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Investigating the contribution of Immunoresponsive gene 1 (Irg1) to macrophage functions and its relevance to disease

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Abstract

Macrophages are essential effectors in the innate immune responses to injury, infection, and disease. Their broad functional plasticity allows them to tailor their behaviours to the specific homeostatic or immune needs of their microenvironment. However, macrophages have also been connected to the propagation of undesirable inflammation and associated disease, as in the case of obesity or neurodegeneration. An understanding of the drivers that determine macrophage phenotype is essential for the interpretation of macrophage roles in different immune environments and to inform how the process of phenotype switching may be modulated for therapeutic benefit. The growing field of immunometabolism proposes that functional changes within macrophages are derived from shifts in metabolic preference. This thesis presents the infection-responsive, mitochondrial enzyme Immunoresponsive gene 1 (Irg1) as a metabolic driver of macrophage activation and effector functions. Generation of Tg(irg1:EGFP)nz4, a unique transgenic zebrafish reporter line that marks expression of irg1 in macrophage-lineage cells, has enabled, for the first time, observation of the in vivo process of macrophage activation. This thesis has further exploited the advantages of the Tg(irg1:EGFP)nz4 transgenic line for transcriptome analysis of activated macrophages responding to LPS with or without the contribution of functional Irg1. This has led to the discovery that Irg1 exerts its effector functions in part by modulating NFκB signalling pathways and inducing the expression of potent inflammatory cytokines, including TNFα and IL-1β. Irg1 is anticipated to mediate these effects through mROS produced by Irg1-driven fatty acid β oxidation, drawing further support for a connection between metabolism and immunity in inflammatory disease. Finally, a novel aqueous screening platform has been developed to facilitate a drug-repurposing screen aimed at identifying modulators of irg1 expression. This
screen has revealed a number of strong candidates for the therapeutic reduction of \textit{irg1} expression. In sum, the \textit{Tg(irg1:EGFP)nz4} transgenic has provided invaluable insight into the process of macrophage activation through investigations into the downstream consequences of Irg1 activity. This transgenic line is anticipated to be of great value for further analyses of macrophage plasticity and effector functions. In addition, small molecules have been identified that may have applications to the therapeutic modulation of Irg1 activity in instances where macrophage-driven inflammation is associated with the propagation of disease. Together, this thesis has advanced understanding of the regulation of inflammatory effector functions in macrophage-lineage cells through Irg1.
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Abbreviations

