Macrophages are well-defined immune cells and their phagocytic capacity in zebrafish relies at least in part on activation of scavenger receptors responsible for recognition and binding mycobacterial ligands (Benard *et al.,* 2014). Neutrophils were found to be the cells most frequently infected with Mtb in a range of airway samples (including sputum, bronchoalveolar lavage and cavity samples) from patients with active pulmonary TB undergoing surgery (Eum *et al.,* 2010). In this study the neutrophils were felt to be permissive for infection rather than to control, however this scenario represents late and uncontrolled disease, rather than the initial infection that I am using zebrafish to model.

Infection into the caudal vein is a common route to establish a systemic infection by injecting numerous pathogens such as: *Salmonella typhimurium, Burkholderia cenocepacia,*

Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae, Listeria monocytogenes and *Mycobacterium marinum* (Brannon *et al.,* 2009; Levraud *et al.,* 2009; Li and Hu, 2012; Meijer, 2016; Mesureur and Vergunst, 2014; Rounioja *et al.,* 2012; van der Sar *et al.,* 2003). However, systemic infection models have some important limitations, such as lack of knowledge of exact bacterial location therefore imaging of host-pathogen interactions in details is technically challenging. In order to investigate neutrophil recruitment to Mm I adapted muscle somite and tail fin models which deploy Mm in proximity to CHT and allow imaging of a higher number of phagocytic cells (Benard *et al.,* 2012; Hosseini *et al.,* 2014). Upon microinjections of Mm into the tail fin and muscle somite I observed neutrophil migration resulted in formation of a single granulomatous lesion as previously reported (Hosseini *et al.,* 2014). I observed increases in neutrophil count during the early stages of Mm infection, with no similar increase in neutrophils recruited at the site of mock infection. This excludes the possibility that the increase in neutrophil number was a consequence of wounding caused by injection or incited by the vehicle control (PVP). These findings confirm interaction between neutrophils and mycobacteria (Meijer *et al.,* 2008).

The interactions of neutrophils with mycobacteria have been studied in several animal models (Alemán *et al.,* 2004; D'Avila *et al.,* 2008; Wolf *et al.,* 2007). Nevertheless, there are many contradictory reports about the roles of neutrophils during mycobacterial infection. Neutrophils are able to phagocytose Mtb in mice (Repasy *et al.,* 2013) and in rats. Neutrophils recruited to the lungs have been shown to reduce bacterial burden and prevent the development of tuberculosis (Sugawara *et al.,* 2004). In humans, neutrophil phagocytosis is extremely fast with uptake and vacuole closure completed within 20 seconds. Upon Mtb uptake a phagocytic vacuole fuses with specific or azurophil granules indispensable for neutrophil ability to eliminate pathogens (Segal and Jones, 1980). The neutrophil phagosomes are markedly different from those observed in macrophages, having neutral pH which results in a high level of oxidative burst and phagosomal maturation dependent on cytosolic calcium (Jankowski *et al.,* 2002; Tapper *et al.,* 2002). The host-protective role of neutrophils and their phagocytic capacity have been demonstrated in neutropenic patients who suffer from numerous bacterial and fungal infections (Amulic *et al.,* 2012). The main obstacle to work with neutrophils is their short life span and intrinsic predisposition to undergo activation (Lowe *et al.,* 2012) however the use of zebrafish has opened up opportunities to study neutrophils in their *in vivo* settings.

Neutrophils have been shown to efficiently phagocytose tissue-localised Mm and contribute to bacteria dissemination during the early stages of infection (Hosseini *et al.,* 2016). My data corroborate that neutrophils, alongside macrophages, are recruited to the site of tail fin infection and interact with mycobacterium before the granuloma starts to form. In addition, the number of neutrophils recruited to the site of infection were significantly higher than macrophages but the ratios of the cells with phagocytosed Mm were comparable. Differences between findings may be due to the different routes of Mm administration and timepoints when neutrophil phagocytic activity was analysed. One potential explanation for differences between systemic models and local infection models is that the local phagocytic cell populations differ. Neutrophils have been shown to require a solid surface for phagocytosis. In the caudal vein systemic model, Mm is injected into the liquid blood environment and is rapidly dispersed across the embryo. Liquid environments favour macrophage phagocytosis (Colucci-Guyon *et al.,* 2011), therefore in systemic models neutrophils may play less of a phagocytic role compared to macrophages. In local infection models, bacteria are introduced into more solid environments, which may preferentially favour neutrophil phagocytosis. In both tail fin and somite injections neutrophils were major phagocytic cells, whereas in the hindbrain this was not found to be the case, supporting this hypothesis. An alternative reason for differences in neutrophil phagocytosis in different injection sites could be the local resident immune cell population. I observed the highest number of neutrophils recruited to a localised site in the somite infection model, and this could be due to its proximity to the CHT where there is a predominance of neutrophils. The tail fin lacks neutrophils in resting state, however challenge of the tail fin tissue, either by wounding (Renshaw *et al.,* 2006), or by infection (Hosseini *et al.,* 2016) leads to rapid neutrophil recruitment, which is what I observed in my tail fin Mm infection. This recruitment of neutrophils to the vasculature might not be observed in a systemic infection model. As demonstrated, Mm injected into the caudal vein or hindbrain was not phagocytosed by neutrophils (Yang *et al.,* 2012). However, HBV similarly to tail fin model is deprived of phagocytic cells (Clay *et al.,* 2007). Hence, the zebrafish tail fin model due to its composition of epithelial cell layers may be used to recapitulate environment of human lungs during Mtb infection. It is noteworthy that different experimental conditions such as neutrophil isolation or higher infection doses of mycobacteria may eventually increase neutrophil phagocytic capacity, and this is something that could be further addressed in my local zebrafish infection models (Persson *et al*., 2008, Abadie *et al*., 2005).

It was previously assumed that neutrophils are a homogenous cell population with a narrow spectrum of activity. Recent studies highlight that, regardless of macrophages, other phagocytic cells play an important role in protection against mycobacteria (Nouailles *et al.,* 2014; Srivastava *et al.,* 2014). Results presented in this chapter are in accordance with previous observations that macrophages and neutrophils are able to phagocytose Mm. Interestingly, Yang et al demonstrated that the subset of neutrophils recruited to the nascent granuloma in response to signals coming from dying macrophages could eliminate mycobacteria via an NADPH oxidase-dependent mechanism. As shown in the same study, neutrophils were infrequently recruited to initial site of infection and did not interact with mycobacteria during the early stages of Mm infection in zebrafish (Yang *et al.,* 2012). My findings confirmed that both muscle and tail fin infection models represent an insightful approach to investigate host-pathogen interactions during early stages of mycobacterial infection in zebrafish (Hosseini *et al.,* 2016). Having validated bacteria preparation methods and investigated neutrophil recruitment in both tail fin and muscle infection models I will now examine the role of different phagocytic cells during the early stages of Mm infection.

Chapter 4: Neutrophils and macrophages play important roles during *Mycobacterium marinum* infection

4.1 Introduction

Macrophages and neutrophils are the host's first line defence against infection and are essential for effective immune responses against invading pathogens. Both macrophages and neutrophils arise from a common stem cell and undergo differentiation through myeloid and granulocyte/macrophage progenitors (Friedman, 2002; Iwasaki and Akashi, 2007). Different transcription factors activated within progenitor cells determine whether cells become macrophages or neutrophils. Numerous studies on heterogeneity, functions and cytokine-dependent phenotypic diversity of macrophages highlight their importance during TB (Cooper and Khader, 2008; McClean and Tobin, 2016). Neutrophils and macrophages, regardless of their distinct characteristics acquired in the process of differentiation, work together to orchestrate appropriate immune responses during infection (Silva, 2010). While much is understood about macrophage responses to infection, neutrophils are difficult to manipulate *ex vivo* due to resulting activation and short lifespan so their roles in *in vivo* mycobacterial infection are poorly understood (Summers *et al.,* 2010).

The body responds to TB infection by producing more neutrophils in a pro-inflammatory response. Neutrophil blood counts in Mtb-exposed patients are significantly higher when compared with unexposed individuals (Martineau *et al.,* 2007). Recent studies on neutrophil-depleted whole blood confirmed that live neutrophils better control Mtb whereas dead neutrophils exhibit immunosuppressive effect (Lowe *et al.,* 2018). In recent years, studies in humans have shown a positive correlation between neutrophil-driven beta interferon (IFN-β) and active disease (Berry *et al.,* 2010; Bloom *et al.,* 2013). Human neutrophils are one of the most infected cells in active TB. However, research on TB patients shows that neutrophils promote lethal inflammation. This is, in part, mediated by increased levels of micro-RNA223, a small noncoding microRNA regulating expression of CXCL2, CCL3 and IL-6 (Dorhoi *et al.,* 2013). Neutrophil-mediated resistance to mycobacterium is associated with high levels of human neutrophil peptides 1-3 (HNP 1-3) (Eum *et al.,* 2010; Martineau *et al.,* 2007). Similar to other diseases, there are conflicting opinions about exact role of neutrophils in the course of TB

infection, and their positive or negative effects on disease outcome are potentially dependent on the stage of pathogenesis that the studies are performed. However, despite being poorly understood *in vivo*, there is emerging evidence that neutrophils play important roles in bacteria elimination during early stages of infection.

Neutrophils are remarkably short-lived white blood cells and, in humans, they are estimated to be produced at a rate of from 5×10^{10}-10×10^{10} per day (Summers *et al.,* 2010). They are produced and stored in the bone marrow from where are deployed into the bloodstream and circulatory system (Yamashiro *et al.,* 2001). Numerous studies have shown that neutrophils function as effective phagocytes and are able to eliminate pathogens using variety of microbicidal mechanisms (Lyadova, 2017). Neutrophils in chronic granulomatous disease (CGD) contribute to host protection during early stages of infection (Lowe *et al.,* 2018). In response to neutrophil activation by Mtb and pro-inflammatory cytokines, proinflammatory processes including degranulation and respiratory burst occur (Corleis *et al.,* 2012; Silva-Miranda *et al.,* 2017; Wright *et al.,* 2013). Neutrophils have been shown to act against Mtb through production of reactive oxygen species (ROS) and secretion of a range of antimicrobial proteases and proteins. However, some oxidants may trigger tissue breakdown and damage by activating matrix metalloproteinases (MMPs) (Nathan, 2002; Weiss, 1989). Inhibition of ROS derived from myeloperoxidase (MPO) reduced neutrophil necrosis resulting in enhanced efferocytosis of neutrophils by macrophages and limiting intracellular mycobacterial growth (Dallenga *et al.,* 2017). ROS have been shown to interfere with NF-κβ, transcription factor important for IL-1β and IL-8 expression (Warnatsch *et al.,* 2017). ROS also mediates production of TNF and macrophage inflammatory protein 2 (MIP-2) which are important pro-inflammatory cytokines (Naik and Dixit, 2011; Sheshachalam *et al.,* 2014). As a part of oxidative mechanisms, neutrophils utilise NOX2 complex, an enzyme activated by IL-8 and leukotriene B4 (TLB4) (Nathan and Cunningham-Bussel; 2013; Singel and Segal, 2016). Additionally, neutrophils are able to eliminate pathogens by extruding structures comprised of chromatin and granular proteins called neutrophil extracellular traps (NETs), which immobilise bacteria and may kill them (Brinkmann *et al.,* 2010; Fuchs *et al.,* 2007). The aforementioned oxidative mechanisms in concert with neutrophil granules represent a vast array of antimicrobial strategies that neutrophils use to restrict mycobacterial growth.

Neutrophils are prominent phagocytic cells and first responders during the early stages of infection (Riedel and Kaufmann, 1997). Early *in vitro* work suggested that human neutrophils are capable to eliminate Mtb (Brown *et al.,* 1987; Jones *et al.,* 1990). Indeed, neutrophils obtained from healthy individuals are able to eliminate Mtb 1 hour after phagocytosis in a TNF-α-dependent manner (Kisich *et al.,* 2002). A study on mice found that neutrophils and dendritic cells were infected after exposure to both Mtb and *M. bovis* BCG (Tsai *et al.,* 2006; Wolf *et al.,* 2007). The pivotal role of neutrophils has been evidenced by Blomgran and Ernst in mice models. A reduced number of neutrophils increased the presence of Mtb-infected dendritic cells in the lungs, but decreased their migration to lymph nodes resulting in a delayed $CD4^+$ T-cell-dependent response (Blomgran and Ernst, 2011). In a study of Sugawara and co-authors, LPS-activated rat neutrophils were able to lower bacteria number and kill Mtb *in vitro* (Sugawara *et al.,* 2004). Similarly, neutrophils in zebrafish play a beneficial role and are able to kill the internalised mycobacteria through NADPH oxidase-dependent mechanisms (Yang *et al.,* 2012). On the other hand the excess of neutrophils is not necessarily beneficial for the host as the prevalence in TB inflammation may contribute to the progression of pathology (Eruslanov *et al.,* 2005). Indeed, numerous *in vivo* studies suggest that neutrophils participate in disease development are responsible for lung damage (Dorhoi *et al.,* 2013; Gopal *et al.,* 2013; Nandi and Behar, 2011) and mediate an increased mycobacterial load in mice (Kimmey *et al.,* 2015). In addition, uptake of Mtb-induced necrotic neutrophils by macrophages results in an increased bacterial growth due to release of bacillus from damaged phagosomes and abrogated phagolysosomal fusion in macrophages (Dallenga *et al.,* 2017). This is an example of the significance of close interaction between neutrophils and macrophages to collectively restrict mycobacterial growth.

The contribution of neutrophils in the outcome of infection has been extensively evidenced (Alemán *et al.,* 2004; D'Avila *et al.,* 2008; Majeed *et al.,* 1998; Perskvist *et al.,* 2002). However, there are conflicting opinions about exact role of neutrophils in the course of infection. The outcome of infection greatly depends on effective recruitment of macrophages, neutrophils and other phagocytic cells to the site of infection as well as their ability to phagocytose mycobacterium (Nathan, 2006; Srivastava *et al.,* 2014; Sunderkötter *et al.,* 2004). Macrophages are well-known phagocytic cells accountable for host protection and control of mycobacterial infection (Guirado *et al.,* 2013). Numerous studies on heterogeneity, functions and cytokine-dependent phenotypic diversity of macrophages highlight their importance during TB (Cooper

and Khader, 2008; McClean and Tobin, 2016). Neutrophils, unlike macrophages, have not been extensively studied in the context of mycobacterial phagocytosis. However, the interplay between neutrophils and macrophages in this process has been already suggested (Tan *et al.,* 2006). High resolution confocal microscopy opens up new possibilities for insight into the details of the neutrophil migration too and phagocytosis of Mm. The zebrafish embryo has emerged as a well-tailored model to study interactions between host cells and pathogen due to its transparency and ease of chemical and genetic manipulation. Therefore, the zebrafish Mm model is a suitable model in which to study the roles of neutrophils during *in vivo* mycobacterial infection.

4.2 Hypotheses and aims

In this chapter I hypothesise that both macrophages and neutrophils are important to control growth of *Mycobacterium marinum* during the early stages of infection in zebrafish larvae.

The aims of experiments presented in this chapter are:

- Characterisation of chemical and genetic methods to manipulate macrophage and neutrophil populations *in vivo*
    - Validation of macrophage depletion method in zebrafish
    - Validation of neutrophil depletion methods in zebrafish
- Determining the effects of neutrophil and macrophage depletion on bacterial burden in systemic infection model
    - Validation and characterisation of macrophage and neutrophil modification methods
        - Investigating the roles of macrophages and neutrophils during the early stages of mycobacterial infection by quantification of bacterial burden following chemical depletion methods
        - Investigating the roles of macrophages and neutrophils during the early stages of mycobacterial infection by quantification of bacterial burden following genetic depletion methods

4.3 Results

4.3.1 Manipulation of macrophage and neutrophil number in zebrafish

Macrophage and neutrophil populations can be considerably reduced using depletion methods (Figure 4.1). The macrophage population can be ablated by injection of the toxic clodronate drug encapsulated in spherical liposomes, which only has its toxic effect once liposomes are phagocytosed and the drug is released in the cell. The microscopic vesicles containing clodronate are predominantly phagocytosed by macrophages, impair mitochondrial function and eventually trigger macrophage death by apoptosis. The clodronate liposome macrophage depletion method has been previously used in mice and zebrafish studies (Pagán *et al.,* 2015; Bader *et al.,* 2017; Wu *et al.,* 2019). The number of neutrophils and macrophages can also be altered using morpholinos (MO), which are typically 25-base oligonucleotides, designed to preclude protein translation of mRNAs and impair functions of microRNA (e.g. *irf8* morpholino (irf8MO) and *pu.1* morpholino (pu.1MO) (Clay *et al.,* 2007; Flynt *et al.,* 2017). The number of neutrophils can be decreased using *csf3r* morpholino (csf3rMO) or cell-type specific systems which rely on expression of bacterial nitroreductase (NTR) in zebrafish with a cell-specific promoter. NTR due to its catalytic function reduces metronidazole (mtz), a harmless prodrug, into a cytotoxic form that triggers death within NTR^+ cells (Curado *et al.,* 2008; Gray *et al.,* 2011).

Here I test the effectiveness of the genetic change of the macrophage and neutrophil number by using clodronate liposomes and *irf8*/*pu.1* MO knockdowns on mycobacterial infection outcomes. In addition, I plan to use csf3rMO to induce neutropenia to investigate the outcome of mycobacterial infection in the absence of neutrophils.

M Φ and N Φ manipulation methods	M Φ	N Φ
pu.1MO	↓	↓
irf8MO	↓	↑
clodronate liposomes	↓	-
metronidazole (mtz) *Tg(lyzC:NTRmCherry)* NTR+	-	↓
csf3rMO	-	↓

Figure 4.1 Summary of macrophage and neutrophil manipulation methods in zebrafish

Macrophage (MΦ) depletion (red arrow) can be achieved using chemical method (clodronate liposomes injections into the caudal vein at 24hpf in zebrafish larvae) or by using genetic approach (morpholino injections at single cell stage; *irf8* morpholino (irf8MO), *pu.1* morpholino (pu.1MO). Neutrophil (NΦ) population can be genetically increased (green arrow) at the expanse of macrophages using *irf8* morpholino (irf8MO) or decreased (red arrow) using *pu.1* morpholino (pu.1MO) and *csf3r* morpholino (csf3rMO) (morpholino injections at single cell stage). Neutrophil number can be also ablated (red arrow) chemically using metronidazole (mtz) in NTR+ cells. No change in the number of macrophages and neutrophils (-).

4.3.2 *Pu.1* morpholino decreased the number of leukocytes in zebrafish

Transient neutrophil and macrophage knockdown using antisense morpholino against *pu.1* has been evidenced (Rhodes *et al.,* 2005). Therefore, the pattern of distribution of macrophages and neutrophils can be transiently modified using morpholino against *pu.1* resulting in ablation both cell types. Macrophage depletion using *pu.1* is well characterised, however to confirm that neutrophil depletion is maintained throughout the period of infection experiments planned I quantified neutrophil numbers 2dpf and 5dpf in zebrafish.

To assess the efficacy of neutrophil depletion the numbers of neutrophils present in whole *Tg(mpx:GFP)*^{i114} fish were quantified using dedicated pixel counting software 2 days following single cell injections with *pu.1* morpholino (Figure 4.2).

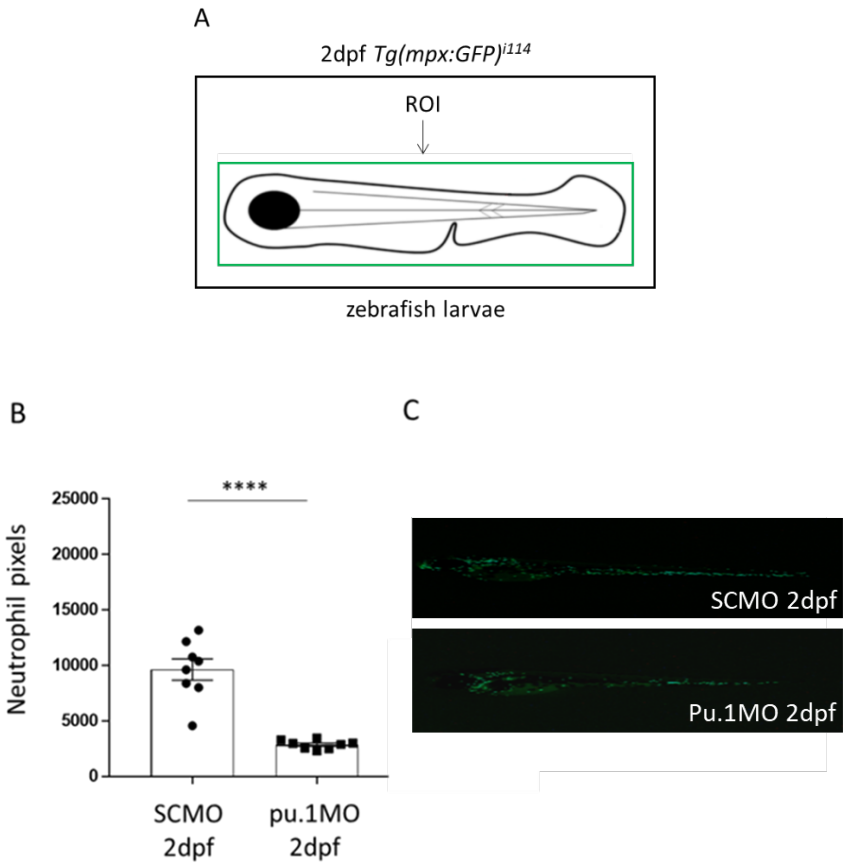


Figure 4.2 *Pu.1* morpholino injections depleted the number of neutrophils

(A) Schematic of zebrafish larvae with marked region of interest (ROI, green frame). (B) Neutrophil manipulations were performed using control morpholino (SCMO) and *pu.1* morpholino (pu.1MO) on *Tg(mpx:GFP)*^{i114} at the single-cell stage. Bar graphs summarise the comparison of pixel count quantified morpholino injections 2dpf. Morpholino injections were carried out 0dpf. Representative images of morpholino injected into the fish correspond with the change in the number of neutrophils. Fish imaging was carried out using dedicated pixel count software. One repeat was performed and three groups were used. Data are shown as mean +/- SEM, n=8 (One-way ANOVA with Bonferroni multiple comparison test ****-p<0.0001). Fish imaging was carried out using dedicated pixel count software. (C) Example fluorescent micrographs of data shown in (B).

Pu.1 MO knockdown significantly decreased green pixel counts and the difference was significant in 2dpf (Figure 4.2B-C). *Pu.1* MO knockdown leads to depletion of both neutrophil and macrophages. The macrophage phenotype is widely reported elsewhere (Mesureur *et al.,* 2017) and confirmed by my eye in my studies (data not shown). These experiments confirm that neutrophils can be decreased using morpholino against *pu.1*.

4.3.3 *Irf8* morpholino increased number of neutrophils in zebrafish

The interferon regulatory factor (IRF) is a family of transcription factors with IRF8 as a key regulator responsible for macrophage/monocyte differentiation (Taniguchi *et al.,* 2001). Transient neutrophil number increase at the expense of macrophages using antisense morpholino against *irf8* has been previously reported using tail fin amputation model (Li *et al.,* 2010). *Irf8* acts downstream of *pu.1*, a transcription factor essential for development of myeloid cells. I tested whether neutrophil numbers can be increased using genetic *irf8* knockdown.

To assess the efficacy of neutrophil increase the numbers of neutrophils present in whole *Tg(mpx:GFP)*^{i114} fish were quantified using dedicated pixel counting software 2 days following single cell injections with *irf8* morpholino (Figure 4.3).

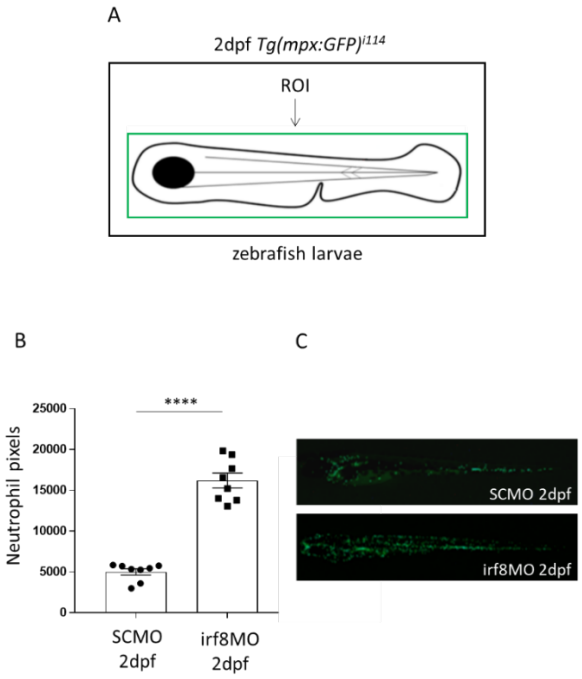


Figure 4.3 *Irf8* morpholino injections increased the neutrophil population

(A) Schematic of zebrafish larvae with marked region of interest (ROI, green frame). (B) Neutrophil manipulation were performed using control morpholino (SCMO), *irf8* morpholino (irf8MO) on *Tg(mpx:GFP)*^{i114} at the single-cell stage. Bar graphs summarise the comparison of pixel count quantified morpholino injections 2dpf. Morpholino injections were carried out 0dpf. Representative images of morpholino injected into the fish correspond with the change in the number of neutrophils. Fish imaging was carried out using dedicated pixel count software. One repeat was performed and three groups were used. Data are shown as mean +/- SEM, n=8-9 (One-way ANOVA with Bonferroni multiple comparison test ****-p<0.0001). Fish imaging was carried out using dedicated pixel count software (C) Example fluorescent micrographs of data shown in (B).

Irf8 morpholino knockdown significantly increased the neutrophil pool at 2dpf (Figure 4.3). These experiments confirmed that neutrophils can be increased using morpholino against *irf8*. I plan to use clodronate liposomes to deplete macrophage number while maintaining normal neutrophil numbers.

4.3.4 Clodronate liposomes reduced the macrophage population in zebrafish

Macrophage pool ablation can be achieved in zebrafish using clodronate liposomes (Bernut *et al.,* 2015). To assess the effectiveness of macrophage depletion in *Tg(mpeg:mCherry)* zebrafish the numbers of macrophages present between yolk sac extension and tail fin were manually counted at 6hpt, 24hpt and 4dpt following injections into the caudal vein at the posterior blood island at 24hpf (Figure 4.4).

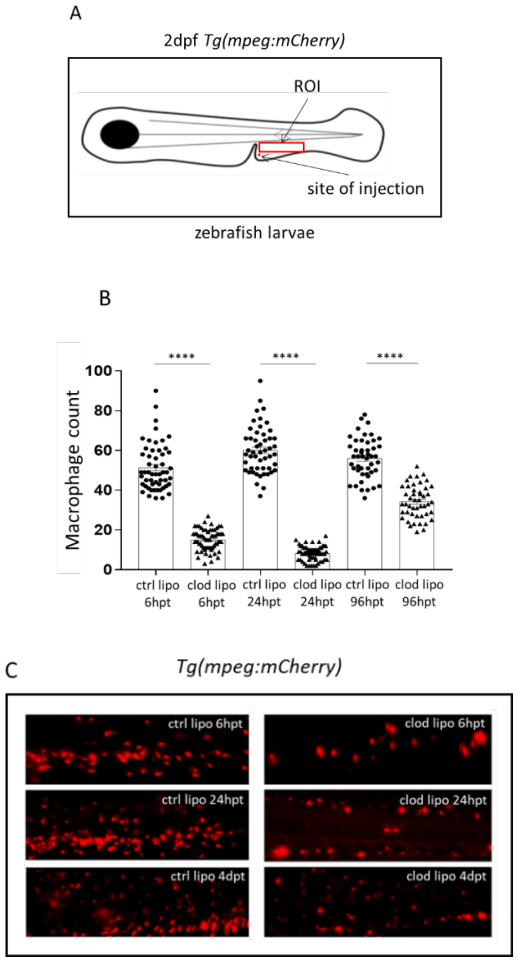


Figure 4.4 Clodronate liposomes injections result in macrophage depletion

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) Macrophage depletion was performed using clodronate liposomes at 24hpf *Tg(mpeg:mCherry)* zebrafish larvae. The number of macrophages present between yolk sac extension and tail fin were manually counted 6hpt 24hpt and 4dpt. After 6 hours post treatment the number of macrophages significantly decreased followed by increased reduction 24 hours post treatment. After 4 days post treatment number of macrophages increased but remained significantly lower when compared with control group at the same timepoint Data are shown as mean +/- SEM, n=47-64 as accumulated from 3 independent experiments. A statistically significant difference in macrophage number was observed between all timepoints (One-way ANOVA with Bonferroni multiple comparison test; ****-$p<0.0001$). (C) Example fluorescent micrographs of data shown in (B).

Clodronate liposomes were effective at macrophage depletion in 6 hours post treatment, followed by increased reduction 24 hours post treatment. After 96 hours post treatment number of macrophages increased but remained significantly lower when compared with a control group (PBS loaded liposomes) (Figure 4.4). These experiments showed that the number of macrophages can be considerably reduced 6 hours after clodronate liposome treatment and this effect was maintained until 24hpt.

4.3.5 Metronidazole does not reduced number of neutrophils in zebrafish

Cell ablation in zebrafish can be achieved using hybrid chemical-genetic method which relies on catalytic activity of bacterial NTR expressed under tissue-specific promoter. This results in reduction of innocuous Mtz to cytotoxic metabolite which induces cell death of NTR^{+} cells (Curado *et al.,* 2008). NTR/Mtz system has been previously used to ablate cardiomyocytes, hepatocytes, pancreatic β-cells and macrophages in zebrafish and larvae (Curado *et al.,* 2007; Gray *et al.,* 2011; Pisharath *et al.,* 2007). I tested whether neutrophil number would be reduced using mtz in NTR^{+} cells in *Tg(lyzC:NTRmCherry)* zebrafish. In the course of experiment 1dpf *Tg(lyzC:NTRmCherry)* fish were kept in metronidazole (mtz) for 36 hours and imaged using confocal microscopy 3dpf (Figure 4.5).

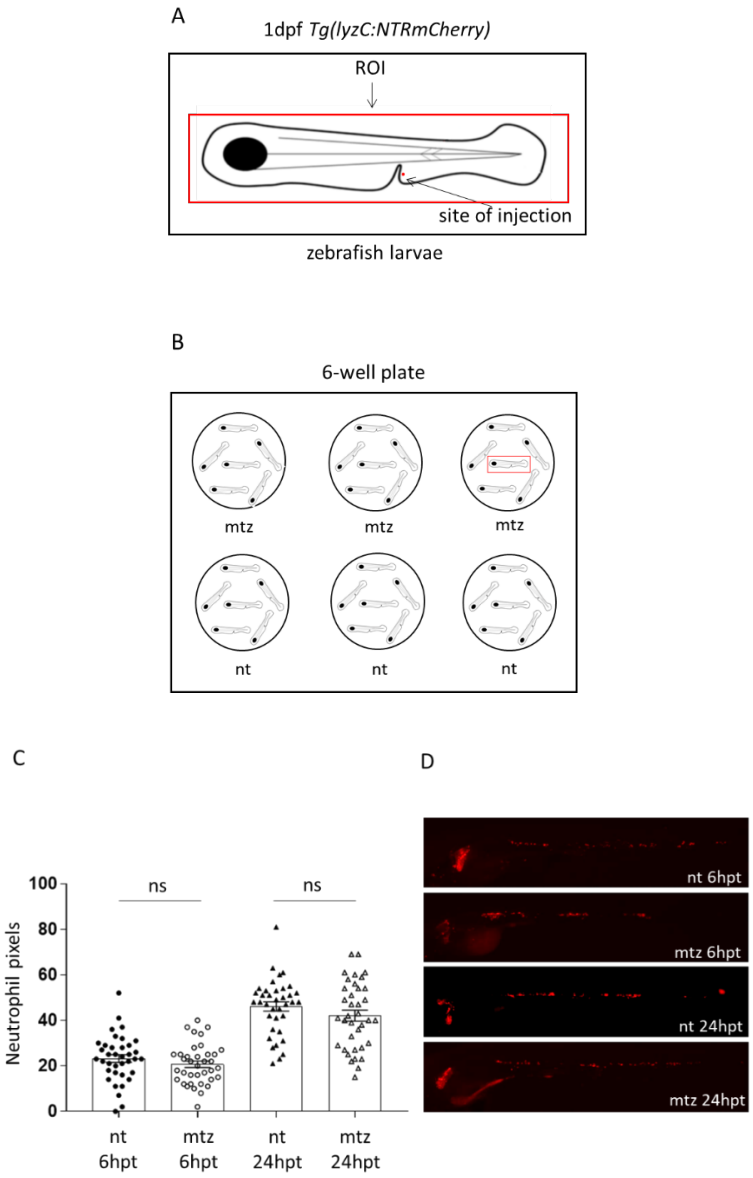


Figure 4.5 Mtz treatment does not lead to neutrophil depletion

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) Schematic of experiment on 6-well cell culture plate with zebrafish immersed in metronidazole (mtz) and non-treated controls (nt). (C) Neutrophil depletion was performed using metronidazole (mtz) on *Tg(lyzC:NTRmCherry)* zebrafish larvae. The number of neutrophils present after whole body count 6 hours post treatment (6hpt) and 24 hours post treatment (24hpt) were counted using dedicated pixel count software. Data are shown as mean +/- SEM, n=37 as accumulated from 3 independent experiments. No significant difference was observed between metronidazole-treated (mtz) and non-treated (nt) zebrafish larvae at 6hpt and 24hpt (One-way ANOVA with Bonferroni multiple comparison test; ns-p>0.05) (D) Example fluorescent micrographs of data shown in (C).

Metronidazole treatment was ineffective in neutrophil depletion (Figure 4.5). Mtz treatment has no effect on neutrophil number when measured 6 hours post treatment (about 20 neutrophils in both mtz and nt group). After 24 hours post treatment numbers of neutrophils increased but

showed no difference when compared with a control group (nt) (about 40 neutrophils in mtz and 50 neutrophils in nt group) (Figure 4.5C-D). These findings are consistent with unreported observations from the Elks/Renshaw lab which has shown that in this line neutrophils round up after mtz treatment and stop migrating from the caudal vein but remain present. I plan to test an alternative neutrophil depletion method using *csf3r* morpholino knockdown.

4.3.6 *Csf3r* morpholino depleted number of neutrophils in zebrafish

In zebrafish, transient neutrophil depletion can be achieved by targeting granulocyte colony-stimulating factor (GCSF) described in the zebrafish literature as Colony-stimulating Factor 3 (CSF3) (Liongue *et al.,* 2009). GCSF regulates neutrophil production and its importance was initially shown in G-CSF-deficient mice with chronic neutropenia and increased susceptibility to apoptosis in depleted neutrophil pool (Lieschke *et al.,* 1994; Liu *et al.,* 1996). Transient neutrophil depletion using antisense morpholino oligonucleotide knockdown strategies against *csf3r* has been previously reported (Stachura *et al.,* 2013). I tested whether neutrophil numbers can be reduced using genetic *csf3r* morpholino knockdown in zebrafish. In the course of experiment 0dpf *Tg(mpx:GFP)*^{i114} fish were injected at single cell stage and imaged using confocal microscopy 1dpf (24hpi) (Figure 4.6).

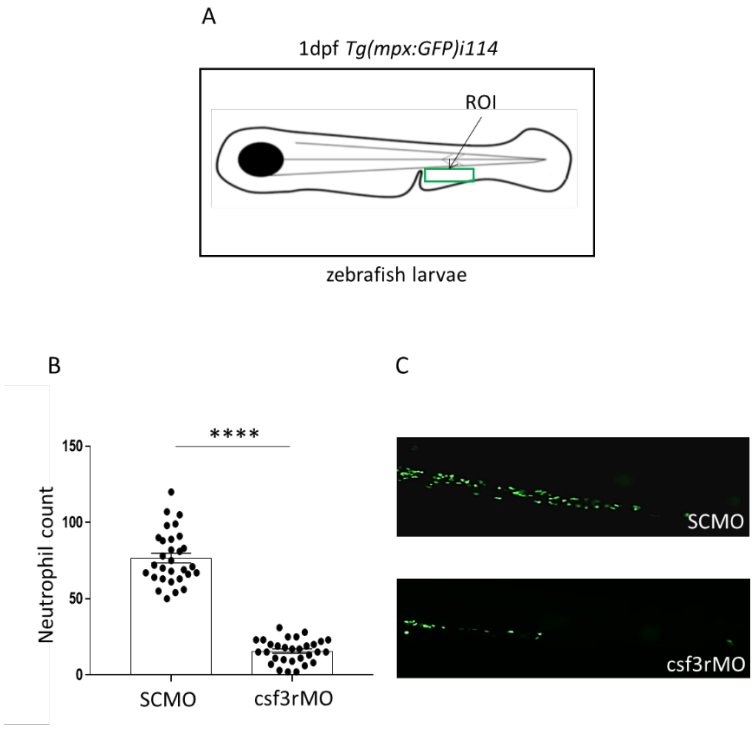


Figure 4.6 *Csf3r* knockdown resulted in neutrophil depletion

(A) Schematic of zebrafish larvae with marked region of interest (ROI, green frame). (B) Neutrophil depletion was performed using control morpholino (SCMO) and *csf3r* morpholino (csf3rMO) on *Tg(mpx:GFP)*^{i114} at the single-cell stage. The number of neutrophils present between yolk sac extension and tail fin after were manually counted 24 hours post injections. Data are shown as mean +/- SEM, n=30 as accumulated from 3 independent experiments). After 24 hours post injection the number of neutrophils significantly decreased. (One-way ANOVA with Bonferroni multiple comparison test ****-p<0.0001. (C) Example fluorescent micrographs of data shown in (B).

Csf3r morpholino knockdown was effective in neutrophil depletion (Figure 4.6). After 24 hours post injections the number of neutrophils decreased and was significantly lower when compared with a control group (SCMO) (about 20 neutrophils in *csf3r* group and 75 neutrophils in control group) (Figure 4.6B-C).

4.3.7 Macrophages and neutrophils are important for mycobacterial control during the early stages of infection

Whilst substantial progress has been achieved in leukocyte manipulation techniques, more detailed understanding of phagocyte roles during infection is required. In order to achieve this, it is important to investigate how bacterial burden at 4dpf after systemic infection is affected by altering numbers of macrophages and neutrophils (Figure 4.7).

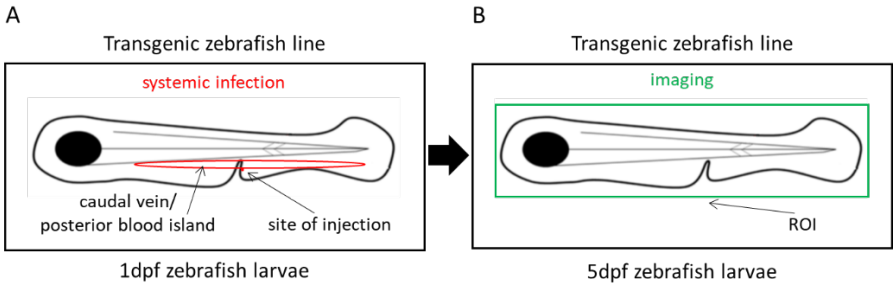


Figure 4.7 *Mycobacterium marinum* systemic infection and imaging techniques in zebrafish model

(A) During systemic infection zebrafish larvae are injected (red dot) with *Mycobacterium marinum* 1 day post fertilisation into the caudal vein/posterior blood island (red ellipse). (B) After 4 days post systemic infection (5 days post fertilisation) zebrafish are imaged in the region of interest (ROI, green frame) using confocal microscopy.

4.3.8 Macrophage and neutrophil depleted larvae have increased mycobacterial burden

Based on results from initial experiments using morpholino genetic knockdowns I planned to test how the depletion of neutrophil and macrophage numbers would effect on the course of the infection and bacterial burden. I hypothesised that *pu.1* MO knockdown would substantially increase bacterial burden in comparison with control MO group. Following neutrophil/macrophage depletion using morpholino against *pu.1,* 1dpf *nacre* fish were infected into the caudal vein at the posterior blood island with 100CFU as previously described (Benard *et al.,* 2012) and imaged at 4dpi (5dpf) (Figure 4.8).

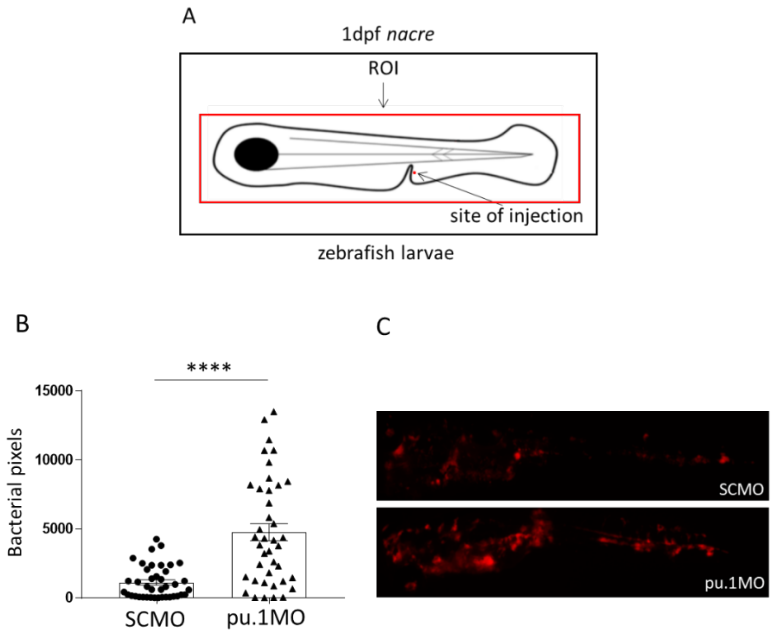


Figure 4.8 *Pu.1* morpholino injections resulted in increased bacterial burden

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of pixel count quantified after control morpholino (SCMO) and *pu.1* morpholino (pu.1MO) knockdown at the single-cell stage and infection 30hpf. MO injected fish were imaged 5dpf. Three repeats were performed MO control (SCMO; n=40); *pu.1* MO (pu.1MO; n=40). (Data are shown as mean +/- SEM, n=40 as accumulated from 3 independent experiments (Unpaired T test ****P<.0001). (C) Example fluorescent micrographs of data shown in (B). Representative images of bacterial burden in pu.1MO-injected fish correspond with the change in the neutrophil pixel count (Figure 4.1).

Depletion of neutrophils and macrophages in *pu.1* injected group led to increased bacterial burden when compared with SCMO group (Figure 4.8). The difference in bacterial pixel count between standard control morpholino (SCMO) and *pu.1*-injected group was significantly different (p=>0.0001) (Figure 4.8B-C). These data confirmed that change in macrophage and neutrophil counts has a significant impact on bacterial burden during Mm infection. I plan to verify whether neutrophil increase at the expense of macrophages would have similar effect on the infection outcome.

4.3.9 Increased neutrophil number at the expense of macrophages increases mycobacterial burden

Based on results from initial experiments using morpholino genetic knockdown and clodronate liposomes, I planned to test how the increase of neutrophils at the expense of macrophages number would effect on the course of the infection and bacterial burden. I hypothesised that *irf8*

morpholino knockdown would substantially increase bacterial burden in comparison with control morpholino group. Following neutrophil/macrophage manipulation using a morpholino against *irf8,* 1dpf *nacre* fish were infected into the caudal vein at the posterior blood island with 100CFU as previously described (Benard *et al.,* 2012) and imaged at 4dpi (5dpf) (Figure 4.9).

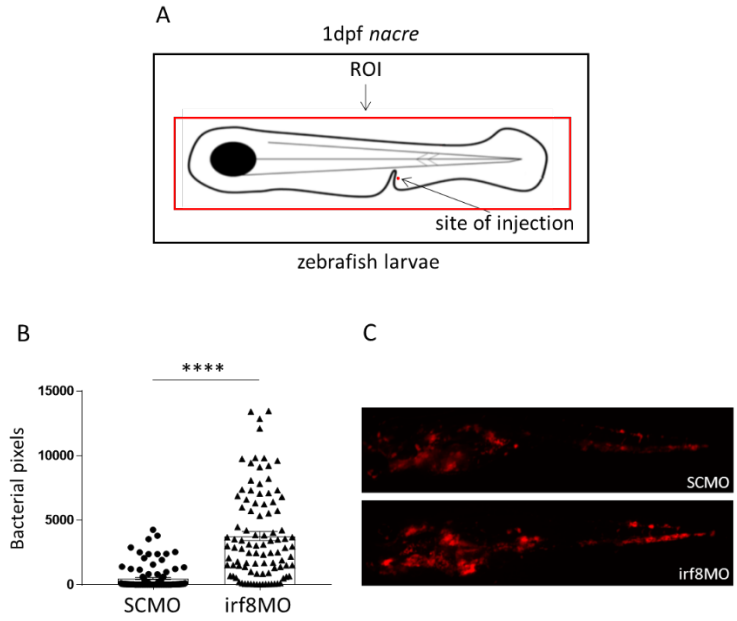


Figure 4.9 *Irf8* morpholino injections results in increased bacterial burden

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of pixel count quantified after control morpholino (SCMO) and *irf8* morpholino (irf8MO) knockdown at the single-cell stage and infection 30hpf. MO injected fish were imaged 5dpf. Irf8MO-injected embryos have significantly higher levels of bacterial burden when compared with group SCMO group. Three repeats were performed MO control (SCMO; n=99); *irf8* MO (irf8MO; n=98). (Data are shown as mean +/- SEM, n=98-99 as accumulated from 3 independent experiments (Unpaired T test ****P<.0001). (C) Example fluorescent micrographs of data shown in (B). Representative images of bacterial burden in irf8MO-injected fish correspond with the change in the neutrophil pixel count (Figure 4.2).

An increase of neutrophils at the expense of macrophages in the *irf8*-injected group led to increased bacterial burden when compared with control morpholino group (SCMO) (Figure 4.9). The bacterial pixel count between SCMO and *irf8*-injected group was significantly different (p=>0.0001) (Figure 4.9B-C). As shown, increased neutrophil population in *irf8*-injected group was insufficient to control infection when macrophages were depleted highlighting the important role of macrophages in Mm infection. These data confirm that changes in macrophage and neutrophil counts have a significant impact on bacterial burden during Mm infection. I plan to verify whether macrophage depletion using clodronate liposomes would have similar effect on the infection outcome.

4.3.10 Macrophage depletion increased mycobacterial burden

I hypothesised that depletion of macrophages would affect zebrafish innate immune response against Mm proving their importance in host response to pathogen. Following macrophage depletion using clodronate liposomes *nacre* zebrafish larvae were infected into the caudal vein at the posterior blood island with 100CFU at 30hpf. Bacterial burden levels in 4dpi infected macrophage-depleted embryos were measured using dedicated pixel counting software (Figure 4.10).

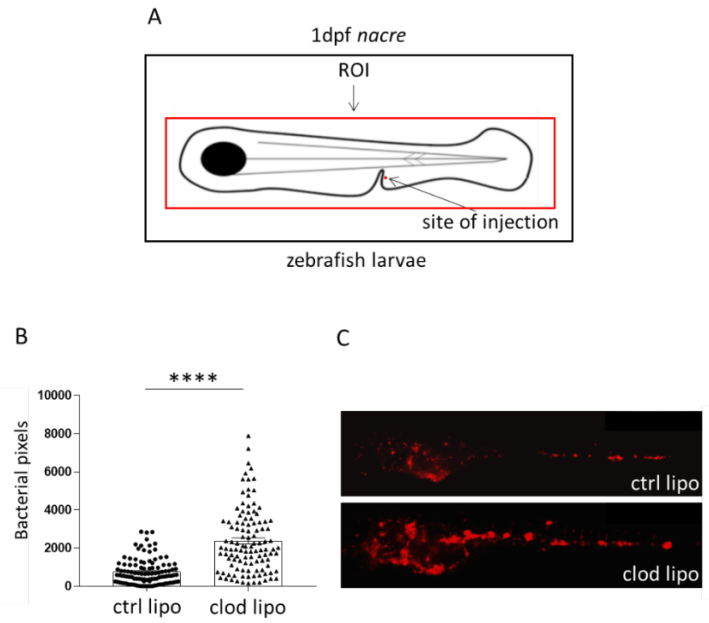


Figure 4.10 Macrophage depletion results in uncontrolled infection in zebrafish larvae

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) Quantification of bacterial burden by fluorescent pixel count on 5dpf (4dpi) nacre larvae after macrophage depletion 24hpf and infection 30hpf. Clodronate-injected embryos (clod lipo) have significantly higher levels of bacterial burden when compared with control liposomes (ctrl lipo). Data are shown as mean +/- SEM, n=109-112 as accumulated from 3 independent experiments (Unpaired T test ****p<.0001). (C) Example fluorescence micrographs of data shown in (B).