aa Amino acid
Arg-1 Arginase 1
ATM Adipose tissue macrophage
ATP Adenosine triphosphate
BAC Bacterial Artificial Chromosome
bp Base pair
C/EBP CCAAT/enhancer binding protein
CAC Citric acid cycle
Cas CRISPR-associated
CHX Cycloheximide
CRE-BP cAMP response element-binding protein
CRISPR Clustered, Regularly-Interspaced, Short Palindromic Repeats
CT Critical threshold
DEPC Diethyl pyrocarbonate
DIG Dioxigenin
DMSO Dimethyl sulfoxide
dpf Days post-fertilisation
EDTA Ethylenediaminetetraacetic acid
ENU N-ethyl-N-nitrosourea
FACS Fluorescence-activated cell sorting
FAD Oxidized flavin adenine dinucleotide
FADH₂ Reduced flavin adenine dinucleotide
FLU Fluorescein
FDA Food and Drug Administration
FPKM Fragments per kilobase of transcript per million mapped reads
HIF-1α Hypoxia inducible factor-1α
hpi Hours post-injection
HSC Haematopoietic stem cell
HSPCs Haematopoietic stem and progenitor cells
IFN-γ Interferon gamma
IL Interleukin
Irg1 Immunresponsive gene 1
iNOS Inducible nitric oxide synthase
kb  kilobase
LPS  Lipopolysaccharide A
MCP-1  Monocyte chemoattractant protein-1
MMP  Matrix metalloproteinase
mROS  Mitochondrial reactive oxygen species
mTOR  Mammalian target of rapamycin
NAD+  Oxidized nicotinamide adenine dinucleotide
NADH  Reduced nicotinamide adenine dinucleotide
NADPH  Reduced nicotinamide adenine dinucleotide phosphate
NLRP3  NOD-like receptor family, pyrin domain containing 3
NO  Nitric oxide
NZGL  New Zealand Genomics Limited
OXPHOS  Oxidative phosphorylation
PBS  Phosphate buffered saline
PBST  Phosphate buffered saline with Tween20
PFA  Paraformaldehyde
PPAR-γ  Peroxisome proliferator-activated receptor gamma
PTU  1-phenyl-2-thiourea
ROS  Reactive oxygen species
rpm  Revolutions per minute
Runx-1  Runt-related transcription factor 1
SREBP  Sterol regulatory element-binding protein
SSC  Saline sodium citrate
STAT3  Signal transducer and activator of transcription 3
TALENs  Transcription Activator-Like Effector Nucleases
TAM  Tumour Associated Macrophage
TGF-β  Transforming growth factor beta
TLR  Toll-like receptor
TNFα  Tumour necrosis factor alpha
TRAF6  Tumour necrosis factor receptor-associated factor 6
USF  Upstream stimulatory factor
VEGF  Vascular endothelial growth factor
WMISH  Whole mount in situ hybridization
ZFNs  Zinc Finger Nucleases
ZIRC  Zebrafish International Resource Center
1 INTRODUCTION

Innate immunity is the body’s first line of defence against invading pathogens. White blood cells are recruited to sites of injury or insult where they respond with antimicrobial molecules and phagocytosis of the offending microbes and damaged tissues (Medzhitov & Janeway, 2000). Notable among these defending cells is the macrophage. Investigations into the form and function of macrophages have progressively revealed a range of applications beyond their inflammatory properties at the site of injury (Mosser & Edwards, 2008). Subtypes have demonstrated roles in wound healing, tissue homeostasis, and even disease progression, with an apparent maintained potential for switching between functional phenotypes (Mosser & Edwards, 2008). The central drivers behind this plasticity are not yet well defined, but data support a role for intracellular metabolic processes in regulating macrophage activities (S. K. Biswas & Mantovani, 2012). The association between immune cell function and intracellular metabolism, as well as the influence immune cells have on metabolic tissues, are the two key concerns of immunometabolism. Interest in this field has been driven by research into obesity-associated inflammation and how it predisposes patients to the development of chronic diseases such as type 2 diabetes, cardiovascular disease, and Alzheimer’s disease (Mathis & Shoelson, 2011). Studies have revealed distinct roles for inflammatory macrophages during the maintenance or development of metabolic disease and suggest that targeting points such as this, at the interface of immunity and metabolism, may produce effective strategies for the treatment of obesity and its associated co-morbidities (Chawla, Nguyen, & Goh, 2011). Immunoresponsive gene 1 (Irg1), a mitochondrial enzyme upregulated in macrophages in response to infection, has recently presented as a promising target to help explore this possibility (Degrandi, Hoffmann, Beuter-Gunia, & Pfeffer, 2009; Hall et al., 2013). Through a greater
understanding of how Irg1 couples metabolic and inflammatory processes within macrophages, it is expected that new pathways controlling macrophage function, that are relevant to the propagation of inflammation and underlying metabolic disease, will be revealed. It is anticipated that this information will lead to the development of novel treatments for metabolic diseases through the therapeutic manipulation of macrophage plasticity.

1.1 Macrophage heterogeneity

Macrophages are white blood cells of the innate immune system first identified for their capacity to take up and degrade a broad array of foreign materials and cellular debris (Metchnikoff & Binnie, 1907). They permeate all tissues of the body and are one of the first populations to respond to either insult or injury, though their phagocytic ability is also indispensible for tissue growth, remodelling, and repair (Mosser & Edwards, 2008). How this incredible pervasiveness and breadth of function can exist within one cell lineage is the subject of innumerable studies and much fascination, but also complicates interpretation of their presence in chronic disease. In this regard, macrophages have been connected to both the resolution and propagation of many illnesses and disease states (Murray & Wynn, 2011). Investigation into the pathways through which these versatile cells communicate and adopt different functionalities is thus of great importance to our understanding of the roles and applications they may have in the advancement of human health.
1.1.1 Developmental origins

In mammals, infiltrating macrophages arise from haematopoietic stem cells (HSCs) in the bone marrow that differentiate into monocytes in the blood and then macrophages following extravasation into tissues (FIG 1.1) (Orkin, 2000). Monocytes are often considered less-differentiated macrophages but this heterogeneous population possesses a range of functional immune properties in addition to its role in supporting or replenishing depleted macrophage and dendritic cell populations (Sunderkotter et al., 2004). These include the scavenging of apoptotic cells and toxins and the ability to respond to and defend against pathogens through the production of inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) (Auffray, Sieweke, & Geissmann, 2009).

FIGURE 1.1: Macrophage ontogeny
Haematopoietic stem cells (HSCs) in the bone marrow give rise to all mature blood cell types by way of progenitor cells. The common lymphoid progenitor (CLP) generates lymphocytes, including B cells, T cells, and natural killer (NK) cells. The common myeloid progenitor (CMP) populates the blood with monocytes, granulocytes, erythrocytes, and megakaryocytes. Once monocytes migrate into the tissues, they differentiate into macrophages. Abbrev. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; MEP, megakaryocytic-erythrocytic progenitor; HSC, haematopoietic stem cell; NK, natural killer.
While the majority of monocytes are short-lived, a portion will leave the blood stream following inflammatory signalling and migrate to the site of stimulation (Van Furth, Diesselhoff-den Dulk, & Mattie, 1973). Here they become infiltrating macrophages, taking on the required phenotype dictated by the microenvironment (Gordon & Taylor, 2005). Further investigations in the blood have identified multiple monocyte phenotypes, suggesting a program of progressive development within the circulation (Sunderkotter et al., 2004). In experiments by Sunderkotter et al., it was noted that monocytes in the early stages of this apparent maturation were preferentially recruited to the tissue during infection (Sunderkotter et al., 2004). A similar observation was made by Nahrendorf and colleagues when investigating the immune response to myocardial infarction. They observed that a population of Ly-6C<sup>hi</sup> monocytes, corresponding to Sunderkotter's immature cells, were selectively recruited during the early inflammatory response (Nahrendorf et al., 2007). In the late response and healing, monocyte recruitment shifted to the Ly-6C<sup>lo</sup>, mature subgroup. Evidence suggests that still other phenotypic variations may exist within the monocytic population that hint at a level of predestination with respect to the specific tissues to which they may eventually home (Arnold et al., 2007; Banati, Hoppe, Gottmann, Kreutzberg, & Kettenmann, 1991; Ingersoll, Platt, Potteaux, & Randolph, 2011; Sunderkotter et al., 2004). Banati et al. first raised this question with their observation of a subpopulation of bone-marrow macrophage-lineage cells that display a potassium ion channel distribution only previously described in microglia, the terminally-differentiated macrophages of the brain (Banati et al., 1991; Ginhoux & Jung, 2014). However, whether specific populations within the heterogeneous monocyte compartment can be preferentially expanded for therapeutic benefit, or how this variation is directed prior to extravasation, remains to be determined.
1.1.2 Tissue distribution

Complementing the on-demand supply and replenishment of macrophages from circulating monocytes are the tissue-resident macrophage populations. Resident macrophages are present throughout the body and, in addition to acting as sentinels scanning for danger, acquire specialised traits that allow them to fulfil homeostatic demands specific to their particular tissue or organ of residence (Davies, Jenkins, Allen, & Taylor, 2013). **Figure 1.2** demonstrates the systemic distribution of some of these populations.