A decrease of macrophages in the clodronate-injected group led to increased bacterial burden when compared with control liposomes (ctrl lipo) (Figure 4.10). The bacterial pixel count between ctrl lipo and clodronate-injected group was significantly different (p=>0.0001). As demonstrated, depletion of macrophage population in clodronate-injected group results in uncontrolled infection confirming the important role of macrophages in Mm infection (Figure 4.10B-C). These data confirm that macrophage depletion has a significant impact on bacterial burden during Mm infection. I plan to verify whether neutrophil depletion using csf3r morpholino would have similar effect on the infection outcome.

4.3.11 Neutrophil depletion increased mycobacterial burden

After validating neutrophil depletion methods I aimed to investigate the effects on the outcome of infection. I hypothesised that depletion of neutrophils would affect zebrafish innate immune

response against Mm proving their importance in host response to pathogen. Following neutrophil depletion using morpholino against *csf3r nacre* zebrafish larvae were infected into the caudal vein at the posterior blood island with 100CFU at 30hpf. Bacterial burden levels in 4dpi infected neutrophil-depleted embryos were measured using dedicated pixel counting software (Figure 4.11).

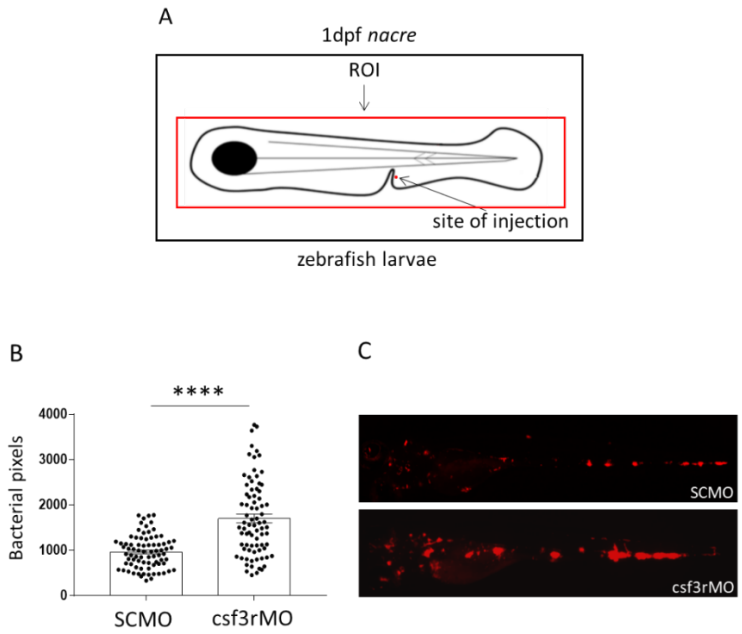


Figure 4.11 Neutrophil depletion results in uncontrolled infection in zebrafish larvae

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) Quantification of bacterial burden by fluorescent pixel count on 5dpf (4dpi) *nacre* larvae after neutrophil depletion at the single-cell stage and infection 30hpf. *Csf3r* morpholino-injected embryos (csf3rMO) have significantly higher levels of bacterial burden when compared with control morpholino group (SCMO). (Data are shown as mean +/- SEM, n=79 as accumulated from 3 independent experiments (Unpaired T test ****p<.0001). (C) Example fluorescence micrographs of data shown in (B).

A decrease of neutrophils in the *csf3r*-injected group led to increased bacterial burden when compared with control morpholino group (ctrl lipo) (Figure 4.11). The bacterial pixel count between *csf3r*-injected and control morpholino-injected group was significantly different (p=>0.0001). As shown, depletion of neutrophil population in *csf3r*-injected group results in uncontrolled infection highlighting the pivotal role of neutrophils in Mm infection (Figure 4.11B-C). These data show that neutrophil depletion has a significant impact on bacterial burden during the early stages of mycobacterial infection.

4.4 Conclusions and chapter discussion

Macrophages and neutrophils are extremely dynamic phagocytic cells involved in the innate immune response (Silva *et al.,* 1989). In humans, neutrophils are the most abundant circulating immune cells and unlike macrophages, have not been extensively studied in the context of TB infection. Our understanding of innate immunity has been improved with the emergence of *in vivo* models where functionality of individual cell subsets can be studied in the context of a whole living organism. Neutrophils are one the first responders to Mtb but in the light of current knowledge their exact roles during TB infection are still poorly defined and controversial (Lyadova, 2017). Better understanding of neutrophil behaviour during the early stages of mycobacterial infection may facilitate the identification of novel host-derived treatment strategies against TB.

In this chapter I validated both genetic and chemical tools to manipulate macrophage and neutrophil populations in Mm zebrafish models. By investigating the host-pathogen interactions following effective leukocyte number modification, I characterised host immune response during the early stages of mycobacterial infection. Here, I demonstrate that depletion of macrophages can be achieved using clodronate liposomes and ablation of these cells leads to uncontrolled infection. Different-route administration of clodronate-loaded liposomes allows for selective depletion of alveolar macrophages (AM) and interstitial macrophages (IM) in murine models, which helped to elucidate the tissue-dependent differences in macrophage plasticity (Rooijen and Hendrikx, 2010). The important roles of macrophages during mycobacterial infection has been extensively reviewed elsewhere (Chacón-Salinas *et al.,* 2005). Macrophages have been shown to play dual role during mycobacterial infection (Flynn *et al.,* 2011). In mice, AM bulk depletion is favourable for the host (Huang *et al.,* 2018; Leemans *et al.,* 2001), whereas decreased numbers of IM leads to impaired resistance and enhanced Mtb growth (Huang *et al.,* 2018; Leemans *et al.,* 2005). The duality of macrophage functions is attributed to their heterogeneity and different activation states (Antonelli *et al.,* 2010; Marino *et al.,* 2015), with diverse Mtb distribution between distinct types of macrophages (Srivastava *et al.,* 2014). In humans, the absence of *irf8* significantly depletes the macrophages pool with an increase of neutrophil number (Hambleton *et al.,* 2011). In BXH-2 mice, which bear a defective *IRF-8*^{R294C} allele even

a low dose of virulent Mtb administrated via i.v. and aerosol routes led to uncontrolled bacteria replication in multiple organs and death (Marquis *et al.,* 2009). Clay et al have demonstrated that ablation of macrophages using *pu.1* MO in zebrafish model promotes mycobacterial dissemination (Clay *et al.,* 2007). My macrophage depletion data corroborate these published findings and highlights the important roles of the macrophage pool in TB infection. In my *in vivo* zebrafish data, all methods that depleted the macrophage pool led to increased bacterial load. Proinflammatory macrophages are engaged in bacterial killing via production of reactive oxygen species (ROS) and nitric oxide (NO), both of which are toxic to mycobacteria (Benoit *et al.,* 2008). Macrophages are specialised to enable mycobacterial killing in these ways, and can do so by rapid lysosome fusion with phagosomes infected with Mtb (Wel *et al.,* 2007). However, mycobacteria have evolved systems to escape these macrophage killing mechanisms (Eum *et al.,* 2010; Repasy *et al.,* 2013), although it is clear from data presented in this chapter and elsewhere that macrophages still have some level of success in TB control.

Neutrophils are first responders to infection and their effective migration towards pathogens depends on intracellular signal transduction triggered by signalling molecules present in the extracellular environment (Futosi *et al.,* 2013; Mayadas *et al.,* 2014). Neutrophils are known to phagocytose bacteria and their protective roles in mycobacterial infection control have been recently reviewed (Chai *et al.,* 2020; Kroon *et al.,* 2018; Warren *et al.,* 2017). Human neutrophils once recruited to the infection site are able to phagocytose and kill Mtb within 1 hour in nonoxidative TNF-α but not IFN-γ-dependent fashion (Kisich *et al.,* 2002). The neutrophil's ability to control Mtb depends on availability of free radicals and level of phagosome acidification among others. An increased levels of glutathione (GSH) in human neutrophils led to enhanced phagosome acidification and limited bacteria growth via TNF-α/IL-6-dependent mechanism (Morris *et al.,* 2013). In patients with pulmonary tuberculosis neutrophils activate other immune cells through the secretion of important chemokines such as MIP-1α , IL-8 and MCP-1 (Hilda *et al.,* 2014). Neutrophil oxidative killing has been shown in zebrafish where Mm is phagocytosed from infected macrophages (Yang *et al.,* 2012), proving that efferocytosis represents an important mechanism against mycobacteria (Briken, 2012). Neutrophil necrosis leads to formation of NETs - DNA structures containing neutrophil elastase, myeloperoxidase (MPO) and metalloproteinases - which trap the invading mycobacteria and limit its growth (Braian *et al.,* 2013; Francis *et al.,* 2014). Mycobacteria infect different cell types and resulting changes within

cells depends on bacteria specie (Ganbat *et al.,* 2016). Therefore, in order to understand how different immune cells contribute to the outcome of mycobacterial infection, effective methods of manipulating the numbers of these cells are needed. In this chapter I show that neutrophil numbers can be reduced using both *csf3r* and *pu.1* morpholino knockdowns. My results recapitulate previous studies using similar morpholino methods and show that the number of macrophages and neutrophils can be manipulated by the use of *irf8* (Li *et al.,* 2010) and *pu.1* (Rhodes *et al.,* 2005) morpholino oligonucleotides. Morpholinos have previously been demonstrated to potentially cause off-target effects (Kok *et al.,* 2015), however the development of embryos injected with *irf8* and *csf3r* morpholinos was not affected. From my observations MO anti-sense knockdown is an effective tool to modulate the number of phagocytic cells which has a knock on effect on the mycobacterial burden.

It is noteworthy that *irf8* MO knockdown results in reduced number of macrophages but increased number of neutrophils, while the absolute number of primitive myeloid cells remains unchanged. This is a subtly different scenario from other methods presented in this chapter which decrease macrophages alone, neutrophils alone, or both together. Using morpholinos against *irf8* show a decrease of macrophage numbers and enhanced numbers of neutrophils (Li *et al.,* 2010) and my results corroborated this. Interestingly, the *irf-8* morpholino increased infection burden, suggesting that neutrophils alone, even if increased in number, do not protect against Mm infection when macrophage numbers are decreased. Therefore, these results highlight the importance of macrophages during mycobacterial infection.

In my *in vivo* zebrafish data, all neutrophil ablation methods using both *csf3r* and *pu.1* morpholinos resulted in increased bacterial burden suggesting they play an active role in Mm control. Up to now, neutrophil bactericidal activity in response to TNF-α activation is controversial as many research groups have reported contradictory findings (Corleis *et al.,* 2012; Kisich *et al.,* 2002; Reyes-Ruvalcaba *et al.,* 2008). Interestingly, several *in vitro* studies have demonstrated that regardless of IFN-γ presence, the neutrophil's ability to eliminate mycobacteria remain limited (Eruslanov *et al.,* 2005). Other *in vitro* studies on NETs have suggested that extracellular traps, previously considered as a neutrophil antimicrobial mechanism, may play the opposite role and support Mtb to proliferate in human lungs (Cardona and Prats, 2016; Ehlers and Schaible, 2012). In contrast, study on rats clearly demonstrate that

neutrophils are actively involved in the killing of bacteria during early stages of infection (Sugawara *et al.,* 2004). Another host-protective effect of neutrophils has been evidenced during the early stages of mycobacterial infection in zebrafish. Neutrophils recruited to emerging granulomas in response to macrophage-secreted signal, killed Mm in an NADPH oxidase-dependent mechanism and impaired recruitment increased number of bacteria (Yang *et al.,* 2012). The importance of neutrophils during Mm infection has been inferred by their migration to the sites of inflammation and contribution in pathogen-induced granuloma formation in zebrafish (Meijer *et al.,* 2008). Tail fin infection of Mm lead to engulfment by neutrophils in early stages to a similar extent as macrophage engulfment. Moreover, there is a growing body of evidence that neutrophils can defend the host during the mycobacterial infection (Lowe *et al.,* 2012) and also control mycobacteria via a HIF-dependent mechanism (Elks *et al.,* 2013). Neutrophil depletion led to increased bacterial burden in studies presented in this chapter, highlighting a potentially important role for neutrophils during the control of Mm infection in zebrafish.

Zebrafish models of tuberculosis have allowed us to develop and validate numerous prospective drugs to combat mycobacteria (Roca and Ramakrishnan, 2013; Tobin *et al.,* 2012). One of the promising, but still not fully elucidated mechanism is increasing nitric oxide production via inducible nitric oxide synthase (iNOS) (Elks *et al.,* 2013; Hall *et al.,* 2013). It has been previously shown that stabilised hypoxia inducible factor 1-alpha (Hif-1α) reduces bacterial burden due to increased levels of potent antimicrobial nitric oxide (NO) predominantly found in neutrophils (Elks *et al.,* 2013). However, it remains unclear whether the NO produced by neutrophils acts on infected macrophages or is used to kill bacteria directly within the neutrophils. Having validated tools to manipulate the macrophage and neutrophil pool within *in vivo* zebrafish, I will now investigate modulation of Hif-1α in these models in the next chapter.

Chapter 5: An important role for neutrophils in mycobacterial infection control after Hif-1α stabilisation

5.1 Introduction

Hypoxia is a physiological condition in which oxygen levels are not sufficient for the needs of the tissue. Local low oxygen tension in tissue areas is a characteristic feature of infection and inflammation (Kennedy *et al.,* 1997). The cellular response to hypoxia is controlled through stabilisation of hypoxia inducible factor alpha (HIF-α) (Cramer *et al.,* 2003). Numerous studies have shown that HIF has a profound impact on the differentiation, polarisation and activation of myeloid cells (Corcoran and O'Neill, 2016; Devraj *et al.,* 2017; Koyasu *et al.,* 2018; Stothers *et al.,* 2018). However, the exact role of neutrophils in the increased control of mycobacteria after Hif activation has yet to be fully elucidated.

During Mtb infection engulfed bacteria are able to survive in macrophages and successfully proliferate. Subsequently, uninfected macrophages and other immune cells, including other myeloid cells like neutrophils, migrate towards the infection site resulting in formation of compact structures called granulomas - a distinctive feature of TB (Cosma *et al.,* 2003). Tuberculous granulomas in human and non-human models have been shown to be extensively hypoxic, though this is not true of all granulomas (Belton *et al.,* 2016; Via *et al.,* 2008). Research into TB has revealed that the hypoxic environment of granulomas is correlated with a higher resistance of Mtb and its response to host and pharmacological treatment (Boshoff and Barry, 2005; Via *et al.,* 2008). According to the classical model, granulomas were considered to play a protective role for the host by limiting bacterial dissemination (Ulrichs and Kaufmann, 2006). These assumptions were supported by results from animal models where inhibited growth of bacteria correlated with activation of adaptive immunity (Saunders and Cooper, 2000; Swaim *et al.,* 2006). In fact, granulomas have been shown to form far earlier than secondary adaptive immune responses emerged (Davis *et al.,* 2002). More recent evidence suggests that they act to shelter the bacteria from host immunity to allow them to survive in a latent form until an immune compromise situation (Davis and Ramakrishnan, 2009). Furthermore, macrophages together with dendritic cells play an essential roles in bacteria ability to establish infection in distant tissues (Clay *et al.,* 2007; Dannenberg, 2003; Wolf *et al.,* 2007). However, neutrophils are also

involved in the granuloma formation and their contribution in controlling mycobacterial infection seems to be underestimated.

Neutrophils are the most abundant type of granulocytes circulating in blood and their beneficial roles during the early stages of Mtb infection have been recently reviewed (Kroon *et al.,* 2018; Lyadova, 2017). Yet, regardless of host-protective roles within granuloma, neutrophils are the subject of controversy mainly due to their contribution to excessive inflammation and subsequent lung destruction (Dallenga *et al.,* 2017). During Mtb infection neutrophils arrive in two different waves and their functions vary depending on presence of adaptive immunity. Neutrophils present in emerging granulomas use oxidative killing and eliminate mycobacteria phagocytosed by macrophages, whereas the second-wave neutrophils are thought to be responsible for regulation of T-cells-dependent immunity within granuloma (Lombard *et al.,* 2016; Yang *et al.,* 2012). Indeed, neutrophils are the most frequently cell types within granulomas (Dorhoi and Kaufmann, 2015). These observations are in line with a very high numbers of neutrophils observed in *in vivo* studies using TB-susceptible animals with developed hypoxic necrotising granulomas (Mattila *et al.,* 2015; Turner *et al.,* 2003). Recently, in a guinea pig model, neutrophils and S100A9 inflammatory protein have been shown to play a key role in regulation of granuloma formation. $S100A9^{+}$ neutrophils were observed in granulomas of human Mtb-infected patients highlighting a potential mechanism by which neutrophils influence the granuloma (Yoshioka *et al.,* 2016). However, despite the fact that neutrophils are well-established immune cells present in the nascent granuloma, in oxygen-deprived conditions their functions are changed and the impact of hypoxia on neutrophil behaviour need to be further investigated.

Hypoxia and myeloid cells are intimately related, with HIF playing important roles in the phenotypes of macrophages and neutrophils. Loss of HIF-1α impairs the capacity of myeloid cells to eliminate bacteria, diminishing their aggregation and motility. Proinflammatory phenotypes of macrophages are tightly correlated with HIF-1α activity (Mills *et al.,* 2016; Tannahill *et al.,* 2013). Additionally, macrophages lacking in HIF-1α showed abolished TNF-α response to LPS during hypoxia (Cramer *et al.,* 2003). Hif-1α has been shown to control macrophage inflammatory response and promote their ability to phagocytose bacteria (Fensterheim *et al.,* 2017; Peyssonnaux *et al.,* 2005). Recent studies revealed that nitric oxide (NO) modulates

macrophage response to pathogen through activation of Hif-1α and repression of NF-κB (Braverman and Stanley, 2017).

Neutrophils are prolific phagocytic cells and first responders during mycobacterial infection, alongside macrophages. Although the roles of HIF-1α in neutrophils in TB is not as well characterised as the role of macrophages, numerous studies demonstrate the importance of this transcription factor in regulating key neutrophil functions and mounting immune responses to inflammatory and infectious stimuli (Thompson *et al.,* 2013). Neutrophils are short-lived effector cells and after activation undergo apoptosis which minimises the potential for damage of the host tissue (Walmsley *et al.,* 2005). However, neutrophil life span is prolonged in oxygen-deprived conditions (Ong *et al.,* 2018). As shown in *in vitro* and *in vivo* studies this extended life span might be associated with PHD3 expression in response to hypoxia and inflammatory stimuli (Walmsley *et al.,* 2011). Studies on human neutrophils incubated in hypoxic conditions or treated with hypoxia mimetics (which stabilise HIF-1α) show that this leads to NF-κB activation and autocrine secretion of the neutrophil survival factor MIP-1β; both of these processes contribute to prolonged neutrophil survival (Walmsley *et al.,* 2005). HIF-1α has been shown to play an essential role against bacteria through forming neutrophil extracellular traps (NETs) (Brinkmann *et al.,* 2004; McInturff *et al.,* 2012). In *ex vivo* studies Völlger at al showed that HIF-1α stabilisation promotes NETs formation in human-blood derived neutrophils (Völlger *et al.,* 2016). The correlation between HIF-1α and neutrophil capacity to limit mycobacterial infection has been demonstrated using zebrafish model. Hif-1α stabilisation in neutrophils during the early stages of infection resulted in bacteria eradication via iNOS which led to increased reactive nitrogen species (RNS) (Elks *et al.,* 2013, 2015; Ogryzko *et al.,* 2019). However, the major obstacle in understanding HIF-1α function in neutrophils is lack of neutrophil-specific HIF-1α deletion methods. In order to overcome this limitations and avoid ablation of HIF-1α in the entire myeloid compartment some approaches from murine model can be adapted. As demonstrated, modulation of neutrophil-specific locus Ly6G with a knock-in allele expressing Cre recombinase enabled to generate mice with neutrophil-specific HIF-1α deletion (Hasenberg *et al.,* 2015).

The high homology to numerous human genes, conserved hypoxia inducible factor (HIF - a key regulator of hypoxic signalling) pathway components, and innate immune cells able to respond to hypoxic stimuli, make zebrafish an exciting model to investigate the roles of neutrophils in the host hypoxia response to infections (Renshaw and Trede, 2012; van der Vaart *et al.,* 2012).

Additionally, the activity of macrophages and neutrophils in zebrafish larvae can be investigated without the impact of adaptive immune response which develops later (2 to 3 weeks post fertilisation) (Lam *et al.,* 2004). Zebrafish are highly amenable to genetic manipulation using techniques such as morpholino oligonucleotide knockdown and CRISPR/Cas9 (Bedell *et al.,* 2011; Hruscha *et al.,* 2013; Hwang *et al.,* 2013). In combination with a caseating granuloma formation process and real-time live imaging techniques, zebrafish seems to be a perfectly tailored model to study the role of HIF-1α in neutrophils during Mtb infection.

5.2 Hypotheses and aims

I hypothesise that neutrophils with stabilised Hif-1α can control *Mycobacterium marinum* (Mm) via nitric oxide in zebrafish larvae.

The aims of experiments presented in this chapter are:

- To determine the roles of neutrophils in infection control after Hif-1α modulation during early Mm infection
    - To characterise the effect of Hif-1α stabilisation on bacterial burden in neutrophil- and macrophage-depleted zebrafish
    - To evaluate the host-protective effect of neutrophil nitric oxide after Hif-1α stabilisation in macrophage-depleted zebrafish
- To determine the effect of Hif-1α modulation on neutrophils at the granuloma formation stage
    - To quantify the neutrophil numbers with internalised Mm within granulomas after Hif-1α stabilisation in macrophage-depleted zebrafish
    - To evaluate neutrophil tyrosine nitration levels within granulomas after Hif-1α stabilisation in zebrafish

5.3 Results

5.3.1 Hif-1α stabilisation is not protective when neutrophils are depleted

After confirming that both neutrophils and macrophages are important to control *Mycobacterium marinum* (Mm) during the early stages of infection (Chapter 4) I investigated whether the protective effect of Hif-1α stabilisation is due to neutrophils. Previous work from the lab has demonstrated that Hif-1α is protective via neutrophil nitric oxide production (Elks *et al.,* 2013), therefore I hypothesised that neutrophil depletion would abrogate the protective effect of Hif-1α stabilisation and result in uncontrolled infection.

Following co-injections with the dominant active (DA) Hif-1α (Elks *et al.,* 2013) and a morpholino against *csf3r*, *nacre* zebrafish larvae were infected into the caudal vein at the posterior blood island with 100CFU at 30hpf. Bacterial burden levels in 4dpi infected neutrophil-depleted embryos were assessed using dedicated pixel counting software (Figure 5.1).

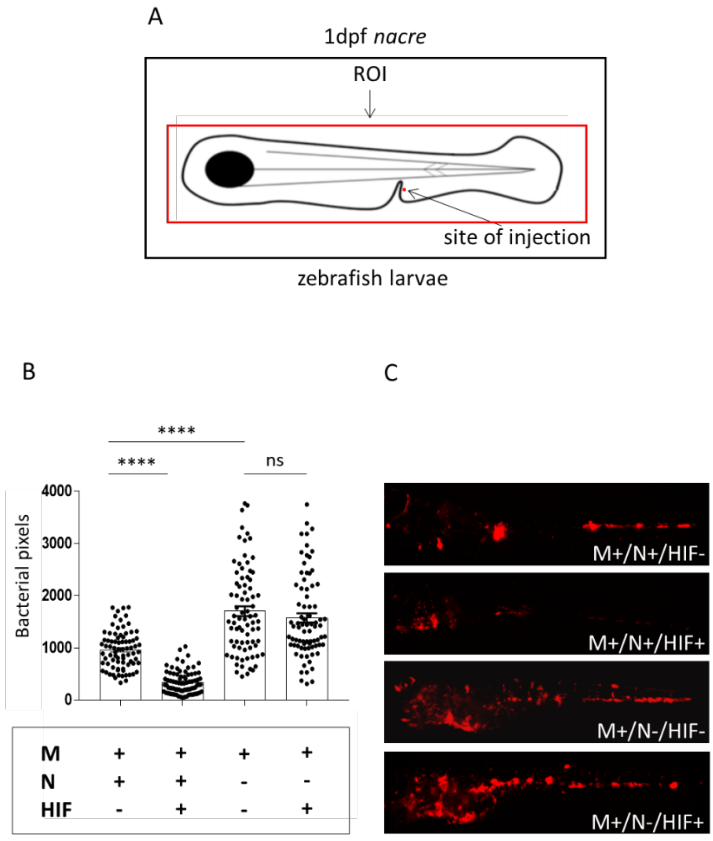


Figure 5.1 Hif-1α protection is lost when neutrophils are depleted

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of pixel count quantified in control infection group (M+/N+/HIF-), Hif-1α-stabilised infection group (M+/N+/HIF+), neutrophil-depleted control infection group (M+/N-/HIF-) and neutrophil-depleted/Hif-1α stabilised group (M+/N-/HIF+). MO injected fish (0hpf) were infected (30hpf) and imaged 5dpf (4dpi). The graph labels: Hif-1α-stabilisation/control (HIF+/HIF-), unchanged macrophage numbers (M+) and unchanged/depleted neutrophil numbers (N+/N-). Data are shown as mean +/- SEM, n=79 as accumulated from 3 independent experiments. Hif-1α-stabilised infection group has significantly lower levels of bacterial burden when compared with control infection group. No significant difference was observed between neutrophil-depleted control infection and neutrophil-depleted/Hif-1α stabilised groups (One-way ANOVA with Bonferroni multiple comparison test; ns-p>0.33, ****-p<0.0001.). (C) Example fluorescence micrographs of data shown in (B).