![Distribution of resident macrophage populations](image)

**FIGURE 1.2: Distribution of resident macrophage populations**

Resident macrophages are maintained in all tissues and organs of the body. Some of these populations demonstrate specialised phenotypes pertaining to the homeostatic requirements of the tissue. Unique terminology is often used to delineate these populations.

Resident macrophages differ from infiltrating macrophages in that they are established during embryogenesis (Ginhoux & Jung, 2014). Originating from the yolk sac and fetal liver, early macrophages seed tissues before the monocytic compartment is fully
developed (Ginhoux & Jung, 2014). The varying origins of some of these different populations are described in Table 1.1.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Terminology</th>
<th>Origin</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Brain</td>
<td>Microglia</td>
<td>Yolk Sac</td>
<td>(Ginhoux et al., 2010)</td>
</tr>
<tr>
<td>Liver</td>
<td>Kupffer Cells</td>
<td>Yolk Sac + Embryonic HSCs</td>
<td>(Schulz et al., 2012)</td>
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<tr>
<td>Skin</td>
<td>Langerhans Cells</td>
<td>Yolk Sac + Embryonic HSCs</td>
<td>(Hoeffel et al., 2012)</td>
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<td></td>
<td>Dermal Macrophages</td>
<td>Adult HSC</td>
<td>(McGovern et al., 2014)</td>
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<tr>
<td>Peritoneum</td>
<td>Peritoneal Macrophages</td>
<td>Embryonic HSCs</td>
<td>(Yona et al., 2013)</td>
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<td>Lung</td>
<td>Alveolar Macrophages</td>
<td>Embryonic HSCs</td>
<td>(Yona et al., 2013)</td>
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<td>Spleen</td>
<td>Red Pulp Macrophages</td>
<td>Embryonic HSCs</td>
<td>(Yona et al., 2013)</td>
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<td></td>
<td>Marginal Zone Macrophages</td>
<td>Unknown</td>
<td>(A-Gonzalez et al., 2013)</td>
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<tr>
<td>Kidney</td>
<td>Kidney Macrophages</td>
<td>Adult + Embryonic HSCs</td>
<td>(Schulz et al., 2012)</td>
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<td>Intestine</td>
<td>Intestinal Macrophages</td>
<td>Adult HSC</td>
<td>(Tamoutounour et al., 2012)</td>
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<tr>
<td>Bone</td>
<td>Osteoclasts</td>
<td>Adult HSC</td>
<td>(Xing, Xiu, &amp; Boyce, 2012)</td>
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It had long been believed that circulating monocytes repopulate, or at least strongly contribute to the repopulation of, resident macrophages following depletion through injury or infection in the adult. However, current studies have shown that this contribution, if any, is often minimal and that resident macrophages have a strong ability to self-renew (Ginhoux & Jung, 2014; Hashimoto et al., 2013). Given the capacity for both resident and infiltrating macrophages to take on a range of phenotypes enabling them to sense and combat infection, questions arise as to what inherent differences between these types selectively allow resident macrophages to self-renew and what other overlooked discrepancies may exist.

1.1.2.1 Microglia

Of particular interest to applications of work presented in this thesis are the resident macrophages of the brain, termed microglia. Glial cells are the non-neuronal cells within the brain that collectively function to promote neuron health (Purves et al., 2001). This
can be structurally, by producing extracellular matrix to facilitate growth and migration (Jakeman, Williams, & Brautigam, 2014); chemically, by regulating extracellular ion and neurotransmitter levels to enable efficient communication (Nagelhus et al., 2013); metabolically, by supplying nutrients (Wyss, Jolivet, Buck, Magistretti, & Weber, 2011); or immunologically, by detecting and destroying immunogens (Aloisi, 2001). Microglia are primarily responsible for the immune defence of the brain, though recent studies indicate additional functions in synaptic network remodelling that may facilitate learning and memory formation (Tremblay, 2011).