Quantification of mycobacterial infection in zebrafish showed that Hif-1α stabilisation (HIF+) reduced bacterial burden when compared with control group (M+/N+/HIF-). Csf3r morpholino injections knockdown granulocyte colony-stimulating factor receptor (GCS3R) which is prerequisite to maintain regular neutrophil numbers during granulopoiesis. Decreasing the number of neutrophils by csfr3 morpholino led to an increased bacterial burden (Figure 5.1). In control morpholino injected larvae, Hif-1α stabilisation led to a significant decrease in bacterial

burden. However, when Hif-1α was stabilised with neutrophils depleted by csf3r morpholino then bacterial burden remain as high as phenol red controls (Figure 5.1B-C). It is worth noting that a high variance of bacterial pixels might be due to incomplete knock down of *csfr3*. Hif-1α stabilisation was not protective for the host when neutrophil numbers were depleted indicating an important role of these cells during the early stages of infection.

5.3.2 Neutrophils activated with Hif-1α are protective against *Mycobacterium marinum* in macrophage-depleted zebrafish

After confirming that Hif-1α stabilisation in neutrophil-depleted embryos is not protective for the host I planned to investigate the role of macrophages during the early stages of infection. In order to manipulate leukocyte numbers, I used clodronate liposomes to deplete macrophage numbers and *irf8* morpholino (irf8 MO) to increase neutrophil numbers at the expense of macrophages.

Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) *nacre* zebrafish larvae were injected into the caudal vein at the posterior blood island at 24hpf to deplete number of macrophages. Alternatively, fish were co-injected with dominant active (DA) Hif-1α and a morpholino against *irf8.* Fish were infected with Mm into the caudal vein at the posterior blood island with 100CFU at 30hpf. Bacterial burden levels in 4dpi infected macrophage-depleted embryos were assessed using dedicated pixel counting software (Figure 5.2).

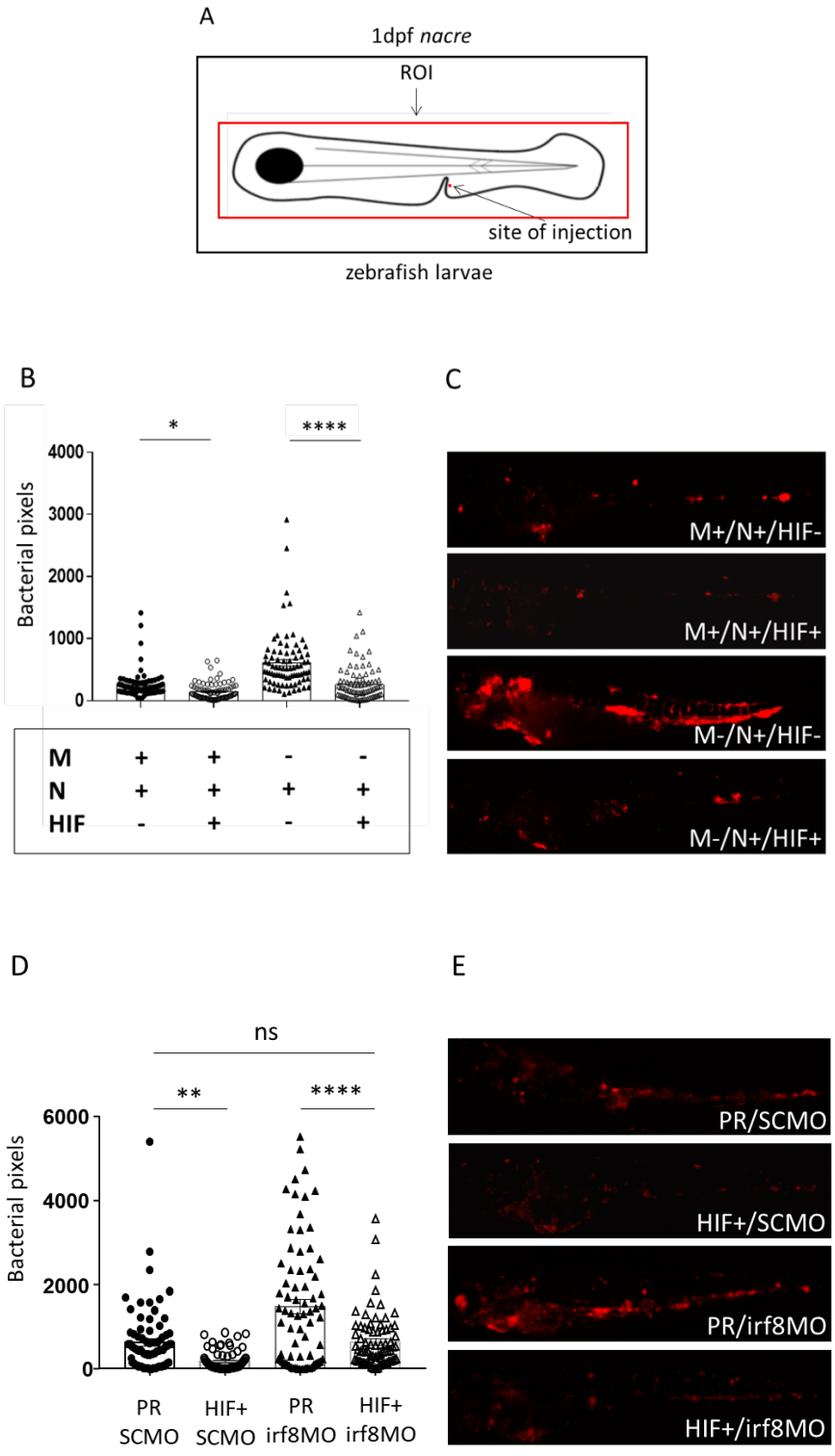

A
1dpf nacre
ROI
site of injection
zebrafish larvae
B
*
****
Bacterial pixels
4000
3000
2000
1000
0
M + + - -
N + + + +
HIF - + - +
C
M+/N+/HIF-
M+/N+/HIF+
M-/N+/HIF-
M-/N+/HIF+
D
ns
**
****
Bacterial pixels
6000
4000
2000
0
PR SCMO
HIF+ SCMO
PR irf8MO
HIF+ irf8MO
E
PR/SCMO
HIF+/SCMO
PR/irf8MO
HIF+/irf8MO

Figure 5.2 Hif-1α remains host protective even when macrophages are depleted

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). The graph summarises the comparison of pixel count quantified in control infection group (M+/N+/HIF-), Hif-1α-stabilised infection group (M+/N+/HIF+), macrophage-depleted control infection group (M-/N+/HIF-) and macrophage-depleted/Hif-1α stabilised group (M-/N+/HIF+). Clodronate liposome-injected fish and controls (24hpf) were infected (30hpf) and imaged 5dpf (4dpi). The graph labels: Hif-1α-stabilisation/control (HIF+/HIF-), unchanged/depleted macrophage numbers (M+/M-) and unchanged neutrophil numbers (N+). Data are shown as mean +/- SEM, n=81 as accumulated from 3 independent experiments (One-way ANOVA with Bonferroni multiple comparison test; *-p<0.039, ****-p<0.0001). Hif-1α-stabilised infection group has significantly lower levels of bacterial burden when compared with control infection group. Macrophage-depleted/Hif-1α stabilised group showed reduced levels of bacterial burden when compared with macrophage-depleted control infection group. (C) Example fluorescence micrographs of data shown in (B). (D) The graph summarises the comparison of pixel count quantified in phenol red control morpholino infection group (PR/SCMO), Hif-1α-stabilised control morpholino infection group (HIF+/SCMO), phenol red neutrophil-increased infection group (PR/irf8MO) and Hif-1α-stabilised neutrophil-increased infection group (HIF+/irf8MO). HIF+/irf8 injected fish and controls (0hpf) were infected (30hpf) and imaged 5dpf (4dpi). Data are shown as mean +/- SEM, n=75 as accumulated from 3 independent experiments (One-way ANOVA with Bonferroni multiple comparison test; **-p<0.0041, ****-p<0.0001). Hif-1α-stabilised control morpholino infection group (HIF+/SCMO) has significantly lower levels of bacterial burden when compared with phenol red control morpholino infection group (PR/SCMO). Hif-1α-stabilised neutrophil-increased infection group (HIF+/irf8MO) showed reduced levels of bacterial burden when compared with phenol red neutrophil-increased infection group (PR/irf8MO). (E) Example fluorescence micrographs of data shown in (D).

Clodronate-liposomes administration results in macrophage depletion as lipid vesicles containing clodronate are phagocytosed, degraded and released clodronate triggers macrophage apoptosis (Figure 5.2). Quantification of bacterial burden showed that Hif-1α stabilisation (M+/N+/HIF+) led to decreased bacterial burden when compared with control group (M+/N+/HIF-). Hif-1α stabilisation in macrophage-depleted larvae (M-/N+/HIF+) remained protective for the host when compared with macrophage-depleted larvae control group (M-/N+/HIF-) (Figure 5.2B-C). Despite bacterial burden not decreasing as low as levels in Hif-stabilised larvae when macrophages are present, the reduction observed in HIF larvae without macrophages is as low as the control situation when macrophages are present. These data indicate that Hif-1α stabilisation can compensate for reduced numbers of macrophages during early stages of mycobacterial infection.

Irf8 morpholino injections upregulate neutrophil numbers at the expense of macrophages, as *irf8* is a regulatory factor responsible for causing differentiation into macrophages from the shared myeloid precursor of both macrophages and neutrophils (Figure 5.2). Bacterial burden analysis showed that in the absence of HIF manipulation (PR groups), *irf8* MO increased infection due to the absence of macrophages. This data confirms the crucial role of macrophages in the protection against Mm and demonstrates that increased numbers of neutrophils are not sufficient to control infection. However, when HIF was stabilised (HIF+) the bacterial burden was significantly lower in the HIF+/irf8MO group compared to the PR/irf8MO group, demonstrating that HIF stabilisation remains protective in the absence of macrophages with increased neutrophil numbers (Figure 5.2D-E).

5.3.3 Neutrophil nitric oxide protects the host in macrophage-depleted larvae after Hif-1α stabilisation

In order to investigate whether effect of Hif-1α stabilisation in macrophage-deprived zebrafish is NO dependent I used an inducible nitric oxide synthase (iNOS) selective inhibitor (L-NIL) which is indispensable for NO production, predominantly present in neutrophils. Based on previous results (Figure 5.2) I hypothesise that, in macrophage-depleted larvae, neutrophil nitric oxide (NO) is required for the protective effect of Hif-1α stabilisation and this protection would be abolished after blocking iNOS.

To determine the role of NO in host protection, *nacre* zebrafish larvae were injected with dominant active (DA) Hif-1α. Following Hif-1α stabilisation zebrafish larvae were injected with clodronate liposomes into the caudal vein at the posterior blood island at 24hpf to deplete number of macrophages. 4 hours before Mm infection L-NIL was added to the embryo water. Next, fish were infected into the caudal vein at the posterior blood island at 30hpf. 24 hours post Mm infection the inhibitor was washed off and bacterial burden levels in 4dpi infected were assessed using dedicated pixel counting software (Figure 5.3).

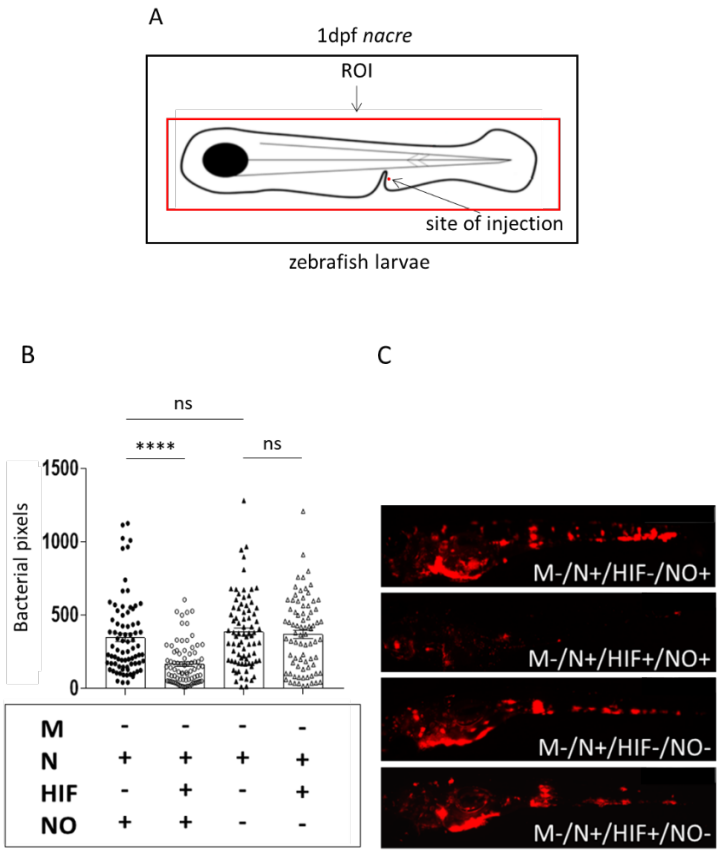


Figure 5.3 Hif-1α protection without macrophages is NO dependent

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of bacterial burden quantified in macrophage-depleted control infection group (M-/N+/HIF-/NO+), macrophage depleted/Hif-1α-stabilised infection group (M-/N+/HIF+/NO+), macrophage-depleted/NO deprived control infection group (M-/N+/HIF-/NO-) and macrophage-depleted/Hif-1α stabilised/NO deprived group (M-/N+/HIF+/NO-). Clodronate liposome-injected fish (24hpf) were infected (30hpf) and imaged 5dpf (4dpi). The graph labels: Hif-1α-stabilisation/control (HIF+/HIF-), depleted macrophage numbers (M-), unchanged neutrophil numbers (N+) and unchanged/deprived nitric oxide (NO+/NO-). Data are shown as mean +/- SEM, n=82 as accumulated from 3 independent experiments. Macrophage-depleted/Hif-1α-stabilised infection group has significantly lower levels of bacterial burden when compared with macrophage-depleted control infection group. No significant difference was observed between macrophage-depleted/NO deprived control infection and macrophage-depleted/Hif-1α stabilised/NO deprived groups (One-way ANOVA with Bonferroni multiple comparison test; ns-$p>0.99$, ****-$p<0.0001$.). (C) Example fluorescence micrographs of data shown in (B).

Inducible nitric oxide synthase (iNOS) is an enzyme essential for NO production and was blocked using a selective iNOS inhibitor N6-(1-iminoethyl)-L-lysine (L-NIL). Quantification of bacterial burden corroborated previous findings (Chapter 5.3.2) that Hif-1α stabilisation remained protective for the host even in macrophage-depleted group (M-) (Figure 5.3). Without Hif-1α stabilisation blocking NO (M-/N+/HIF-/NO-) has no effect on bacterial burden when compared

with control infection group (M-/N+/HIF-/NO+). These data demonstrate that NO is required for the protection as using L-NIL (NO-) abrogated protective effect of Hif-1α stabilisation (M-/N+/HIF+/NO-) resulting in bacterial burden comparable to levels observed in control group (M-/N+/HIF-/NO-) (Figure 5.3B-C). These results highlight the importance of NO in Hif-1α-dependent protection and indicate that in the absence of macrophages nitric oxide is responsible for restricting bacterial growth during the early stages of infection.

5.3.4 Nitric oxide level is decreased after CRISPR targeting the ATG of *nos2ab* gene

In order to understand the role of inducible nitric oxide synthase (iNOS) in neutrophils I planned to use a system based on the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated proteins (Cas) to knockdown *nos2*, zebrafish gene of iNOS. *Nos2* competes with arginase for L-arginine which is a substrate essential for NO production.

It has been previously demonstrated that tyrosine nitration levels are elevated in neutrophils following Mm infection and that this response could be inhibited by a morpholino against *nos2a*, one of the zebrafish genes for iNOS (Elks *et al.,* 2013). CRISPR/Cas system has been shown to be an effective gene knockout tool in zebrafish (Cade *et al., 2012;* Hwang *et al.,* 2013) and has been used to knockdown genes in F0 injected larvae (termed CRISPants) (Isles *et al.,* 2019). I hypothesised that CRISPant *nos2ab* knockdown would reduce nitric oxide level in zebrafish.

Following injections with single guide RNA (sgRNA) targeting ATG at single cell stage 5dpf *nacre* larvae were fixed and nitrotyrosine levels were immune-labelled using a rabbit polyclonal anti-nitrotyrosine antibody (Elks *et al.,* 2013). After staining fish were imaged in caudal hematopoietic tissue (CHT), the site of embryonic hematopoietic stem and progenitor cells (HSPC) expansion in zebrafish (Wolf *et al.,* 2017) (Figure 5.4).

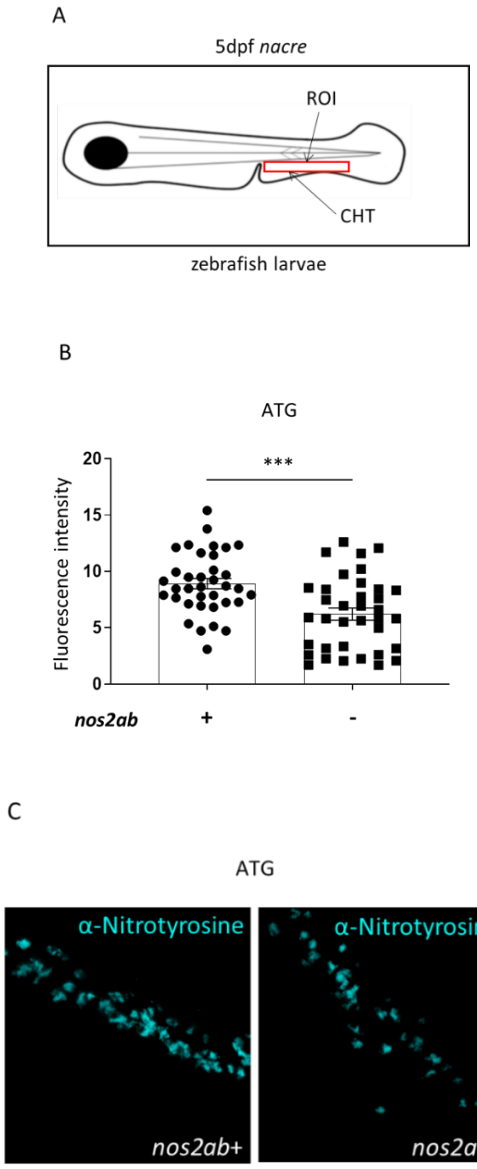


Figure 5.4 CRISPant ATG *nos2ab* knockdown modestly reduced the nitric oxide level in zebrafish

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame). (B) The graph summarises the comparison of nitric oxide levels in zebrafish CHT after targeting ATG of both forms *nos2* gene using CRISPR/Cas system. At single cell stage (0hpf) fish were injected with active Cas9 only (*nos2ab*+) or single guide RNA (sgRNA) targeting ATG/Cas9 (*nos2ab*-). Following injections fish were fixed, nitrotyrosine levels were immune-labelled (5dpf) and imaged. Three repeats were performed and two groups were used: (*nos2ab*+; n=36) and (*nos2ab*-; n=36). The results obtained from CRISPR experiments showed that nitric oxide level is decreased after targeting ATG *nos2ab (nos2ab*-) when compared with control group (*nos2ab*+) (Unpaired T test; ***-p<0.0003). (C) Example fluorescence confocal z-stacks of the caudal vein region of embryos stained with Alexa-633 labelled anti-nitrotyrosine antibody (B). Representative images of nitric oxide (cyan) in CHT at 5dpf. Images of zebrafish CHT taken using confocal microscope Leica DMi8 SPE TCS.

In order to silence *nos2*, which is zebrafish gene of inducible nitric oxide synthase (iNOS) 2 guides RNAs per gene were designed. Fluorescent intensity analysis of anti-nitrotyrosine labelled larvae demonstrated that targeting ATG of *nos2ab* (nos2ab-) reduced nitric oxide level in zebrafish CHT and comparison with control group (nos2ab+) showed that decrease was statistically significant ($p<0.0003$) (Figure 5.4B-C). CRISPants with guides targeting the ATG were not sufficient in completely depleting NO levels, and so alternative guides sites to target were investigated.

5.3.5 Neutrophil nitric oxide production is abolished after *nos2ab* CRISPant knockdown of exon 1

Targeting ATG of *nos2* gene using CRISPR/Cas system reduced level of nitric oxide in CHT. Nonetheless, this method is not sufficient to knockdown NO production completely as nitrotyrosine levels are not completely ablated. In order to test CRISPR/Cas system further I plan to use alternative sgRNA to target exon 1 of *nos2* and then verify whether a, b or both forms need to be targeted to successfully reduce NO production to basal levels. I hypothesised that *nos2* knockdown targeting exon 1 would result in gene disruption and impaired nitric oxide production in neutrophils.

Following injections with single guide RNA (sgRNA) targeting exon 1 at single cell stage 5dpf *Tg(mpx:GFP)*^{i114} larvae were fixed and nitrotyrosine levels were immune-labelled using a rabbit polyclonal anti-nitrotyrosine antibody (Elks *et al.,* 2013). After staining, fish were imaged in caudal hematopoietic tissue (CHT) (Wolf *et al.,* 2017) (Figure 5.5).

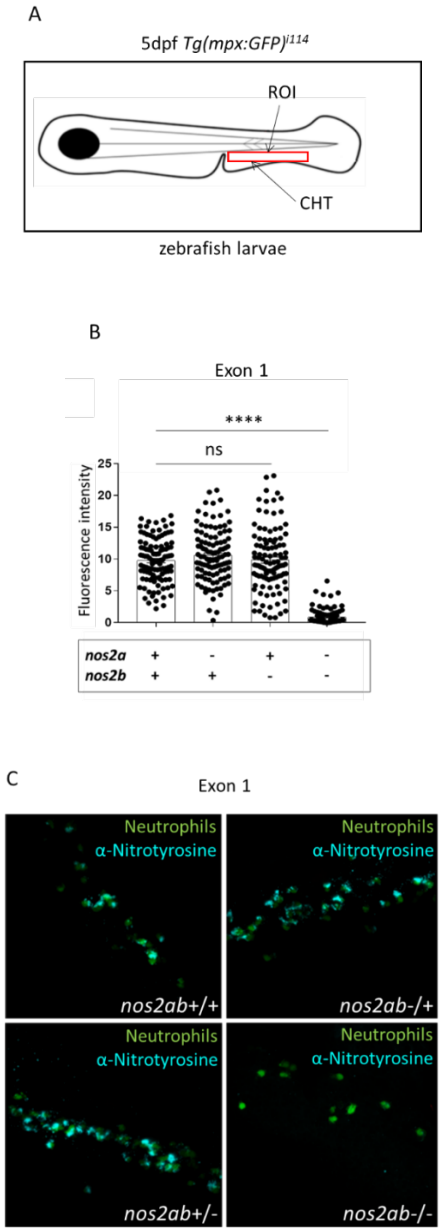


Figure 5.5 Targeting exon 1 of both forms of *nos2* gene impaired nitric oxide level in zebrafish

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame). (B) The graph summarises the comparison of nitric oxide levels in zebrafish CHT after targeting exon 1 of *nos2a* (*nos2ab*-/+), *nos2b* (*nos2ab*+/-), *nos2ab* (nos2-/-) and control group (nos2+/+) using CRISPR/Cas system. At single cell stage (0hpf) fish were injected with active Cas9 only (*nos2ab*+) or single guide RNA (sgRNA) targeting ATG/Cas9 (*nos2ab*-). Following injections fish were fixed, nitrotyrosine levels were immune-labelled (5dpf) and imaged. Data are shown as mean +/- SEM, n=108 as accumulated from 3 independent experiments. The results obtained from CRISPR experiments showed that neutrophil nitric oxide production is impaired only after targeting exon 1 of a and b forms of *nos2* gene (nos2-/-/HIF+) (One-way ANOVA with Bonferroni multiple comparison test; ns-p>0.27; ****-p<0.0001). (C) Example fluorescence micrographs of data shown in (B). Representative images of nitric oxide (cyan) in neutrophils (green) present in CHT at 5dpf. Images of zebrafish muscle taken using confocal microscope Leica DMi8 SPE TCS.

As a result of genome duplication in zebrafish two *nos2* genes are present (*nos2a* and *nos2b*) and each of them exhibits different expression during development with shared gene expression predominantly observed in response to proinflammatory cytokines and bacterial compounds. Quantification of fluorescent intensity of anti-nitrotyrosine antibody staining demonstrated that targeting exon 1 of *nos2* gene successfully impaired neutrophil nitric oxide production in zebrafish (Figure 5.5). Interestingly, analysis of nitrotyrosine levels show that CRISPant knockdown is efficient in decreasing nitric oxide levels to basal levels, but only when both a and b forms of *nos2* gene are targeted ($p<0.0001$ when compared with both control group *nos2+/+* and using guides targeting exclusively one isoform of *nos2*: *nos2ab-/+* and *nos2ab+/-*) (Figure 5.5B-C). These data demonstrate loss of nitrotyrosine signal (cyan) suggesting that nos2 knockdown efficiently impaired NO production.

5.3.6 Hif-1α stabilisation is not protective for the host after CRISPR targeting exon 1 of *nos2ab* gene

Hif-1α stabilisation leads to host protection during the early stages of infection and this phenomenon is iNOS-dependent (Elks *et al.,* 2013). In order to verify whether host protection associated with Hif-1α stabilisation and resulting increased NO production is lost I targeted exon 1 of *nos2ab* simultaneously and used bacterial burden as a readout of systemic infection. I hypothesise that targeting exon 1 of a and b forms of *nos2* would result in increased severity of systemic infection in zebrafish.

Following injections/co-injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) and single guide RNA (sgRNA) targeting exon 1 at single cell stage *Tg(mpx:GFP)*^{i114} zebrafish larvae were infected into the caudal vein at the posterior blood island with 100CFU at 30hpf. Bacterial burden levels in 4dpi infected embryos were assessed using dedicated pixel counting software (Figure 5.6).

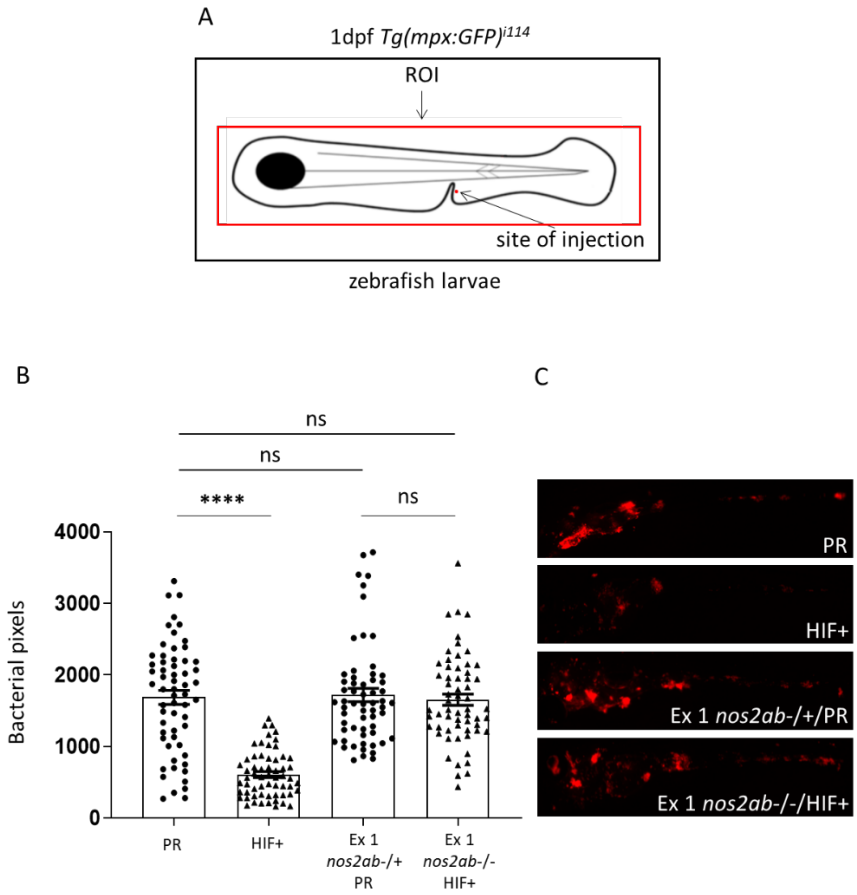


Figure 5.6 Hif-1α stabilisation is not protective in CRISPants targeting exon 1 of *nos2ab*

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of pixel count quantified in control infection group (PR), Hif-1α-stabilised infection group (HIF+), *nos2ab* control infection group (Ex 1 *nos2ab*-/+/PR) and Hif-1α-stabilised *nos2ab*-targeted infection group (Ex 1 *nos2ab*-/-/HIF+). At single cell stage (0hpf) fish were injected with phenol red (PR), dominant active (DA) variant of Hif-1α only (HIF+), active Cas9 only (Ex 1 *nos2ab*+/+) or co-injected with dominant active (DA) variant of Hif-1α/single guide RNA (sgRNA) targeting exon 1 *nos2ab* (Ex 1 *nos2ab*-/-/HIF+). Following injections zebrafish larvae were infected into the caudal vein at the posterior blood island at 30hpf. Data are shown as mean +/- SEM, n=60 as accumulated from 3 independent experiments. The results obtained from CRISPR/infection experiments showed that Hif-1α stabilisation is not protective for the host after targeting exon 1 of *nos2ab* (One-way ANOVA with Bonferroni multiple comparison test; ns-p>0.81; ****-p<0.0001). (C) Example fluorescence micrographs of data shown in (B). Images of zebrafish muscle taken using confocal microscope Leica DMi8 SPE TCS.

Targeting exon 1 of both a and b forms of *nos2* gene using CRISPR/Cas system results in gene disruption (Chapter 5.3.5). Indeed, antibody detection of nitrotyrosine confirmed that *nos2ab* knockdown significantly reduced NO levels. As previously corroborated (Chapter 5.3.3) Hif-1α stabilisation leads to host protection during the early stages of infection and this phenomenon

is iNOS-dependent. Bacterial burden analysis showed that Hif-1α stabilisation reduced bacterial burden and the difference between bacterial pixel counts were statistically significant ($p<0.0001$). Comparison of mycobacterial infection after *nos2* gene disruption demonstrated that *nos2* control group (Ex 1 *nos2ab*-/+/PR) has equivalent bacterial burden ($p>0.81$) with control infection group (PR) (Figure 5.6B-C). Quantification of bacterial burden shows that disruption of the *nos2* gene (Ex 1 *nos2ab*-/-/HIF+) resulted in abrogated Hif-1α protection observed in Hif-1α-stabilised infection group (HIF+). This data confirms that the host beneficial effect of HIF activation and NO-dependent protection is lost when *nos2* and NO production is knocked down.

5.3.7 Hif-1α stabilisation does not affect neutrophil numbers recruited to a muscle somite infection during the first hours of *Mycobacterium marinum* infection

My data above (Chapter 5.3.1-5.3.3) show that in macrophage-depleted larvae, neutrophils are able to control bacteria when there is stabilised Hif-1α. However, NO may not be the only defense mechanism that is altered by HIF stabilisation. To address whether HIF stabilisation affects neutrophil migration to the infection site I needed to move away from a systemic model, and switch to a local infection model. To investigate whether Hif-1α stabilisation has an effect on the neutrophil recruitment to mycobacterial infection I adapted a previously established muscle infection model (Oehlers *et al.,* 2015).

Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) *Tg(mpx:GFP)*^{i114} zebrafish larvae were injected into the muscle somite with 200CFU at 2dpf as was previously established (Oehlers *et al.,* 2015) from a glycerol stock aliquot. At 2hpi, neutrophils present in the muscle somite were counted and the same imaging procedure was repeated 4hpi and 6hpi (Figure 5.7).

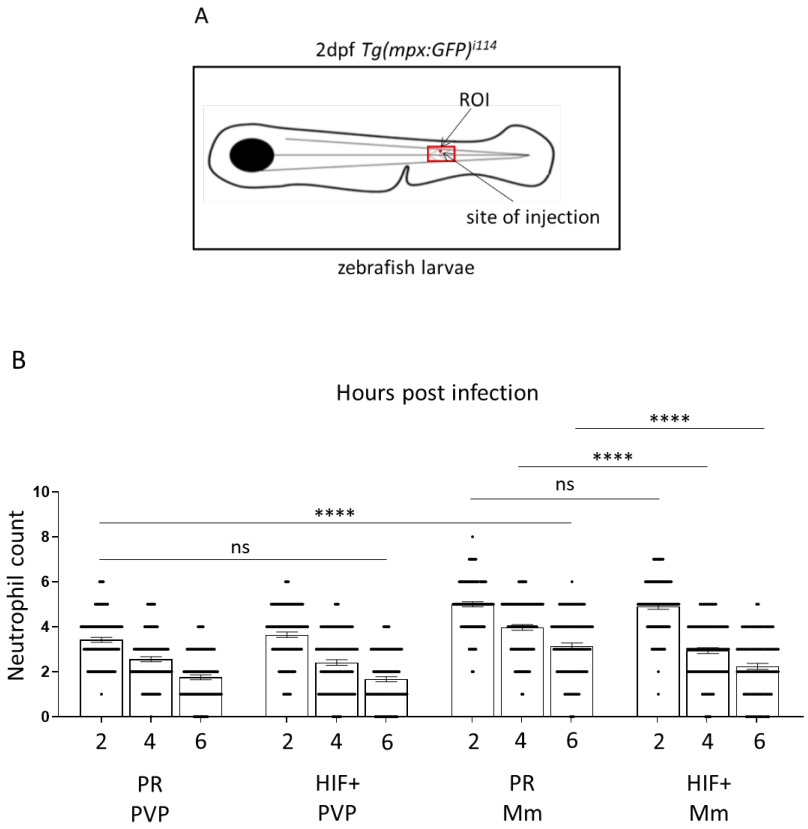


Figure 5.7 Hif-1α stabilisation does not change neutrophil numbers recruited to a muscle infection model

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of neutrophil recruitment to muscle somite quantified in phenol red PVP control injection group (PR/PVP), Hif-1α-stabilised control injection group (HIF+/PVP), control infection group (PR/Mm) and Hif-1α-stabilised infection group (HIF+/Mm) during the first 6 hours post infection. Local injections with Mm were performed at 2dpf and neutrophil counts were carried out manually in the region of interest 2hpi, 4hpi 6hpi using a fluorescent stereomicroscope Leica MZ10F. Data are shown as mean +/- SEM, n=102 as accumulated from 3 independent experiments. The results from neutrophil recruitment experiments showed that Hif-1α stabilisation have diversified effects on neutrophil numbers in muscle somite and observed differences vary depending on time when neutrophils were counted (One-way ANOVA with Bonferroni multiple comparison test ns-$p>0.9$; ****-$p<0.0001$).

Quantification of neutrophils present in muscle somite demonstrated that without infection (PR/PVP and HIF+/PVP groups) neutrophil numbers were similar (no significant difference at any time point) which suggest that Hif-1α stabilisation has no effect on neutrophil recruitment in the absence of bacteria (Figure 5.7B). Following Mm infection, neutrophil counts significantly increased at 2, 4 and 6hpi when compared with non-infected control group (PR/Mm vs PR/PVP). This data confirms that presence of bacteria enhanced neutrophil recruitment to local infection in muscle somite. Similarly, in Hif-1α stabilised groups (HIF+/PVP and HIF+/Mm) mycobacterial

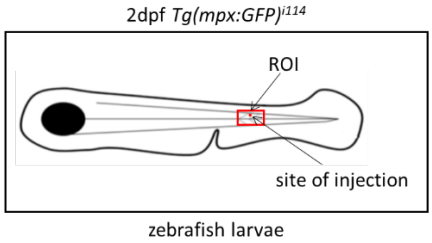
infection resulted in increased neutrophil numbers at each time point (2hpi $p<0.001$, 4hpi $p=0.023$ and 6hpi $p=0.010$). Quantification of neutrophils present in muscle somite after infection (HIF+/Mm and PR/Mm groups) showed that at 2hpi Hif-1α stabilisation has no effect on neutrophil numbers ($p>0.999$). As infection continued (4hpi and 6hpi) Hif-1α stabilisation modestly decreased neutrophil numbers ($p<0.0001$ for both time points), however this difference equates to less than a single neutrophil (4hpi; mean=3.97 vs 2.94; 6hpi; mean=3.14 vs 2.23 neutrophils) and so it remains unclear whether this difference would be biologically relevant or not.

5.3.8 The total number of neutrophils within granulomas was not affected by macrophage depletion or Hif-1α stabilisation

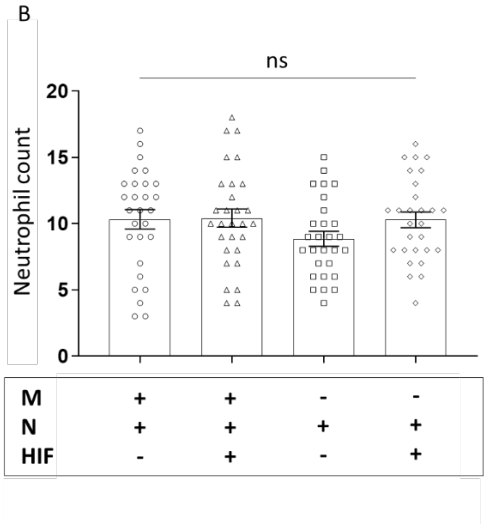
The granuloma is a complex, host-derived structure and a hallmark of TB. Although granuloma constitution is relatively well defined, the early stages of this process, as well as initial interactions of mycobacteria with macrophages and neutrophils less well understood. To determine whether Hif-1α stabilisation or macrophage depletion affects the total numbers of neutrophils within granulomas I used clodronate liposomes to deplete macrophage numbers.

Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) *Tg(mpx:GFP)*^{i114} zebrafish larvae were injected into the caudal vein at the posterior blood island at 24hpf to deplete number of macrophages with clodronate. Following macrophage depletion zebrafish larvae were injected into the muscle somite with 200CFU at 2dpf as was previously established (Oehlers *et al.,* 2015) from a glycerol stock aliquot. Fish were imaged at 5dpf and the acquired images were used to assess neutrophil number within granuloma (Figure 5.8).

A


2dpf Tg(mpx:GFP)i114
ROI
site of injection
zebrafish larvae

B


ns
Neutrophil count
20
15
10
5
0
M + + - -
N + + + +
HIF - + - +

C

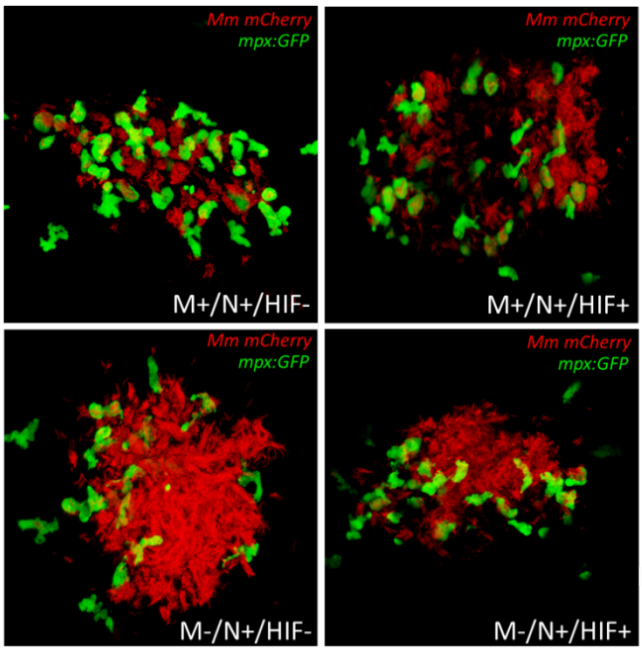

Mm mCherry
mpx:GFP
M+/N+/HIF-
M+/N+/HIF+
M-/N+/HIF-
M-/N+/HIF+

Figure 5.8 Hif-1α stabilisation and macrophage depletion do not affect neutrophil numbers in granulomas in a muscle infection model

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of neutrophil recruitment to granuloma in muscle somite quantified in control infection group (M+/N+/HIF-), Hif-1α-stabilised infection group (M+/N+/HIF+), macrophage-depleted control infection group (M-/N+/HIF-) and macrophage-depleted/Hif-1α stabilised group (M-/N+/HIF+). Clodronate liposome-injected fish (24hpf) were infected 2dpf and imaged 5dpf. The graph labels: Hif-1α-stabilisation/control (HIF+/HIF-), unchanged/depleted macrophage numbers (M+/M-) and unchanged neutrophil numbers (N+). Data are shown as mean +/- SEM, n=28 as accumulated from 3 independent experiments. All analysed groups have even number of neutrophils within granuloma (One-way ANOVA with Bonferroni multiple comparison test; ns-p>0.27). (C) Example fluorescence micrographs of data shown in (B). Representative images of neutrophils (green) in granulomas (red) in the muscle somite at 5dpf. Images of zebrafish muscle taken using confocal microscope Leica DMi8 SPE TCS.

Neutrophil counts showed that Hif-1α stabilisation (HIF+) has no effect on neutrophil numbers in granulomas when compared with control group (HIF-) (Figure 5.8). Likewise, quantification of neutrophils after macrophage depletion (M-) confirmed that absence of macrophages does not affect neutrophil numbers in granulomas. In macrophage-deprived and Hif-1α-stabilised zebrafish larvae (M-/N+/HIF+) the number of neutrophils also remained unchanged. These data demonstrate that neutrophils, regardless of Hif-1α stabilisation and macrophage depletion form part of the early granuloma with no statistically significant difference across all groups. Above results confirm that local infection model is accurate to investigate granuloma formation and assess the effect of Hif-1α stabilisation on neutrophils.

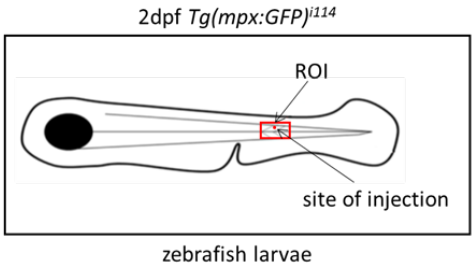
5.3.9 The proportion of neutrophils containing *Mycobacterium marinum* within granulomas is increased after Hif-1α stabilisation

Understanding the role of neutrophils in processes associated with granuloma formation is important to establish new host-derived strategies which enable restrict mycobacterial growth. To investigate whether Hif-1α stabilisation or macrophage depletion affects the amount of Mm contained within neutrophils within granulomas I planned to use clodronate liposomes.

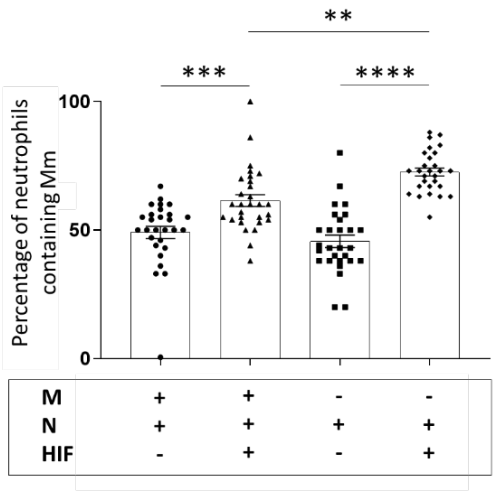
Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013), *Tg(mpx:GFP)*^{i114} zebrafish larvae were injected into the caudal vein at the posterior blood island at 24hpf to deplete number of macrophages. Following macrophage depletion zebrafish larvae

were injected into the muscle somite with 200CFU at 2dpf as was previously described (Chapter 5.3.8) from a glycerol stock aliquot. Fish were imaged at 5dpf and the acquired images were used to assess proportion of neutrophils with internalised Mm within granuloma (Figure 5.9).

A


2dpf Tg(mpx:GFP)i114
ROI
site of injection
zebrafish larvae

B


**
***
****
Percentage of neutrophils containing Mm
100
50
0
M + + - -
N + + + +
HIF - + - +

C

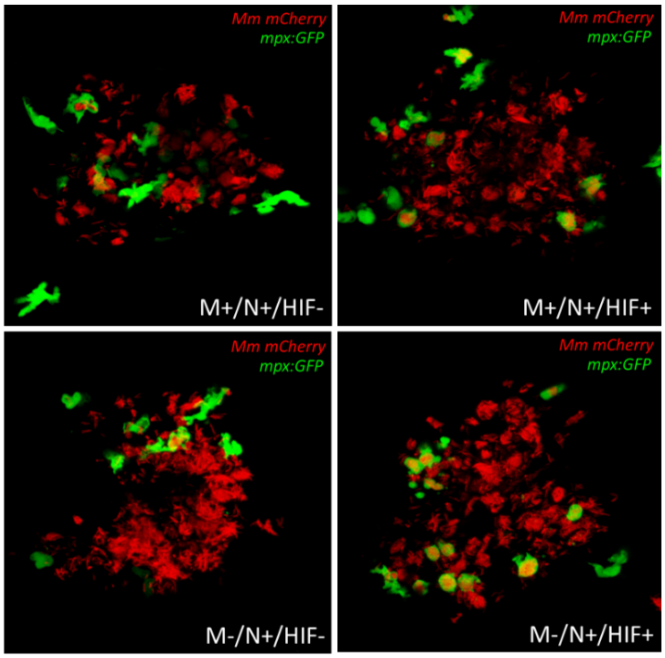

Mm mCherry
mpx:GFP
M+/N+/HIF-
M+/N+/HIF+
M-/N+/HIF-
M-/N+/HIF+

Figure 5.9 Hif-1α stabilisation increased the percentage of neutrophils with internalised *Mycobacterium marinum* within granulomas regardless of macrophage depletion

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of neutrophils containing Mm within granuloma in muscle somite quantified in control infection group (M+/N+/HIF-), Hif-1α-stabilised infection group (M+/N+/HIF+), macrophage-depleted control infection group (M-/N+/HIF-) and macrophage-depleted/Hif-1α stabilised group (M-/N+/HIF+). Clodronate liposome-injected fish (24hpf) were infected 2dpf and imaged 5dpf. The graph labels: Hif-1α-stabilisation/control (HIF+/HIF), unchanged/depleted macrophage numbers (M+/M-) and unchanged neutrophil numbers (N+). Data are shown as mean +/- SEM, n=28 as accumulated from 3 independent experiments. The results obtained from muscle infection experiments showed that Hif-1α stabilisation has increased neutrophil internalisation of Mm at 5dpf in both control and macrophage-depleted group (One-way ANOVA with Bonferroni multiple comparison test; **- $p<0.0016$; ***-$p<0.0006$; ****-$p<0.0001$). (C) Example fluorescence micrographs of data shown in (B). Representative images of neutrophils (green) recruited to granuloma (red) in the muscle somite at 5dpf. Images of zebrafish muscle taken using confocal microscope Leica DMi8 SPE TCS.

Neutrophils are prolific phagocytic cells and their ability to contain bacteria represents an essential mechanism limiting mycobacterial infection. Both macrophage depletion and Hif-1α stabilisation have been shown to have no effect on neutrophil numbers within granulomas (Figure 5.9). Quantification of neutrophils within granulomas showed that Hif-1α stabilisation (M+/N+/HIF+) increased the percentage of neutrophils containing Mm when compared with the control group (M+/N+/HIF-), which represents an important feature that might be advantageous for the host by restricting mycobacterial growth. This Hif-1α-associated neutrophil internalisation of Mm was also augmented after macrophage depletion (M-/N+/HIF+). However, Hif-1α stabilisation (HIF+) in macrophage-depleted zebrafish larvae (M-/N+/HIF+) resulted in an increased percentage of neutrophils containing Mm when compared with control group (M+/N+/HIF+). These data confirms that Hif-1α stabilisation increase neutrophil contained Mm and this process does require normal macrophage numbers. This suggest an important role of Hif-1α stabilisation on neutrophils and may indicates that HIF manipulation is favourable for the host during early granuloma stages.