Microglia are unique among resident macrophage populations in that they are derived exclusively from yolk sac primitive macrophages, while other tissues see additional contributions from the fetal liver or adult haematopoiesis (Ginhoux et al., 2010). Hypotheses exist suggesting that this is due to the development of the blood-brain barrier, which physically isolates the brain from fetal macrophage invasion (Ginhoux & Jung, 2014). The blood-brain barrier, while providing a formidable defence against infection, creates unique challenges once a pathogen does find its way through, as a number of immune resources easily available to the rest of the body (such as many antibodies) are unable to cross into the brain (Yu et al., 2011). This means that microglia play a more crucial role in host defence and must be able to act very quickly and effectively. It has been suggested that this is facilitated by a potassium channel pattern unique to microglia that enables the detection of very small changes in extracellular potassium (Kettenmann, Hoppe, Gottmann, Banati, & Kreutzberg, 1990). Support for this comes from the observation that rat microglia display reversible activation characteristics (including MHC Class II expression) in response to stimuli as limited as non-lethal neuron depolarisation events within the brain (Gehrmann, Matsumoto, & Kreutzberg, 1995). Recruitment of microglia to a site of injury within the brain has also
been observed following the development of calcium ion gradients and is dependent on extracellular adenosine triphosphate (ATP), though the molecular pathways responsible for microglial recognition and response to this signal are not yet understood (Sieger, Moritz, Ziegenhals, Prykhozhij, & Peri, 2012).

1.1.2.2 Intestinal macrophages

Immune defence in the intestine is presented with a unique challenge due to the presence of commensal bacterial populations (Smythies et al., 2005). Resident macrophages need to be vigilant in their patrol of the intestinal lining while avoiding a state of chronic inflammatory stimulation. Thus, intestinal macrophages develop a unique phenotype characterised by high phagocytic and bactericidal potential yet limited production of pro-inflammatory cytokines (Smythies et al., 2005).

1.1.2.3 Alveolar macrophages

Similar to the intestine, the lung has a heightened exposure to immunostimulatory particles by virtue of its function as an interface between the body's internal and exterior environment (Wright, 2004). Pulmonary surfactant produced by alveolar epithelial cells assists in the defence of the lung against inhaled antigens through the direct antimicrobial effects of its constituent molecules, while also facilitating gas exchange and proper tissue structure (Wright, 2004). In addition to their function as immune sentinels, alveolar macrophages are responsible for surfactant catabolism, ensuring efficient turnover and preventing excessive accumulation (B. C. Trapnell & Whitsett, 2002). When surfactant metabolism is not properly managed, as in instances of alveolar
Macrophage dysfunction, surfactant accumulation can lead to pulmonary alveolar proteinosis, a range of conditions typified by reduced gas exchange efficiency and increased risk of infection (B. C. Trapnell & Whitsett, 2002).

1.1.2.4 Red pulp and marginal zone macrophages

Macrophages of the spleen are critical to this organ’s function in blood filtration, through their removal of aging erythrocytes from the circulation and the subsequent breakdown and recycling of contained haemoglobin and iron (Kohyama et al., 2009). Red pulp macrophages are particularly proficient in these tasks, with a loss of this population leading to localised iron-overload (Kohyama et al., 2009). A second spleen-resident population, known as marginal zone macrophages, contributes to the process of blood filtration through modulation of the immune response to apoptotic cell uptake (McGaha, Chen, Ravishankar, van Rooijen, & Karlsson, 2011). In instances of depletion of marginal zone macrophages, mice have shown increased inflammatory cytokine production in the spleen and a greater susceptibility to developing autoimmunity (McGaha et al., 2011).

1.1.2.5 Osteoclasts

Osteoclasts are the resident macrophage population in bone (Xing et al., 2012). Their unusual development requires the fusion of mononuclear pre-osteoclasts at the bone surface, resulting in multinucleated, mature macrophages (Xing et al., 2012). This fusion is crucially dependent on RANK-L expression on the surface of adjacent stromal cells or osteoblasts, which directly interacts with RANK on the pre-osteoclasts in conjunction with c-Fms receptor ligation by M-CSF (Lacey et al., 1998). Mature osteoclasts are
responsible for bone resorption, the degradation of bone in order to maintain appropriate mass (Popoff & Schneider, 1996). Loss of osteoclast function leads to excessive bone mass and a reduced marrow cavity, while enhanced osteoclast function results in dramatically reduced bone density (Mizuno et al., 1998; Popoff & Schneider, 1996). These conditions are named osteopetrosis and osteoporosis, respectively.

1.1.3 Macrophage diversity

Macrophages and their monocyte precursors form a critical component of the innate immune system – the body’s indiscriminate defence system against a broad range of invading substances (Gordon & Taylor, 2005). They are best known for their high phagocytic potential but have also been correlated with efficient wound healing and tissue repair (Enderlin Vaz da Silva, Z, Lehr, & Velin, 2014; Mosser & Edwards, 2008; Murray & Wynn, 2011). Given the diversity of potential inflammatory environments they may encounter, macrophages have developed an equally broad capacity for functional calibration (Mosser & Edwards, 2008; Murray & Wynn, 2011). This is suggested by the varied specialisations of tissue-resident macrophages and demonstrated through the flux in cytokine and signalling molecule profiles they produce over time in varied circumstances (Epelman, Lavine, & Randolph, 2014; Murray & Wynn, 2011).

1.1.3.1 Spectra of macrophage function and plasticity

Macrophage phenotype has long been compartmentalised into one of two fairly rigid categories: M1 or M2, corresponding to pro-inflammatory/classically-activated and anti-
inflammatory/alternatively-activated respectively (Nathan, Murray, Wiebe, & Rubin, 1983; Stein, 1992). These two categories, however broad, are insufficient to describe all the intricacies of macrophage form and function that are being continuously discovered through the increasing power of in vivo studies. This presents two difficulties. First, use of these categories complicates analyses of the literature, as inconsistent or inadequate nomenclature makes it difficult to assess exactly which cell type is being studied. Second, they may erroneously imply a fixed, end-stage differentiation status to cells that are described in these ways. Rather, what is becoming more apparent is that macrophages retain a high level of plasticity throughout their existence and that these categories may only serve as useful landmarks along a much larger continuum of functionality (Mosser & Edwards, 2008; Murray et al., 2014).

1.1.3.2 The M1-M2 phenotype continuum

The M1 phenotype, also known as “classically activated,” has been traditionally characterised by the production of pro-inflammatory mediators including TNFα, IL-1β, IL-6, IL-12, and inducible nitric oxide synthase (iNOS) (Mosser & Edwards, 2008). The primary functions of this state are amplification of the pro-inflammatory immune response, phagocytosis of pathogens, and stimulation of T cells in the adaptive immune system to produce interferon gamma (IFN-γ) (Mosser & Edwards, 2008). This cycle is somewhat self-amplifying, as M1 macrophages are activated through Th1 cytokines such as IFN-γ in combination with lipopolysaccharide A (LPS) (Edwards, Zhang, Frauwirth, & Mosser, 2006; Nathan et al., 1983). Conversely, the strongly anti-inflammatory M2 “alternatively activated” phenotype is activated through exposure to Th2 cytokines, including IL-4, IL-10, and IL-13, and is associated with resolution of inflammation and subsequent tissue remodelling (Mosser & Edwards, 2008; Stein, 1992). The
transcription profile of M2 macrophages includes anti-inflammatory molecules such as transforming growth factor beta (TGF-β) and IL-10 (Fadok et al., 1998; Gerber & Mosser, 2001). One of the most commonly noted differences between M1 and M2 macrophages is their relative production of IL-10 and IL-12 (Martinez, Sica, Mantovani, & Locati, 2008). Very low levels of IL-10 and high levels of IL-12 are distinctive of M1 macrophages, while the opposite is true of M2 macrophages (Martinez et al., 2008).