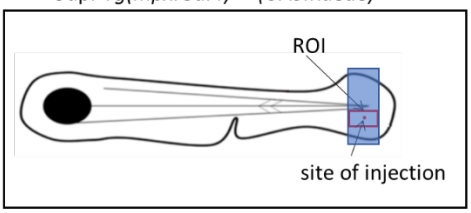
5.3.10 The number of neutrophils remains the same within tail fin granulomas after Hif-1α stabilisation

Neutrophils have been shown to be able to internalise Mm in the absence of macrophages and this process was more pronounced after Hif-1α stabilisation. To understand whether Hif-1α stabilisation changes the number of neutrophils within granuloma and has the effect on migration of new neutrophils to existing tail fin granuloma between day 4 and day 5 I used *Tg(mpx:Gal4)*^{i222}*(UAS:Kaede)*^{s1999t} (Robertson *et al.,* 2014) referred later as *mpx*:Kaede. This zebrafish transgenic line has neutrophils with photoconvertible fluorescent protein which changes colour from green to red after exposure to blue light (Dixon *et al.,* 2012).

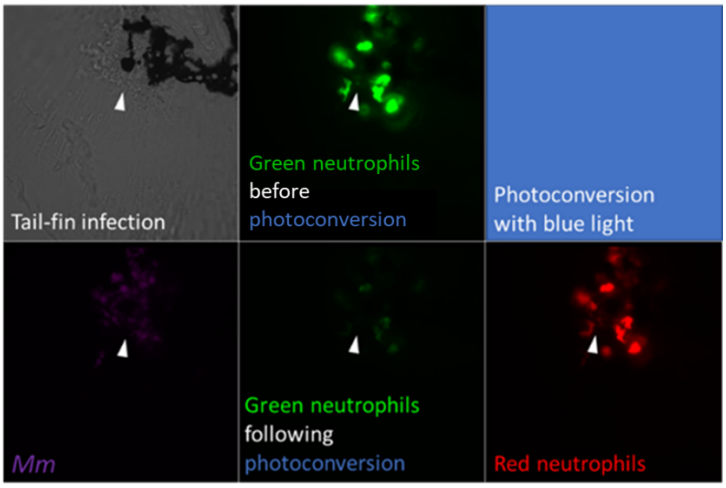
However, it would be technically challenging to exclusively photoconvert the muscle somite granuloma alone, without photoconverting neutrophils in the surrounding tissue. Previously it has been demonstrated that the tail fin infection model allows tracking of phagocytic cells during granuloma formation (Hosseini *et al.,* 2016). As an alternative I planned to use a tail fin infection model and photoconvert neutrophils within the granuloma without altering fluorescent pigment of neutrophils outside the granuloma.

Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) *mpx*:Kaede zebrafish larvae were injected into the tail fin with 500CFU at 3dpf as previously described (Oehlers *et al.,* 2015) from a glycerol stock aliquot. At 4dpf (1dpi) larvae were screened for tail fin granuloma, imaged and green neutrophils within granuloma were photoconverted. At 5dpf (2dpi) previously photoconverted *mpx*:Kaede fish were reimaged and neutrophil counts present within tail fin granuloma were compared (Figure 5.10).

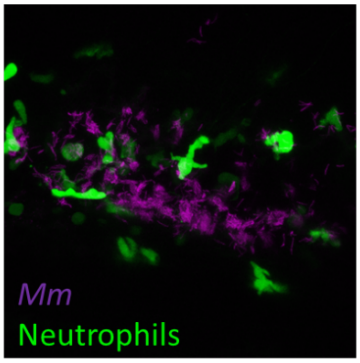
A


3dpf Tg(mpx:Gal4)i222(UAS:Kaede)s1999t
ROI
site of injection
zebrafish larvae

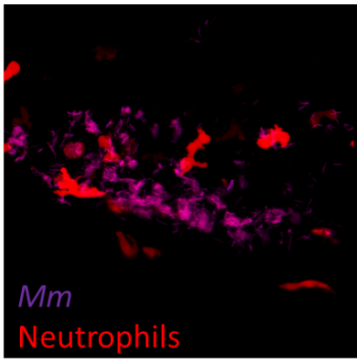
B


Tail-fin infection
Green neutrophils
before
photoconversion
Photoconversion
with blue light
Mm
Green neutrophils
following
photoconversion
Red neutrophils

C


Mm
Neutrophils

D


Mm
Neutrophils

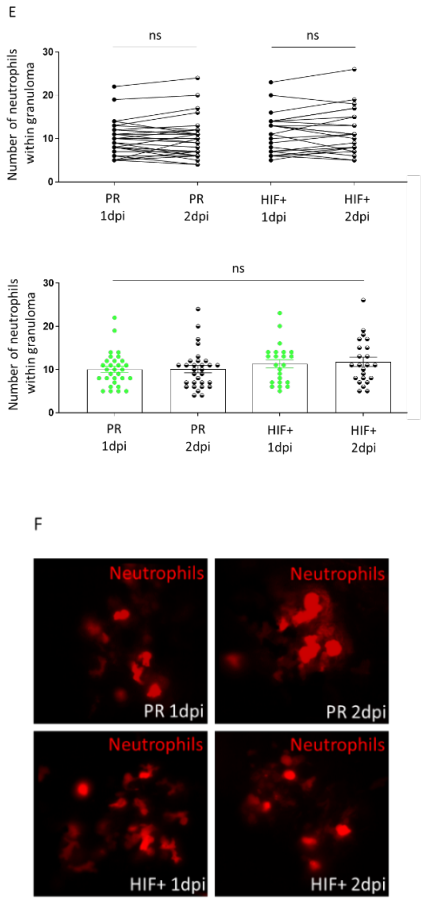


Figure 5.10 Hif-1α stabilisation does not change the number of neutrophils within granulomas between 1 and 2dpi

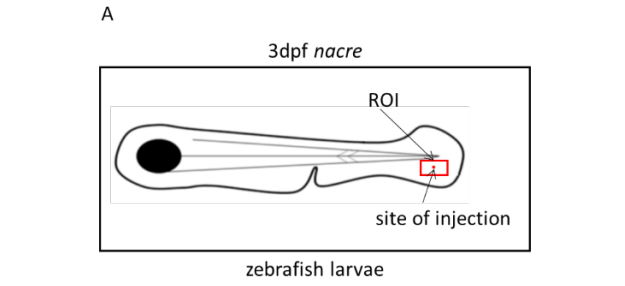
(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame), site of injection (red dot) and photoconverted area (blue box). (B) Representative images of green neutrophils photoconverted to red neutrophils and Mm (magenta) in the tail fin at 1dpi. White arrowheads indicate the infection points. (C) Representative image of granuloma before photoconversion with neutrophils (green) recruited to granuloma (magenta) in the tail fin at 1dpi. (D) Representative image of granuloma after photoconversion with neutrophils (red) recruited to granuloma (magenta) in the tail fin at 1dpi. (E) The graph summarises the comparison of neutrophils recruited to granuloma between 1 and 2dpi in tail fin quantified in control infection group (PR) and Hif-1α-stabilised infection group (HIF+). PR/HIF+ injected fish (0hpf) were infected into tail fin 3dpf, screened for granuloma at 1dpi and photoconverted. At 2dpi photoconverted fish were reimaged and numbers of neutrophils were compared. Three repeats were performed and two groups were used: (PR; n=30) and (HIF+; n=23). The results obtained from photoconversion experiments showed that between 1 and 2dpi neutrophil numbers remained the same in control group (PR) and Hif-1α stabilisation (HIF+) has no effect on neutrophil numbers recruited to granuloma between 1 and 2dpi (Unpaired T test; ns-p>0.97 (PR); ns-p>0.76 (HIF+)). (F) Example fluorescence micrographs of data shown in (E). Representative images of neutrophils (red) recruited to granuloma in the tail fin between 1dpi and 2dpi.

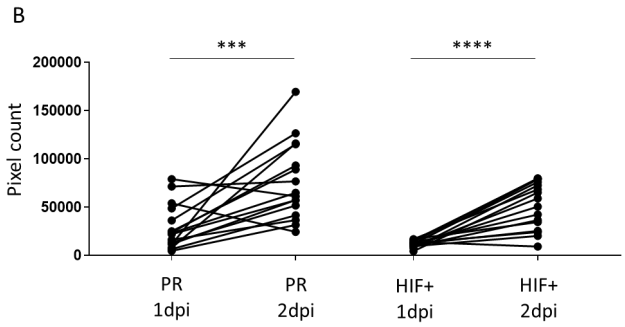
Using *mpx*:Kaede fish facilitates the distinction of neutrophils that arrived to forming granulomas between 1dpi and 2dpi as colour of neutrophils is changed from green to red after photoconversion (Figure 5.10A-C). Quantification of photoconverted neutrophils showed that between 1 and 2 days post infection in control group (PR) neutrophil numbers within tail fin granuloma were unchanged (ns-p>0.97 PR). Similarly, neutrophil counts in Hif-1α-stabilised groups (HIF+) confirmed that number of neutrophils recruited to tail fin granuloma remained stable (ns-p>0.76) (Figure 5.10E). Together, the above results demonstrate that neutrophil recruitment to tail fin granuloma occurs predominantly during the early stages of granuloma formation as neutrophil numbers remain unchanged between 1-2dpi. Following photoconversion the neutrophils within granuloma remained red indicating the presence of the same population of neutrophils between 1-2dpi (Figure 5.10F). Hif-1α stabilisation has no effect on neutrophil recruitment and these data correspond with the results obtained in chapter 5.3.8 which showed that Hif-1α stabilisation does not increase the neutrophil numbers within muscle somite granuloma when compared with controls.

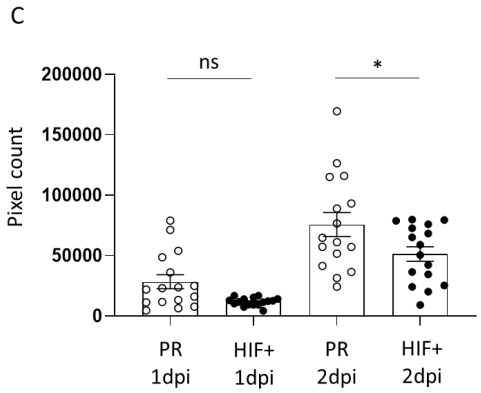
5.3.11 Granuloma size is reduced after Hif-1α stabilisation

Quantification of neutrophils recruited to both muscle somite granuloma (Chapter 5.3.8) and tail fin granuloma (Chapter 5.3.10) demonstrated that Hif-1α stabilisation does not increase neutrophil numbers and has no effect on migration of new neutrophils to existing granuloma. To verify whether Hif-1α stabilisation have an impact on granuloma size I imaged and compared the same granulomas between 1-2dpi. Granulomas tends to grow through increased recruitment of both innate and adaptive immune cells alongside bacterial proliferation. I hypothesised that Hif-1α stabilisation would change granuloma size.

Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) *nacre* zebrafish larvae were injected into the tail fin with 500CFU at 3dpf (Oehlers *et al.,* 2015) from a glycerol stock aliquot. At 1dpi larvae were screened for tail fin granulomas and imaged. At 2dpi the same tail fin granulomas were reimaged and compared with granulomas captured on the previous day (Figure 5.11).


A
3dpf *nacre*
ROI
site of injection
zebrafish larvae


B
***
****
Pixel count
200000
150000
100000
50000
0
PR 1dpi
PR 2dpi
HIF+ 1dpi
HIF+ 2dpi


C
ns
*
Pixel count
200000
150000
100000
50000
0
PR 1dpi
HIF+ 1dpi
PR 2dpi
HIF+ 2dpi

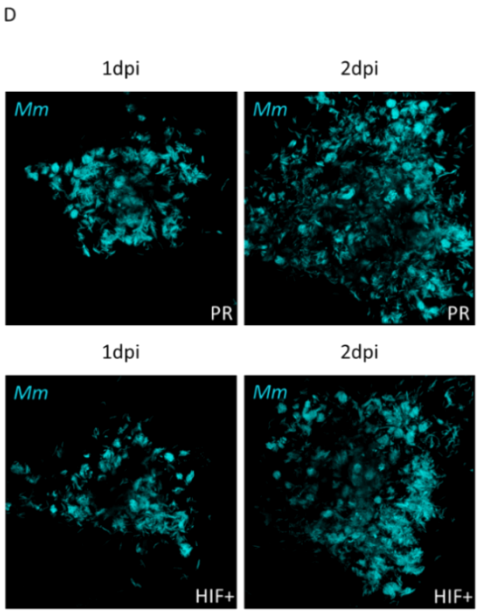


Figure 5.11 Hif-1α stabilisation decreased the size of granuloma at 2dpi

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of granuloma sizes between day 4 and day 5 in tail fin quantified in control infection group (PR) and Hif-1α-stabilised infection group (HIF+). PR/HIF+ injected fish (0hpf) were infected into tail fin 3dpf, screened for granuloma at 4dpf (1dpi) and imaged. At 5dpf (2dpi) fish were reimaged and granuloma sizes were compared. Data are shown as mean +/- SEM, n=16 as accumulated from 3 independent experiments (One-way ANOVA with Bonferroni multiple comparison test; ***-p<0.0003; ****- p<0.0001). (C) The graph summarises the comparison of granuloma sizes at 4dpf between control infection group (PR) and Hif-1α-stabilised infection group (HIF+) and at 5dpf between the same groups. Data are shown as mean +/- SEM, n=16 as accumulated from 3 independent experiments (One-way ANOVA with Bonferroni multiple comparison test; ns-p>0.14; *-p<0.02). The results obtained from tail fin granuloma comparison showed that size of granuloma constantly increases over time for both examined group (PR and HIF+). At 4dpf Hif-1α stabilisation (HIF+) has no effect on granuloma size but 5dpf the sizes of granuloma were reduced when compared with control group (PR). (D) Representative images of granulomas (cyan) in the zebrafish tail fin at 4 and 5dpf shown in (B&C). Images of zebrafish muscle taken using confocal microscope Leica DMi8 SPE TCS.

During TB infection invading bacteria is phagocytosed by macrophages which serve as a protective niche in which bacteria is able to survive, multiply and recruit other cells to eventually establish complex structure known as the granuloma. This compound structure tends to grow through enhanced recruitment of both innate and adaptive immune cells alongside bacterial proliferation. Quantification of bacterial pixel count confirmed that bacteria within granulomas proliferate showing a significant difference in size between 1-2dpi (p<0.0003 PR

group) (Figure 5.11B). The above data demonstrates that at 1dpi Hif-1α stabilisation tends to decrease initial size of granuloma but bacterial burden analysis showed no statistically significant differences between HIF+ and PR groups (ns-p>0.14) (Figure 5.11C). Further analysis of bacterial pixels confirmed that Hif-1α stabilisation does not completely inhibit mycobacterial growth, as granulomas were enlarged when compared between 1-2dpi (****-p<0.0001). However, at 2dpi Hif-1α stabilisation resulted in reduced size of granulomas when compared with control group (*-p<0.02) (Figure 5.11C-D). It is important to note that granuloma measurements take into account the area not the volume of the analysed bacteria. The above data indicates that HIF activation may be protective for the host by limiting bacterial growth within established granulomas.

5.3.12 Neutrophil nitric oxide remains high within granulomas after Hif-1α stabilisation

Nitric oxide (NO) is a potent antimicrobial and increased levels in immune cells allows RNS mediated killing of bacteria and provides better protection for the host. It has been previously shown that NO production in neutrophils is reduced within granulomas when compared with NO signal colocalised in neutrophils outside of granuloma. Mm infection initially increased nitrotyrosine levels of infected and non-infected neutrophils which may suggest that bacteria counteract the host's nitrosative defense mechanism (Elks *et al.,* 2014). To investigate whether Hif-1α stabilisation has an effect on nitric oxide (NO) production in neutrophils within muscle somite granuloma I used anti-nitrotyrosine antibody staining.

Following injections with dominant active (DA) variant of Hif-1α (Elks *et al.,* 2013) *Tg(mpx:GFP)*^{i114} zebrafish larvae were injected into the muscle somite with 200CFU at 2dpf as was previously established (Oehlers *et al.,* 2015) from a glycerol stock aliquot. At 5dpf, larvae were screened for granulomas, fixed and nitrotyrosine levels were immune-labelled using a rabbit polyclonal anti-nitrotyrosine antibody (Elks *et al.,* 2013) and imaged (Figure 5.12).

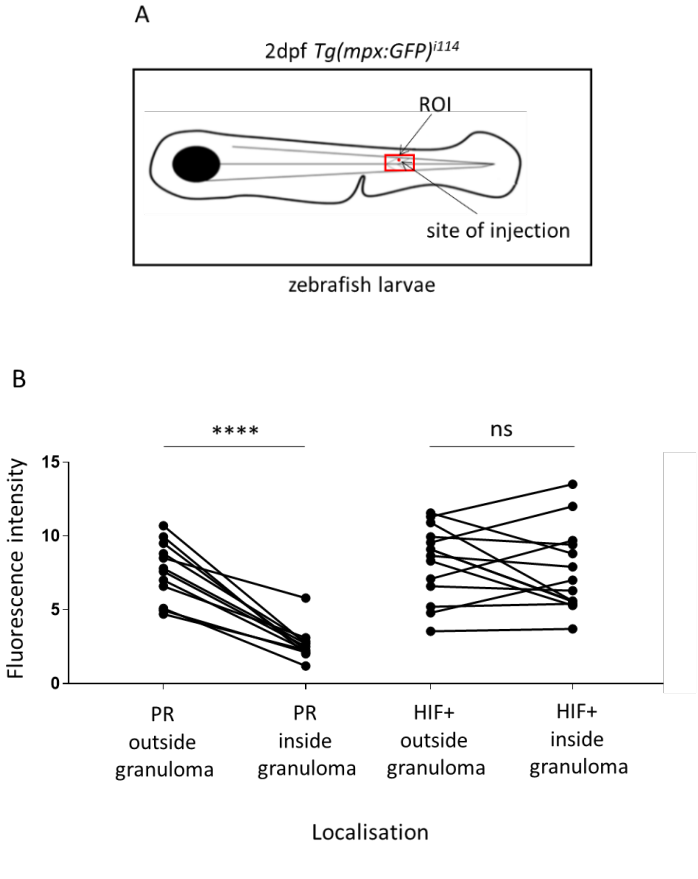

A
2dpf Tg(mpx:GFP)i114
ROI
site of injection
zebrafish larvae
B
****
ns
Fluorescence intensity
15
10
5
0
PR outside granuloma
PR inside granuloma
HIF+ outside granuloma
HIF+ inside granuloma
Localisation
C
O = outside granuloma I = inside granuloma
Mm
Neutrophils
α-Nitrotyrosine
PR
α-Nitrotyrosine
PR
Mm
Neutrophils
α-Nitrotyrosine
HIF+
α-Nitrotyrosine
HIF+

Figure 5.12 Hif-1α stabilisation maintained high levels of nitric oxide in neutrophils within granulomas

(A) Schematic of zebrafish larvae with marked region of interest (ROI, red frame) and site of injection (red dot). (B) The graph summarises the comparison of nitric oxide level in neutrophils outside granuloma (O) and inside granuloma (I) in muscle somite quantified in control infection group (PR) and Hif-1α-stabilised infection group (HIF+). PR/HIF+ injected fish (0hpf) were infected into muscle somite (2dpf), fixed and nitrotyrosine levels were immune-labelled (5dpf) and granulomas were imaged. Three repeats were performed and two groups were used: (PR; n=12) and (HIF+; n=16). The results obtained from granuloma experiments showed that Hif-1α stabilisation has maintained high level of nitric oxide in neutrophils inside granuloma when compared with PR control group (One-way ANOVA with Bonferroni multiple comparison test; ns-$p>0.70$; ****-$p<0.0001$). (C) Example fluorescence micrographs of data shown in (B). Representative images of nitric oxide (cyan) in neutrophils (green) recruited to granuloma (red) in the muscle somite at 5dpf. Images of zebrafish muscle taken using confocal microscope Leica DMi8 SPE TCS.

Inducible nitric oxide synthase (iNOS) is an enzyme required for nitric oxide production and host ability to combat invading mycobacteria greatly depends on this antimicrobial. Analysis using anti-nitrotyrosine antibodies staining enable to determine NO levels in neutrophils. During the early stages of mycobacterial infection in zebrafish, Mm initially increase nitrotyrosine levels of infected and non-infected neutrophils. This data demonstrates that in control group (PR) neutrophils outside muscle granuloma (O) have a higher level of nitric oxide when compared with neutrophils within granulomas (I) with $p<0.0001$ (Figure 5.12). This phenomenon is probably due to mycobacterial counteract strategy which targets the host's nitrosative defense mechanism. However, quantification of cell fluorescence of each neutrophil inside (I) and outside granuloma (O) confirmed that Hif-1α stabilisation (HIF+) prevents the reduction of nitric oxide level observed in control group (PR). Above findings indicate that HIF activation promotes NO-dependent neutrophil protection.

5.4 Conclusions and chapter discussion

Early interactions of host immune cells with mycobacteria determine whether infection is controlled or develops to form granulomas with the potential to cause active disease. Mtb has the ability to evade leukocyte defence mechanisms, surviving within these cells and proliferating (Eum *et al.,* 2010; Repasy *et al.,* 2013). Macrophage manipulation by the bacteria allows establishment of a permissive niche in which Mtb can grow and build granulomas. In this chapter I characterised neutrophil behaviour after Hif-1α modulation during the early stages of *Mycobacterium marinum* (Mm) infection. By studying the host-pathogen interactions I investigated the effect of Hif-1α stabilisation on bacterial burden after leukocyte number manipulation. Here, I show that neutrophil depletion leads to abolished Hif-1α protection in zebrafish larvae. Furthermore, I revealed that following Hif-1α stabilisation neutrophil nitric oxide is host-protective in macrophage-depleted zebrafish. I decreased nos2 levels to show that this abrogates host-beneficial effect of Hif-1α stabilisation. Subsequently, I utilised local infection models to demonstrate that Hif-1α stabilisation does not increase neutrophil numbers recruited to granuloma. Finally, I established that Hif-1α stabilisation remains high tyrosine nitration levels in neutrophils, increases neutrophil capacity to internalise Mm and restricts granuloma growth. However, the details of how neutrophils contribute to TB are still the subject of debate and several recent reviews addressed this question (Kroon *et al.,* 2018; Liu *et al.,* 2017; Remot *et al.,* 2019). In the light of current knowledge, the differential roles of neutrophils during TB depends upon the stage of disease. My data support the idea that neutrophils are considerably involved in mycobacterial infection (Lyadova, 2017; Warren *et al.,* 2017) and highlights the role of Hif-1α in antimicrobial activity.

In TB, neutrophils are considered a “double-edged sword”, being the first line of defence during infection but also participating in disease exacerbation, in part due to excessive inflammation (Dallenga and Schaible, 2016; Mayadas *et al.,* 2014). Neutrophils are able to phagocytose Mtb *in vitro* and *in vivo* (Ganbat *et al.,* 2016; Kisich *et al.,* 2002; Lombard *et al.,* 2016). In *in vitro* studies neutrophil do kill Mtb (Jones *et al.,* 1990; Martineau *et al.,* 2007), however some studies have shown only a limited capacity of neutrophils to eliminate Mtb, even after stimulation with IFN-γ (Denis, 1991), and TNF-α (Corleis *et al.,* 2012; Reyes-Ruvalcaba *et al.,* 2008). In human and mouse Mtb infection studies it has been shown that neutrophils are the most infected cells in the

airways of the patients with active pulmonary TB and cause severe lung inflammation (Eruslanov *et al.,* 2005; Eum *et al.,* 2010). In a mouse model neutrophil depletion resulted in decreased lung tissue pathology, reduced mycobacterial CFU counts and increased survival (Yeremeev *et al.,* 2015). However, the details how neutrophils retain their antimicrobial functions in hypoxic environment during the early stages of mycobacterial infection remains unclear.