Between the extremes of typical M1 and M2 classifications lie a myriad of phenotypic subgroups that address the specific needs of immunoprivileged organs and tissues and the rapidly changing microenvironment at the site of insult (Mosser & Edwards, 2008). As these additional subgroups are discovered and defined, not only does it become more difficult to fit observed phenotypes to a polar scale, but it also becomes more challenging to accurately describe and discuss them. Mosser and Edwards have suggested shifting to a “colour-wheel” analogy to more accurately describe various “shades” of function and the ease of transition between them (Mosser & Edwards, 2008). They propose three functional categories positioned similarly to the three primary colours: classically activated macrophages, wound-healing macrophages, and regulatory macrophages. This third classification describes a subgroup that specifically stimulates adaptive immune responses while reducing innate immune activation through the promotion of T cell IL-4 production instead of IFN-γ (Anderson & Mosser, 2002; Gerber & Mosser, 2001; Mosser & Edwards, 2008). These cells have a cytokine profile similar to M1 macrophages in that they maintain production of TNFα, IL-1β, and IL-6, however their marked decrease in IL-12 and increase in IL-10 indicates a shift towards a more M2-like phenotype (Anderson & Mosser, 2002; Gerber & Mosser, 2001; Mosser & Edwards, 2008).
While the proposed colour-wheel model addresses the issue of acknowledging macrophage plasticity and the transient and intermediary phenotypes that it may produce, it does little to clarify how these different populations may be described or compared in the literature. Murray et al. have proposed a system of nomenclature to address this, based on the characteristic phenotypes arising from macrophages activated by a range of specific stimuli (Murray et al., 2014). For example, pro-inflammatory macrophages may be described as “M(IFN-γ)” or “M(LPS),” indicating their expression profile is recapitulated by naïve macrophages that have been conditioned with either IFN-γ or LPS in isolation. While both of these stimuli produce “inflammatory” effector cells, differences exist, such as the higher iNOS production of the M(IFN-γ) population, that may convey information useful for inter- or intra-experimental analyses (Murray et al., 2014). Similarly, macrophages with anti-inflammatory, “M2-like” phenotypes may demonstrate variable cytokine profiles and metabolic preferences that are more accurately and meaningfully described by comparison with naïve macrophages stimulated with IL-4, immune complexes, or IL-10, than with a broad “M2” designation. In these instances, Murray et al. suggest using the terminology M(IL-4), M(Ic), or M(IL-10) respectively (Murray et al., 2014). As further subgroups are discovered, this system would provide a more empirical framework for the description, interpretation, and comparison of new discoveries. Given the inconsistent terminology of existing literature, for the purpose of this study the generalised terms of “M1-type” and “M2-type” will be used to describe macrophage populations with predominantly pro- or anti-inflammatory phenotypes respectively, in conjunction with specific, relevant subpopulations as described.

The necessity and plausibility of such a wide range of macrophage functions becomes apparent when considering the breadth of their involvement and distribution.
throughout the body. For example, homeostatic functions such as the resorption of bone by osteoclasts and the unique immune tolerance of intestinal macrophages required by their constant interaction with commensal bacteria understandably necessitate different expression profiles and functionalities (Popoff & Schneider, 1996; Smythies et al., 2005). While this remarkable functional potential enables effective immune defence in a wide range of contexts, it also presents many routes for the manipulation or errant activation of undesirable phenotypes if not carefully monitored.