Hypoxia inducible factor (HIF) transcription factors are the master regulators of the myeloid response to hypoxia, first discovered almost thirty years ago (Semenza *et al.,* 1991). Almost two decades ago Cramer et al showed that Hif-1α is essential for myeloid cell infiltration and activation *in vivo* during inflammation in mice, while deletion of Hif-1α does not impair macrophage development (Cramer *et al.,* 2003). After the exposure to microenvironmental signals such as bacterial compounds and cytokines macrophages adopt proinflammatory programs and this process depends on Hif-1α activity (Mills *et al.,* 2016; Rius *et al.,* 2008; Tannahill *et al.,* 2013). Hif-1α is an essential hub that integrates hypoxic and immunogenic signals during infection and inflammation. Low oxygen levels induce Hif-1α stabilisation which shapes metabolic and phenotypic profiles of myeloid cells and determine their capacity to form immune response against pathogen. Increasing lines of evidence indicate that Hif activation promotes immune cells ability to combat bacteria (Devraj *et al.,* 2017; Taylor and Colgan, 2017). However, Hif-1α has a broad-spectrum effect on host immune cells and its dysfunctions may contribute to pathogenic inflammatory processes (Stothers *et al.,* 2018). Therefore, the exact mechanism by which Hif-1α stabilisation regulates neutrophil activity and shapes Mtb infection outcome needs to be elucidated.

Neutrophils are the first responders to invading pathogens and Hif-1α plays an important role in regulation of their inflammatory response (Thompson *et al.,* 2013). Activated neutrophils undergo apoptosis within 72 hours of stimulation which reduces their damaging effect on surrounding tissues. In human and mice hypoxia-induced neutrophil survival is mediated by Hif-1α-dependent NF-κB activity (Walmsley *et al.,* 2005). Neutrophil survival is also strengthened upon Hif-1α-dependent NF-κB stabilisation and secretion of macrophage inflammatory protein-1β (MIP-1β) (D'Ignazio *et al.,* 2016). In patients with heterozygous germline mutation in the Hippel Lindau protein (pVHL) neutrophils exhibit delayed apoptosis and enhanced phagocytosis of *Streptococcus pneumoniae* suggesting that the HIF-1α/VHL pathway regulates the innate immune response (Walmsley *et al.,* 2006). In mice, neutrophils, HIF promotes expression of

cathelicidin peptides and granule proteases (Peyssonnaux *et al.,* 2005). In zebrafish, Hif-1α stabilisation during the early stages of *Mycobacterium marinum* (Mm) infection promotes reduction of bacterial burden due to elevated levels of reactive nitrogen species (RNS) in inducible nitric oxide synthase (iNOS)-dependent fashion (Elks *et al.,* 2013; Harvie and Huttenlocher, 2015). In my *in vivo* zebrafish data, host protection against Mm due to Hif-1α stabilisation was abrogated following neutrophil depletion which corroborates the importance of neutrophils in host protection during TB. *In vitro* and *in vivo* studies show that in murine peritoneal macrophages capacity to phagocytose bacteria increased in a HIF-1α-dependent manner (Anand *et al.,* 2007). Recently, *in vitro* studies suggested that HIF-1α-deficient macrophages have impaired ability to phagocytose bacteria (Fensterheim *et al.,* 2017). Above results highlight strong association between Hif-1α and increased survival and phagocytic capacity of innate immune cells, however it remains unclear how Hif-1α protects the host in the absence of macrophages.

In order to investigate whether the protective effect of Hif-1α stabilisation is due to neutrophils I depleted macrophage pool. Macrophages play a pivotal role in TB and due to their heterogeneity they can promote or restrict Mtb infection (Srivastava *et al.,* 2014). Depletion of alveolar macrophages (AMs) exerts protective effects in pulmonary tuberculosis in mice (Huang *et al.,* 2018; Leemans *et al.,* 2001). In contrast, mice deprived of interstitial macrophages (IMs) have enhanced mycobacterial outgrowth which prove that different macrophage linages respond divergently to Mtb infection (Huang *et al.,* 2018; Leemans *et al.,* 2005). In zebrafish, the absence of macrophages leads to increased bacterial burden indicating macrophage control of early Mm infection (Clay *et al.,* 2007). Even when macrophages are depleted, Hif-1α stabilisation is able to help the host immune response control infection and reduce bacterial burden. In my experiments, without Hif-1α stabilisation, an increased number of neutrophils at the expense of macrophages augmented infection, corroborating a pivotal role of macrophages in this process. However, Hif-1α stabilisation reduced bacterial burden in irf-8 larvae indicating that macrophage depletion is compensated by elevated number of neutrophils when Hif-1α is stabilised.

Nitric oxide (NO, nitrogen monoxide) is a short-lived key signalling molecule synthesised by iNOS (NOS2) in response to bacterial compounds or proinflammatory cytokines and in neutrophils regulates cell migration, apoptotic rate and antimicrobial activities (Saini and Singh, 2018). Recently it has been shown that during mycobacterial infection iNOS activates Hif-1α in NO-

dependent manner and promotes macrophage antimicrobial activity (Braverman and Stanley, 2017). In my data, blocking NO production using an iNOS inhibitor led to higher infection levels. Ogryzko et al demonstrated that Hif-1α stabilisation increases levels of macrophage and neutrophil-derived proinflammatory cytokine Il-1β, upregulating NO production in neutrophils (Ogryzko *et al.,* 2019). My data shows that Hif-1α stabilisation in macrophage-depleted larvae deprived of nitric oxide is not protective for the host, while when NO is present then even in the absence of macrophages and Hif-1α stabilisation remains protective. Previous data from my lab has shown that neutrophils are the cells that produce the most nitric oxide during early infection (Elks *et al.,* 2013) so my data suggest that Hif-1α protection is still present without macrophages due to neutrophil NO. Nevertheless, more in-depth analysis of iNOS activity is required to facilitate better understanding of the processes associated with NO-dependent host protection during the early stages of mycobacterial infection.

Zebrafish has two *nos2* genes, *nos2a* and *nos2b*, which arose as a consequence of genome duplication. Nos2 expression vary across gene variants with *nos2a* predominantly observed in immune tissues whereas *nos2b* is particularly expressed in tissues surrounding the oral cavity in zebrafish embryos (Holmqvist *et al.,* 2004; Lepiller *et al.,* 2009). Both *nos2* genes exhibit higher homologies with vertebrate *nos2* than with constitutively expressed *nos* genes (Lepiller *et al.,* 2009). Previously, it has been shown that Hif-1α stabilisation results in increased leukocyte NO production and that both pharmacological and morpholino genetic inhibition of Nos2 enzyme demonstrated that this process is iNOS dependent (Elks *et al.,* 2013). Gene knockdown can be also achieved using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technologies (Albadri *et al.,* 2017; Liu *et al.,* 2019). My results show that targeting ATG of *nos2ab* gene using CRISPR decreased NO levels in zebrafish, recapitulating previous studies using drugs and morpholino knockdown. However, the presence of a significant amount of NO following *nos2ab* knockdown indicated that targeting ATG is insufficient to silence *nos2* completely. In order to disrupt *nos2* and impair NO production I alternatively targeted exon 1. From my observations targeting exon 1 is an efficient method to knockdown *nos2* and abrogates NO production but only when both a and b forms of *nos2* are targeted. One of the reasons why targeting *nos2a* and *b* are required to inhibit NO production may be due to gene redundancy and functional compensation mechanisms (El-Brolosy *et al.,* 2018; El-Brolosy *et al.,* 2019; Peng, 2019). My bacterial burden analyses demonstrate that Hif-1α stabilisation is not protective for the host

after targeting exon 1 of nos2ab supporting the view that *nos2* is prerequisite for NO-dependent early protection in zebrafish. However, in order to confirm this mechanism is neutrophil driven an additional method such as neutrophil specific CRISPR interference (CRISPRi) could be used to deplete nos2 in future experiments.

The outcome of Mtb infection depends on complex interactions between pathogen and host immune cells such as alveolar macrophages (AM), dendritic cells (DC), natural killer cells (NK) and neutrophils among others (Berry *et al.,* 2010; Lowe *et al.,* 2012; Nouailles *et al.,* 2014). In *in vivo* TB models, neutrophils are the first responders present in Mtb-infected lungs and their impaired recruitment leads to the augmented infection (Srivastava *et al.,* 2014; Yang *et al.,* 2012). In this chapter I demonstrate that Hif-1α stabilisation has no effect on neutrophil numbers in the muscle somite in the absence of infectious challenge. Following Mm injections neutrophil recruitment increases in response to invading pathogens, which is widely documented phenomenon observed in several zebrafish infections of Gram-negative bacteria *Salmonella Typhimurium* (Tyrkalska *et al.,* 2016), *Shigella flexneri* (Mazon-Moya *et al.,* 2017), *Pseudomonas aeruginosa* (McCarthy *et al.,* 2017), *Burkholderia cenocepacia* (Mesureur *et al.,* 2017) Gram-positive bacteria *Listeria monocytogenes* (Levraud *et al.,* 2009), *Staphylococcus aureus* (Prajsnar *et al.,* 2012) and *Mycobacterium marinum* (Yang *et al.,* 2012). It is worth noting that initial numbers of neutrophils in the muscle somite during the first hours of infection are low. Similarly, in a zebrafish tail fin infection Hosseini et al demonstrated that numbers of neutrophils recruited to the site of infection are initially low (Hosseini *et al.,* 2016). However, these data identify significant participation of neutrophils in the early granuloma.

Almost two decades ago Davis et al using zebrafish model shed new light on the mechanisms contributing to granuloma formation showing that mycobacterium-macrophage interactions are sufficient to initiate this process (Davis *et al.,* 2002). Mtb-infected cells have altered metabolic functions which results in their impaired capacity to distribute signalling molecules important for mounting an adequate immune response (Gleeson *et al.,* 2016; Qualls and Murray, 2016). In humans, as well as TB susceptible animals including non-human primates, nascent granulomas have been demonstrated to be hypoxic (Belton *et al.,* 2016; Mattila *et al.,* 2015; Via *et al.,* 2008). In neutrophils, as well as in other immune cells the metabolic switch in response to hypoxic conditions is chiefly governed by (HIF-α) (O'Neill and Pearce, 2016). However, the exact role of Hif-α in neutrophil recruitment to granulomas as well as their ability to contain and clear bacteria

is still poorly understood. In my *in vivo* zebrafish data, the numbers of neutrophils within granulomas following macrophage depletion remain similar when compared with granulomas with unchanged numbers of macrophages. Likewise, Hif-1α stabilisation has no effect on neutrophil counts within granulomas. Similarly, in macrophage-deprived Hif-1α-stabilised zebrafish larvae the numbers of neutrophils remained stable. Interestingly, Hif-1α stabilisation significantly increases internalised Mm in immune cells of the granuloma. After Hif-1α stabilisation the percentage of neutrophils containing Mm has increased indicating that these cells may inhibit neutrophils to efficiently internalise Mm. Interestingly, macrophage depletion also increased the amount of bacteria contained by neutrophils in Hif-1α stabilised zebrafish larvae. Other macrophage and neutrophil depletion studies aiming at deciphering their roles in granuloma formation remain controversial (Remot *et al.,* 2019). Large discrepancies and contradictory results coming from neutrophil data may simply reflect the 'double-edged sword' nature of these cells and distinct functions dependent on the disease stage. In mice neutrophil depletion has been demonstrated to limit granuloma growth without altering bacterial counts (Seiler *et al.,* 2003). In guinea pigs using tasquinimod - a specific inhibitor which blocks neutrophil marker protein S100A9 - impaired formation of organized granuloma (Yoshioka *et al.,* 2016). A large number of neutrophils are commonly present in low-oxygen level necrotising granulomas in Mtb-infected animals (Mattila *et al.,* 2015). Neutrophils are highly motile phagocytic cells and one of the first responders towards various infections (Amulic *et al.,* 2012). Neutrophil motility relies on CXCR4 - a G protein-coupled chemokine receptor and mutation of this receptor results in neutrophil retention in the hematopoietic tissue (Walters *et al.,* 2010). Neutrophil recruitment depends on recombinant human interleukin-8 (IL-8 or CXCL2) - a neutrophil-specific chemotactic, human leukotriene B_4 (LTB_4) and the synthetic N-formylated peptide N-formyl-methionine-leucine-phenylalanine (fMLP). Neutrophils are recruited, partially in response to signals originating from dying macrophages present within the granuloma (Yang *et al.,* 2012), that form by 2-4dpi (Davis *et al.,* 2002). Neutrophils sporadically migrate through the Mm granuloma and leave uninfected but in the most cases they become infected in early granulomas through phagocytosis of infected macrophages. Within granulomas the majority of neutrophils are nonmotile and more spherical due to contained Mm, whereas less common motile neutrophils move through the granuloma and are able to phagocytose and kill bacteria multiple times (Yang *et al.,* 2012).

My *in vivo* zebrafish data show that neutrophil recruitment to nascent granulomas take place during the early stages of granuloma formation and neutrophil numbers remain equal regardless tissue differences. At these infection stages, granuloma are predicted to be normoxic and do not stain with Hypoxyprobe (Cronan *et al.,* 2016). The early stages of local Mm infection in zebrafish are accompanied with macrophage and neutrophil migration towards the site of infection and granuloma development (Hosseini *et al.,* 2016). Here, I show that Hif-1α stabilisation restricts granuloma growth and these observations might be important from therapeutic point of view. Taken together, Hif-1α stabilisation does not increased the arrival of new neutrophils to established tail fin granuloma. These findings are in line with my previous observations (shown in chapter 5.3.8), demonstrating no effect on neutrophil counts within muscle somite granulomas. However, Hif-1α transcription factor have a pleiotropic effect on a variety of immune cell functions, metabolic reprogramming in response to changing oxygen levels and antimicrobial properties (O'Neill and Pearce, 2016; Shi *et al.,* 2016; Taylor and Colgan, 2017). It has been shown that within zebrafish granulomas, mycobacteria target host TLR-dependent nitrosative defense mechanism resulting in attenuated immune response (Elks *et al.,* 2014). Taking into account that Hif-1α stabilisation is responsible for increased production of potent antimicrobial NO (Elks *et al.,* 2013), the role of Hif-1α in neutrophils within granuloma is yet to be fully answered.

Mycobacteria are able to take advantage of the host cells to survive, successfully proliferate and disseminate (Remot *et al.,* 2019). Numerous studies have shown that neutrophils participate in pro- and anti-inflammatory response, which to a large extent determine the outcome of TB infection (Domingo-Gonzalez *et al.,* 2015; Etna *et al.,* 2014). A correlation between Hif-1α and bactericidal activity of innate immune cells is well established (Knight *et al.,* 2018; Knight and Stanley, 2019; Rius *et al.,* 2008; Tannahill *et al.,* 2013). Neutrophil life-span is significantly increased in low-oxygen environment present in human TB granuloma (Ong *et al.,* 2018). It has been shown that hypoxic conditions augment neutrophil degranulation and enhance tissue damage in a Hif-1α-independent fashion (Hoenderdos *et al.,* 2016). However, little is known about the effect of Hif-1α stabilisation on neutrophil-released immune mediators and their impact on granuloma formation. In this chapter I demonstrate that Hif-1α stabilisation prevents the reduction NO levels observed in neutrophils within zebrafish granuloma. My *in vivo* zebrafish data corroborate that HIF activation favours NO-dependent neutrophil protection highlighting

the important role of Hif-1α transcription factor in neutrophil response to mycobacteria. In *in vitro* and *in vivo* studies Braverman et al showed that macrophages lacking Hif-1α are incapable of mediating IFN-γ-dependent protection against Mtb. In addition, Hif-1α-deprived macrophages were unable to control a metabolic shift - from oxidative phosphorylation to aerobic glycolysis – called the Warburg effect, which is prerequisite to IFN-γ-dependent bacteria control (Braverman *et al.,* 2016). In mice, macrophage NO - whose production is iNOS-dependent - has a robust bactericidal activity and acts as a key signalling molecule required for adequate Hif-1α functionality. Furthermore, NO has been shown to inhibit NF-κB activity to avert excessive inflammation. It is noteworthy that in oxygen-deprived granulomas NO also plays an anti-inflammatory role by decreasing neutrophil migration (Braverman and Stanley, 2017). Recent studies in NADPH oxidase 2 (NOX2)-deficient mice uncovered that reactive oxygen species (ROS)-deficiency results in intensified neutrophilic granulomatous inflammation indicating that IL-1β blocking might be an interesting avenue in the development of a new treatment regimen (Chao *et al.,* 2017). Neutrophils are able to sense ROS generated in response to microbes of different size to tune IL-1β expression and implement distinct inflammatory program via the selective oxidation of NF-κB (Warnatsch *et al.,* 2017). The importance of IL-1β has been confirmed in mice, as neutrophils deprived of IL-1R are unable to generate ROS and control infection (Di Paolo *et al.,* 2015). In zebrafish, Hif-1α triggers production of IL-1β in macrophages and neutrophils which results in elevated levels of neutrophil NO and enhanced host protection (Ogryzko *et al.,* 2019). Results presented in this chapter corroborate the important role of Hif-1α in the neutrophil response to pathogens and adds more evidence for the involvement of neutrophil NO in host protection against mycobacteria.

6 Final discussion

Tuberculosis (TB) remains the leading cause of death from an infectious disease among adults worldwide with more than 10 million new cases each year (WHO Global Tuberculosis Report, 2019, 2019). Although the global incidence of TB has been slowly decreasing the disease burden remains significant with an increasing prevalence of multi drug-resistant Mtb strains. Host-pathogen interactions during early stages of Mtb infection are critical for both infection control and establishment a primary progressive disease with the potential risk of latent infection development. Innate immune cells such as macrophages and neutrophils represent a first-line defence against invading pathogens and their capacity to restrict and eliminate mycobacteria eventually determines the course of disease. Whilst the roles of macrophages and intracellular fate of mycobacteria are well-established, the exact contribution of neutrophils during Mtb infection remains elusive.

Neutrophils are responsible for both mounting adequate protection in response to infection and orchestrating pro- and anti-inflammatory signals. They play a dual role during mycobacterial infection, on the one hand, they participate in infection control and granuloma formation, on the other hand, they contribute to tissue damage and infection dissemination. Neutrophils are able to phagocytose mycobacteria, however, their ability to kill Mtb is still a matter of debate as both *in vitro* and *in vivo* studies have shown contradictory results (Lyadova, 2017). These discrepancies highlight the burning need for the development of more reproducible and insightful methods which more fully explain the complexity of neutrophil functions during mycobacterial infection. Better understanding of these processes may help to identify new targets for host-derived strategies and improve current therapeutic interventions against multi-drug resistant Mtb strains.

Here, I investigated neutrophil behaviour against the fish pathogen *Mycobacterium marinum* (Mm) as a model for human tuberculosis. I explored how manipulation of Hif signalling impacts granuloma formation and improved host response during mycobacterial infection in zebrafish. Due to its optical transparency and genetic tractability as well as a high homology of innate immune cells zebrafish model has emerged as a powerful tool which helped to elucidate the details of TB pathogenesis (Meijer, 2016). Live imaging techniques in zebrafish allow for tracking of individual cells and their interactions with pathogens which has given profound insight into

the early events of Mtb infection. In this study, I examined the contribution of macrophage and neutrophil populations during early infection and utilising different chemical and genetic approaches and determined how the presence of macrophages and neutrophils impact on Mm infection. My studies have helped to characterise the effect of Hif-1α stabilisation on bacterial burden and in combination with high resolution imaging l examined the nitric oxide-dependent antimicrobial activity of neutrophils and their contribution to granuloma formation.

6.1 Characterisation of the neutrophil recruitment to *Mycobacterium marinum* and phagocytosis in zebrafish

Neutrophils are prominent phagocytic cells and in humans account for 50%-70% of all circulating leukocytes. Following infection they are first responders recruited to the infection site where recognise, engulf and eliminate invading pathogens (Mayadas *et al.,* 2014). Neutrophil exit from the blood and recruitment is a multifactorial ordered process involving interactions between numerous neutrophil receptors, microbe-derived products, endothelial cell receptors, signals generated by dying cells and a wide range of cytokines and chemokines. After neutrophil arrival bacteria are recognized directly or through Fcγ and complement receptors and phagocytosed (Lyadova, 2017). The primary evidence of classical leukocyte extravasation cascade derived from intravital microscopy (IVM) (Megens *et al.,* 2011). Mice lacking in activating Fcγ receptors (FcγRs) have impaired neutrophil accumulation which can be restored by neutrophil-selective transgenic expression of the two uniquely human neutrophil FcγRs: FcγRIIA and/or FcγRIIIB (Tsuboi *et al.,* 2008). Neutrophil recruitment has been demonstrated to rely on complement component 5a (C5a), a potent chemoattractant that decreases the threshold for FcγR-mediated neutrophil activation (Tsuboi *et al.,* 2011).

Neutrophils are a first responder to invading pathogens and after rapid recruitment to sites of mycobacterial infection they phagocytose bacilli (Lowe *et al.,* 2012). In *in vivo* experiments using zebrafish larvae allow for visualisation of neutrophil recruitment towards infection site at the singe-cell level. Drawing upon the advantages of live imaging I demonstrated that neutrophils are a predominant phagocytic cell type recruited to different infection sites during early stages of infection. My findings are in line with previous observations in zebrafish where neutrophils have been shown to efficiently phagocytose Mm whereas macrophages were chiefly involved in efferocytosis - phagocytosis of dead cells that contain bacterial content (Hosseini *et al.,* 2016).

Several *in vitro* and *in vivo* studies have also shown that neutrophils phagocytose mycobacteria and utilise different mechanisms to clear Mtb infection (Kroon *et al.,* 2018). In my zebrafish data I demonstrate that both macrophages and neutrophils phagocytose Mm within 2 hours post infection, which supports the notion that both cell types are involved in mycobacteria uptake during the early stages of infection. This is in line with studies which showed that neutrophils are able to kill Mtb in human lesions within 1 hour of phagocytosis, augmented by tumour necrosis factor alpha (TNF-α) (Kisich *et al.,* 2002). In mice, neutrophils manipulated to be genetically susceptible to TB exhibit increased phagocytic capacity for mycobacteria (Eruslanov *et al.,* 2005). Intranasal infection of mice with virulent Mtb or the live attenuated vaccine strain Bacillus Calmette Guérin (BCG) results in neutrophil recruitment and phagocytosis (Lombard *et al.,* 2016). Neutrophils are therefore innate immune cell type in the initial response to mycobacterial infection with early recruitment and phagocytosis events.

Macrophages are the main phagocytic cells understood to control mycobacterial infection mycobacterial infection (Weiss and Schaible, 2015), however numerous studies suggest that other phagocytic cells especially neutrophils are also important in restricting mycobacteria (Nouailles *et al.,* 2014; Srivastava *et al.,* 2014). Results included in this thesis evidence the important role of phagocytic cells in limiting bacterial growth *in vivo*. Low mycobacterial burden during the early stages of infection was due to initial control by phagocytic cells; without professional phagocytes Mm infection proliferates unchecked to cause large burdens. With the progression of infection, host phagocytic cells lose the ability to completely control mycobacteria and the granuloma forms. These findings are in line with well-known dichotomous roles of macrophages which vary depending on the stage of mycobacterial infection investigated (Clay *et al.,* 2007). Similarly, impaired neutrophil recruitment to Mm in zebrafish results in increased bacterial burden (Yang *et al.,* 2012). In mice, neutrophils have been shown to phagocytose Mtb (Repasy *et al.,* 2013), and their presence in rat lungs decreased bacterial burden and inhibited progression of TB (Sugawara *et al.,* 2004). According to studies in patients with active pulmonary TB neutrophils are the most frequently infected with Mtb taking into account the range of airway samples such as sputum, bronchoalveolar lavage and cavity samples (Eum *et al.,* 2010). However, there are some important limitations to work with neutrophils such as short life span and an inherent tendency to undergo activation (Lowe *et al.,* 2012). The zebrafish model has overcame these obstacles and emerged as a promising alternative to study neutrophils in their *in vivo*

settings, with its fully sequenced genome and macrophages and neutrophils that mimic their mammalian counterparts enable us to use it as a model system for a number of bacterial diseases (Torraca and Mostowy, 2018).

I validated local infection models and demonstrated that neutrophil migration to Mm resulted in the formation of a single granulomatous lesion. In contrast to human tuberculous granulomas, larval zebrafish lesions contain few lymphocytes, however formation of granulomatous lesions are similar to early TB granulomas and if infection were allowed to proceed to adulthood then the lymphocytes would become associated and all the features of the hallmark TB granuloma would be present (Swaim *et al.,* 2006). Heterogeneity of tuberculosis lesions is large and following the same granuloma over time has allowed for identification of several distinct types of lesions in a single individual (Ehlers and Schaible, 2013). In my *in vivo* data I shown elevated numbers of neutrophils following Mm infection which corroborate that mycobacteria and neutrophil interact during the early stages of mycobacterial infection (Meijer *et al.,* 2008). Zebrafish models of local infection appear to be enlightening tools for future study of bacterial populations, phagocyte dynamics, granuloma formation process and the effect of Hif manipulation on neutrophil activity. Their usefulness as a model for human TB has been already demonstrated (Benard *et al.,* 2012; Hosseini *et al.,* 2016; Oehlers *et al.,* 2015). Furthermore, local infection models enable fate tracking of single phagocytic cells in tissue-localised mycobacterial infection in terms of some key mechanisms, such as efferocytosis (Erwig and Henson, 2008) and extrusion of Mm aggregates (Pagán and Ramakrishnan, 2018).

6.2 Investigating the role of neutrophils and macrophages during Mycobacterium marinum infection

The course of mycobacterial infection mainly depends on the collective interplay between macrophages and neutrophils as well as their individual capacity to phagocytose bacteria (Tan *et al.,* 2006). Macrophages play a crucial role in host protection during Mtb infection and they diverse types and phenotypes depend on local environment and developmental origins (McClean and Tobin, 2016). Data on neutrophil functions during mycobacterial infection are inconsistant suggesting that these cells exert both positive and negative influences on the host response to TB (Kroon *et al.,* 2018). Similarily, evidence for neutrophil capacity to kill Mtb are not uniform

(Lyadova, 2017). Therefore, more in-depth studies are required to fully explain how neutrophils interact with Mtb and elucidate they contribution to the infection outcome.

In my *in vivo* zebrafish data, I confirmed that concomitant knockdown of both macrophages and neutrophils led to exacerbated infection outcome. Similarly, increased numbers of neutrophils at the expense of macrophages resulted in more robust mycobacterial burden. Elevated neutrophil numbers were insufficient to overcame the lack of macrophages. A reduced number of these cells leads to uncontrolled bacterial growth which highlights their significance in mycobacteria control. Indeed, the importance of macrophages in host protection during TB has been extensively evidenced (Cooper and Khader, 2008; Guirado *et al.,* 2013; McClean and Tobin, 2016). Here I investigated the infection outcome after macrophage depletion using chemical method. Similarly to genetic approach, macrophage-deprived zebrafish are not able to control mycobacteria which is another line of evidence to show an indispensable role of these cells during the early stages of infection. Based on my observations form macrophage depletion models I aimed to explore how solely neutrophil ablation would affect infection outcome.

Neutrophil depletion has been previously investigated in human blood of pulmonary TB patients and results clearly show more than 3-fold reduction capacity to restrict mycobacterial growth (Martineau *et al.,* 2007). Another studies on the impact of live and dead neutrophils demonstrated that viable neutrophils enhance Mtb control in blood, whereas necrotic neutrophils have opposite effect (Lowe *et al.,* 2018). However, as discussed previously the roles of neutrophils change over time with their detrimental effect for the host if excessive in numbers during the later stages of mycobacterial infection (Lyadova, 2017). My findings demonstrate that reduced number of neutrophils caused augmented infection levels, emphasising an important role of neutrophils during the early stages of infection. The general conclusion from these studies supports that both macrophages and neutrophils being crucial for host defence against mycobacteria in the zebrafish model.

My macrophage depletion experiments add further to evidence showing that macrophages play a pivotal role in mycobacterial control (Khan *et al.,* 2019). Macrophage act as the site of primary infection and shape both innate and adaptive immunity (Marakalala *et al.,* 2018). Of note, different subtypes of macrophages have a distinct impacts on mycobacterial infection and they are likely to have large heterogeneity and a vast array of activation states during complex infections like TB and granuloma formation (Marino *et al.,* 2015).

Mice alveolar macrophages (AM) promote mycobacterial infection serving as an effective niche for the bacteria, whereas interstitial macrophages (IM) have been demonstrated crucial for host resistance against Mtb (Huang *et al.,* 2018). Likewise, neutrophils represent diversified cell population which largely contribute to host protection mainly due to their antimicrobial properties in TB (Chai *et al.,* 2020; Kroon *et al.,* 2018). My zebrafish data on macrophages and neutrophils suggest that both cell types play host-protective roles during the early stages of mycobacterial infection. This conclusion is based on observations that depletion of neutrophils augment infection and this outcome is more pronounced following macrophage depletion. However, mycobacteria have evolved to survive within macrophages and in the case of failed bacterial clearance, macrophages provide protection to the pathogen and promote its dissemination. In order to improve understanding of detailed roles of phagocytic cells during Mtb infection, more advanced and informative approaches are required. Work included in this thesis sheds light on role of macrophages and neutrophils during the early stages of mycobacterial infection in zebrafish, which may be helpful to establish novel treatment strategies to test in other pre-clinical models. Finally, some findings observed in the zebrafish model may be supportive in development of novel drug targets to tackle mycobacterial infection. One of the promising avenues is increasing inducible nitric oxide synthase (iNOS). Hif-1α stabilisation has emerged as a powerful approach to elevate levels of potent antimicrobial nitric oxide (NO) (Elks *et al.,* 2013). However, further studies are needed to elucidate the full translational impact of Hif manipulation on neutrophils and host protection during mycobacterial infection.

6.3 Elucidating the role of Hif-1α stabilisation on neutrophils in mycobacterial infection

Hypoxia is a condition where cells or tissues lack adequate oxygen levels and is present in some areas of both infection and sterile inflammation due to elevated oxygen uptake by host immune cells and bacteria. Hypoxia inducible factor (HIF) is a heterodimeric transcription factor and master regulator of cell metabolism responsible for adaptation to oxygen-deprived environment. HIF is largely regulated at the protein level and following stabilisation drives expression of numerous genes associated with glycolysis, angiogenesis and host cell survival in hypoxia (Nizet and Johnson, 2009). The link between myeloid cells, such as macrophages and neutrophils, and HIF is well-documented (Corcoran and O'Neill, 2016; Devraj *et al.,* 2017; Stothers *et al.,* 2018). Upregulation of Hif-1α supports nitric oxide (NO) production via iNOS-dependent mechanism

resulting in reduced mycobacterial burden (Elks *et al.,* 2013). However, the details how Hif-1α impacts on neutrophil antimicrobial activity and tyrosine nitration levels within granuloma had yet to be elucidated.

Risk of TB infection is inversely and independently correlated with peripheral blood neutrophil counts in contacts of patients with pulmonary TB (Martineau *et al.,* 2007). In humans, neutrophils are able to kill Mtb withing 1 hour of phagocytosis in nonoxidative fashion but after exposure to human neutrophil peptides 1-3 and this process is enhanced following TNF-α stimulation (Kisich *et al.,* 2002). Viable neutrophils enhance control of Mtb in human blood, whereas necrotic neutrophils have the opposite effect (Lowe *et al.,* 2018). Neutrophils have considerably enriched genes of inflammasome pathway which is crucial for interleukin 1 (IL-1)-dependent cytokine production (Chen *et al.,* 2016). In mice, IL-1 is responsible for TNF-dependent control of Mtb infection (Di Paolo *et al.,* 2015). NO is important in TB control modulating the destructive innate inflammatory response by inhibiting NLRP3 inflammasome-dependent IL-1β processing (Mishra *et al.,* 2013). Recent studies corroborated that HIF-1α is involved in differentiation, polarisation and activation of myeloid cells (Koyasu *et al.,* 2018). Activation of Hif-1α is crucial for myeloid cell infiltration and activation *in vivo* through a mechanism independent of VEGF, which demonstrates direct regulation of survival and function in the inflammatory microenvironment (Cramer *et al.,* 2003). The lack of macrophages in zebrafish larvae results in enhanced bacteria outgrowth highlighting their importance during mycobacterial infection (Clay *et al.,* 2007). Similarly, my results show that in the absence of macrophages the host is not able to restrain bacteria which leads to uncontrolled infection. However, my *in vivo* data demonstrate that Hif-1α remains protective for the host irrespective of macrophage presence. Hif-1α has been shown to enhance phagocytic capacity of innate immune cells (Zinkernagel *et al.,* 2008). Braverman and Stanley provided evidence that iNOS activates Hif-1α in NO-dependent manner and elevate macrophage bactericidal activity (Braverman and Stanley, 2017). My findings suggest that Hif-1α stabilisation enhances NO-related host protection in neutrophils. Recently, Hif-1α has been shown to increase macrophage-derived IL-1β which enhanced NO levels in neutrophils (Ogryzko *et al.,* 2019). Macrophage stimulation with microbial products leads to increased Hif-1α and subsequent change of macrophage phenotype up to 7 days, which demonstrates potential for trained immunity (Arts *et al.,* 2016; Cheng *et al.,* 2014). Recently, trained innate immunity has been demonstrated in TB

where Joosten et al have shown that CXCR3 ligands are critical factors in mycobacterial growth inhibition (Joosten *et al.,* 2018). Increasing lines of evidence suggest that Hif activation increases capacity of immune cells to restrict bacteria (Devraj *et al.,* 2017; Taylor and Colgan, 2017), however more work is required to elucidate HIF-1α-dependent protection during mycobacterial infection.

Neutrophils, like macrophages represent heterogenous cell population and their intra-individual subsets display functional and phenotypic differences (Mishalian *et al.,* 2017; Scapini *et al.,* 2016; Silvestre-Roig *et al.,* 2016). Neutrophil numbers are correlated with TB development and recent studies demonstrate that frequency of low-density granulocytes (LDG) is associated with the severity of tuberculosis (Deng *et al.,* 2016). Neutrophils amplify the innate immune response and confer protection at the early stages of Mtb infection (Pokkali and Das, 2009). Here, I demonstrated that host protection against Mm infection associated with Hif-1α-stabilisation is abolished as a result of impaired functionality of zebrafish iNOS gene (*nos2*). My findings are in line with observations in mice homozygous for a disrupted *NOS2* allele. MacMicking et al demonstrated that $NOS2^{-/-}$ mice are highly susceptible to Mtb infection and enable mycobacteria to replicate faster in the lungs than reported for other gene-deficient hosts. High susceptibility has been shown NRAMP1-independent and NOS2 inhibition in wild-type mice led to progression of chronic tuberculosis (MacMicking *et al.,* 1997). Other studies in mice with non-functional iNOS also linked increased susceptibility to Mtb infection with inhibited activity of NO (Chan *et al.,* 1992; Darwin *et al.,* 2003). NO has been shown to control tuberculosis immunopathology by inhibiting NLRP3 inflammasome-dependent IL-1β processing (Mishra *et al.,* 2013). Augmenting endogenous NO production in chronic infective lung disease caused by *P. aeruginosa* is anti-inflammatory (Hopkins *et al.,* 2006). Recently, it has been demonstrated that Hif-1α function requires NO production, and that Hif-1α and iNOS are linked by a positive feedback loop that amplifies macrophage activation (Braverman and Stanley, 2017). In zebrafish two *nos2* genes exist: *nos2a* and *nos2b* and their expression is tissue-dependent where *nos2a* is associated with immune tissues and *nos2b* is predominately expressed in the surrounding of the oral cavity (Holmqvist *et al.,* 2004; Lepiller *et al.,* 2009). My data show that targeting ATG both forms of the *nos2* gene (*nos2a* and *nos2b*) is required to attenuate the hosts ability to produce NO whereas an alternative knockout of exon 1 leads to gene disruption and abolished NO production in zebrafish. These results could be explained by gene redundancy, which defines phenomenon

where 2 gene homologues play same or similar roles and is perceived as host-beneficial adaptation in case of impaired function one of the 2 copies (Peng, 2019). Genome editing techniques such as TALEN and CRISPR/Cas9 are well established (Chang *et al.,* 2013; Huang *et al.,* 2012; Hwang *et al.,* 2013). Rossi et al reported that gene compensation originates from deleterious mutation but not gene knockdown (Rossi *et al.,* 2015). Recent report evidenced the same phenomenon in nid1$^{-/-}$ zebrafish mutant (Zhu *et al.,* 2017). Gene analysis of zebrafish mutants performed by El-Brolosy et al revealed that genetic compensation is triggered by mutant mRNA degradation (El-Brolosy *et al.,* 2018). My results show that *nos2* gene knockdown can be achieved using CRISPR/Cas9 technologies, however a stable full knockout would confirm these findings. One insightful method to investigate tissue-specific gene knockdowns is CRISPR interference (CRISPRi), which allow for gene disruption in desired cell subset such as neutrophils or macrophages (Long *et al.,* 2015).

Macrophages and neutrophils are extremely dynamic phagocytic cells involved in the innate immune response (Silva *et al.,* 1989). Their key function in the phagocytosis of Mtb has been the subject of extensive research (Berry *et al.,* 2010; Srivastava and Ernst, 2014). However, the bacteria has the ability to evade leukocyte defence mechanisms, surviving within these cells and proliferating (Eum *et al.,* 2010; Repasy *et al.,* 2013). Apart from macrophages and neutrophils other cells of both the innate and adaptive immune system, such as T-cells, B-cells and dendritic cells are recruited to the site of infection forming the hallmark structure of TB – the caseating granuloma (Flynn *et al.,* 2011; Ulrichs and Kaufmann, 2006). The word “caseating” usually, but not exclusively, refers to active human tuberculosis and is related with a necrosis process (Adams, 1976). Identification of necrotising granulomas is extremely important from a diagnostic point of view as this process contribute to transmission and morbidity of TB (Cosma *et al.,* 2003; Mukhopadhyay *et al.,* 2012). In TB patients and animals susceptible to mycobacterial infection encompassing mice, guinea pigs, rabbits and non-human primates (NHP), granulomas exhibit low-oxygen concentration (Belton *et al.,* 2016; Mattila *et al.,* 2015; Via *et al.,* 2008). Hif-1α is a heterodimeric transcription factor responsible for cell adaptation to hypoxic environment of granuloma (O’Neill and Pearce, 2016). In my *in vivo* zebrafish data neutrophil contribution in nascent granulomas remains unchanged irrespective neither macrophage ablation nor Hif-1α stabilisation. Equally, Hif-1α stabilisation in macrophage-deprived granuloma has not diminished neutrophil population. TB granulomas used to be thought of as being protective for the host, but

more recent evidence suggests that they act to shelter the bacteria from host immunity to allow them to survive in a latent form until an immune compromise situation (Davis and Ramakrishnan, 2009). Macrophages play a key role in granuloma formation (Ehlers and Schaible, 2012; Guirado and Schlesinger, 2013; Reece and Kaufmann, 2012; Russell *et al.,* 2009), whereas neutrophils are regarded less important (Basaraba, 2008; Orme, 2011; Orme, 2014; Turner *et al.,* 2003). Here, I demonstrate that Hif-1α stabilisation increased the amount of intracellular Mm within neutrophils, however exact contribution of both neutrophils and macrophages in granuloma formation remains elusive (Remot *et al.,* 2019). In both mice and guinea pigs neutrophil ablation impairs formation organized granulomas (Seiler *et al.,* 2003; Yoshioka *et al.,* 2016). My data on neutrophil recruitment to granuloma is in line with observations of Hosseini et al indicating that this process occurs during the early stages of infection (Hosseini *et al.,* 2016) and numbers of recruited cells do not fluctuate. Comparison of different granulomas demonstrated that TB susceptible mice have elevated numbers of necrotic neutrophils (Kondratieva *et al.,* 2010; Marzo *et al.,* 2014; Nandi and Behar, 2011), whilst lower susceptibility is associated with smaller populations of non-necrotic neutrophils (Vesosky *et al.,* 2010). A significant body of data corroborate a pivotal role of HIF pathway in regulation of neutrophil functions (Thompson *et al.,* 2013). My *in vivo* zebrafish data suggest that Hif-1α stabilisation limits granuloma expansion and does not augment new neutrophil recruitment to established granulomas. In zebrafish granulomas, Mm infection initially elevates nitrotyrosine levels of infected and non-infected neutrophils. However, mycobacteria is able to impair TLR-dependent nitrosative defense mechanisms leading to compromised host immune response (Elks *et al.,* 2014). My results show that Hif-1α stabilisation maintains elevated levels of neutrophil NO, which may represent a counterstrategy against Mm. Hif-1α stabilisation has been previously demonstrated to limit Mm burden due to increased levels of NO via iNOS-dependent mechanism (Elks *et al.,* 2013). These findings are in line with well-established correlation between Hif-1α and antibacterial functions of innate immune cells, but in this case murine macrophages (Knight and Stanley, 2019; Tannahill *et al.,* 2013). NO modulates macrophage responses to Mtb through activation of Hif-1α and NF-κB repression (Braverman and Stanley, 2017). Finally, in zebrafish Hif-1α-induced expression of IL-1β protects against Mm which represents a host protective axis during mycobacterial infection (Ogryzko *et al.,* 2019). Taken together, data presented in this thesis shed a new light on details of host-pathogen interactions, demonstrated previously unknown host-protective neutrophil behaviours, both during the early stages of infection and within granuloma

that supports Hif-1α activation in neutrophils being an untapped therapeutic possibility against TB.

6.4 Clinical impact and future work

The ever-increasing number of multi-drug resistant Mtb strains and unsuccessful attempts to develop new vaccines have led researchers to focus on host-derived strategies (HDTs), which represent attractive alternative for existing TB treatment. Neutrophil studies have proven difficult and contradictory, probably due to their dual role during TB infection and highlight host-beneficial potential (Dallenga and Schaible, 2016; Kroon *et al.,* 2018; Lyadova, 2017; Remot *et al.,* 2019). Neutrophils have long been considered as simple foot soldiers and their role during infection was restricted to pro-inflammatory signalling. However, advances in cell biology demonstrate that neutrophils are heterogenous phagocytes comprised of different subsets possessing distinct properties and functions (Liew and Kubes, 2019; Warren *et al.,* 2017). Granulomas are complex structures and pathological hallmarks of TB. The hypoxic milieu, where complex interplay between host immune cells and pathogen take place require cell adaptation to low oxygen levels, the process chiefly regulated by HIFs (Stothers *et al.,* 2018). Numerous studies on HIF highlighted hydroxylase inhibitors as a promising avenue in the development of a novel treatment regimen against life-threatening diseases (Böttcher *et al.,* 2018; Chen *et al.,* 2019). In zebrafish, Schild et al demonstrated that in comorbid models Hif-1α stabilisation is protective against Mm infection and leads to postponed neutrophil inflammation resolution (Schild *et al.,* 2020). Recent advances have led to the establishment of a new approach to combat Mtb which is based on antibiotic delivery using pH-sensitive polymersomes. The nanoscopic polymersomes which amplified the effectiveness of antibiotics, were able to penetrate granulomas and also kill intracellular pathogens such as *Staphylococcus aureus*, *Mycobacterium bovis* and *Mycobacterium tuberculosis* (Fenaroli *et al.,* 2020). Necrotic neutrophils largely contribute to excessive inflammation and targeting these cells might be an appropriate tactics against Mtb (Dallenga *et al.,* 2017). More attention should be given to immunometabolism as metabolic changes seem to be an important feature of TB granuloma (Qualls and Murray, 2016). In order to establish new interventions combating TB more complex models fully recapitulating hypoxic granuloma are needed. In the future, knowledge taken from angiogenesis studies could be applied as multiple lines of evidence link this process with granuloma growth (Polena *et al.,*

2016). Because of the similarity between granuloma and tumour formation, analogous solutions derived from cancer therapy should be also considered (Remot *et al.,* 2019). Altering the HIF pathway has been promising in development novel strategies tackling TB (Balamurugan, 2016), and my *in vivo* zebrafish data corroborate that Hif-1α manipulation represents an untapped therapeutic approach which should be exploited to improve current TB treatment.

One of the potential therapeutic approach against TB is iron chelation to artificially trigger Hif-1α mediated proinflammatory and glycolytic pathways in host immune cells during Mtb infection. Iron chelator-induced inhibition of PHD proteins would results in Hif-1α stabilisation. In consequence, the molecular cascade during Mtb infection would be triggered resulting in a potentially boosted pro-inflammatory response of the infected host immune cells and improved clearance of bacteria (Phelan *et al.,* 2018). The addition of iron itself could have an opposite effect and previous studies have shown that iron promotes intra- and extracellular growth of Mtb H37Rv in J774A.1 macrophages and substantially reduce TNF-α, IL1α, IL1β and IL6 transcripts and TNF-α protein levels during Mtb infection (Serafín-López *et al.,* 2004). Hif-1α also enhances the expression and secretion of MMP-1 which contributes to uncontrolled destruction of lung tissue in TB patients (Belton *et al.,* 2016). The effect on tissue damage is not something that I addressed in this thesis, however, damage to infected tissue could be performed in the zebrafish model using collagen markers to identify tissue damage and remodelling during Mm pathogenesis and after HIF manipulation (Thompson *et al.,* 2014). Improved understanding of Mtb infection and HIF1α-dependent molecular mechanism from the work in this thesis paves the way to discovery novel HDTs and more efficient treatment strategies against TB.

In future work it would be useful to generate transgenic zebrafish lines of leukocyte specific HIF-α variants that can be used to reveal yet undiscovered modulatory potential of hypoxia signalling components in controlling mycobacterial infection during TB. Additionally, it would be interesting to investigate whether nitric oxide is able to kill mycobacteria in hypoxia and validate these *in vivo* findings in mammalian/human cell models. In the first place, I would incubate human neutrophils in hypoxia to measure NO level and also perform hypoxic neutrophil killing assays of various mycobacteria species such as *M. marinum*, *M. abscessus* and *M. smegmatis*. Furthermore, If I had more time in the lab I would use a recently established double transgenic zebrafish lines *Tg(mpx:GFP;mpeg:mCherry)* and *Tg(lysC:mCherry;CFMS:GFP)* and address the question about the number of phagocytes which undergo apoptosis. The level of apoptosis can

be studied at single-stage resolution and is evaluated by quantification of fluorescence using annexinV:YFP protein which binds the phospholipid phosphatidylserine - a marker of apoptosis present on outer plasma membrane of cell. Finally, in the future I would study lysosomal acidification which occurs during leukocyte phagocytosis of pathogens and compare this to activation of Hif/NO signalling. Mycobacteria are known to be able to circumvent phagolysosome acidification, but it is unknown *in vivo* when this occurs in early infection and whether Hif signalling plays a role in this. The acidity of different membrane compartments vary and can be measured using pH-sensitive dye (pHrodo™). The increase of fluorescence reflects acidification level after phagocytosis of Mm and is quantified to determine the phagocytic capacity to digest bacteria. I would also use LysoTracker™ dye, an alternative method to label lysosomes in live cells. Importantly, this staining technique would allow me to visualise acidic compartments and can also be used together with other fluorescent dies. Taken together, my findings in this chapter highlight the important roles of Hif-1α in neutrophil-driven NO mediated host protection during the early and granuloma stages of mycobacterial infection in zebrafish.

6.5 Final conclusions and closing remarks

To summarise, I have utilised zebrafish model to describe leukocyte behaviour against the fish pathogen *Mycobacterium marinum* (Mm) and have investigated how manipulation of Hif signalling affects granuloma formation and enhances the immune response during initial stages of mycobacterial infection. In this study, I explored the contribution of macrophage and neutrophil populations in Mm infection and by altering their numbers I determined the impact on infection outcome. My studies allow for better understanding of Hif-1α-dependent host protection and identifies previously unknown neutrophil behaviour during infection. I have shown that the absence of macrophages, which normally leads to uncontrolled overwhelming infection, can be rescued by Hif-1α manipulation via nitric oxide-dependent mechanism in neutrophils. Finally in-depth characterisation of granuloma formation reveals that Hif-1α stabilisation maintains a high levels of nitric oxide in neutrophils within granulomas and promotes restriction of bacterial growth. Together these findings demonstrate pivotal roles of neutrophil-driven NO mediated host protection and highlights Hif-1α stabilisation as a promising avenue in development of new host-derived treatment strategies against TB.

Lack of novel vaccines against TB and increasing multi-drug resistance of Mtb strains is a global threat which requires more advanced approaches. Host-derived strategies have emerged as an important alternative to combat Mtb (Young *et al.,* 2020), providing opportunities for alternative interventions. Neutrophils play a dual role during mycobacterial infection, however increasing lines of evidence support the belief that targeting these potent phagocytic cells could be host beneficial (Kroon *et al.,* 2018; Liu *et al.,* 2017; Lyadova, 2017). However, neutrophils have a short life span and an intrinsic predisposition to undergo activation, which has significantly complicated the elucidation of their functions during Mtb infection. Zebrafish is a powerful model which has enabled for a profound insight into the roles of individual cell types during the course of many infections (Torraca and Mostowy, 2018). Studies included in this thesis corroborate that the zebrafish represents an informative model to study host-pathogen interactions and have improved our understanding of Hif-dependent host protection during mycobacterial infection.

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