1.2 Macrophage-mediated inflammation and metabolic disease

The potential for immune dysregulation, and especially that of macrophages, is of central concern to the growing field of immunometabolism. Immunometabolism studies immune responses in the context of metabolic processes (Mathis & Shoelson, 2011). This field is built upon the observation that inflammation is a hallmark of chronic metabolic disorders such as obesity and diabetes, and these pro-inflammatory environments in turn mobilise and activate immune cells (Mathis & Shoelson, 2011). The first suggestion of crosstalk between these seemingly disparate systems was raised by Gökhan Hotamisligil, who observed that many immune-associated cytokines, including IL-1, IL-6, interferon, and TNFα, have effects on lipid metabolism and so sought to investigate their potential involvement in obesity (Hotamisligil, Shargill, & Spiegelman, 1993). In 1993, it was reported that adipose tissue in obese rodents exhibited an increase in TNFα expression (Hotamisligil et al., 1993). This induction was associated with the systemic insulin resistance seen in obesity, leading to the hypothesis that TNFα played a role in the high incidence of co-morbid diabetes. TNFα was later shown to mediate insulin resistance by inducing the phosphorylation of insulin receptor
substrate 1, which contributed to inhibiting the activity of the insulin receptor (Hotamisligil et al., 1996). In turn, insulin resistance has since been shown to reinforce this vicious cycle by limiting uptake and storage of pro-inflammatory, circulating FFAs, thus increasing susceptibility to inflammatory disease (Guilherme, Virbasius, Puri, & Czech, 2008).

These data integrate metabolism and immune response, and position the immune system as a sensor for metabolic dysregulation. By virtue of their function as immune sentinels and their wide range of effector phenotypes, it is unsurprising that macrophages are strongly associated with many inflammatory environments and metabolic disease states (S. K. Biswas & Mantovani, 2012; Murray & Wynn, 2011). However, the nature of this involvement has been less obvious, as macrophages have controversially been identified as both mediators of disease progression and disease resolution (Murray & Wynn, 2011). Approaching the question of macrophage phenotype selection from the perspective of immunometabolism, and the effects that altered metabolic environments may have on macrophage responses, may help us reach a greater understanding of the mechanisms leading to disease progression and how these cells may be manipulated for therapeutic benefit.

1.2.1 Obesity

One of the first observations of a connection between immunity and metabolism was made during research into obesity, with these discoveries laying the foundation for the field of immunometabolism (Hotamisligil et al., 1993; Hotamisligil et al., 1996). Obesity affects nearly a third of adults in New Zealand (Ministry of Health, 2013) and is associated with chronic, low-grade inflammation perpetuated by the recruitment,
proliferation, and inflammatory polarisation of macrophages within adipose tissue (Lumeng, Bodzin, & Saltiel, 2007; Weisberg et al., 2003). Obese individuals carry a high risk of developing multiple obesity-associated conditions such as cardiovascular disease, type 2 diabetes, and cancer (Calle, Rodriguez, Walker-Thurmond, & Thun, 2003; Esser, Legrand-Poels, Piette, Scheen, & Paquot, 2014; Hubert, Feinleib, McNamara, & Castelli, 1983). Adipose tissue macrophage (ATM) numbers have been found to directly correlate with increasing body mass index and adipocyte size. This suggests that understanding and controlling pro-inflammatory macrophage activation and expansion may assist in treating obesity and limiting the risk of developing associated disease (Heilbronn & Campbell, 2008; Weisberg et al., 2003).

1.2.1.1 Adipose tissue metabolism in health

In healthy individuals, adipose tissue tightly regulates both systemic glucose and lipid balance (Kalderon, Mayorek, Berry, Zevit, & Bar-Tana, 2000; Rosen & Spiegelman, 2006). This is controlled through the production of “adipokines,” peptide hormones and cytokines released from adipocytes, and the removal of free fatty acids from the circulation through intercellular storage as triglycerides (Guilherme et al., 2008). These adipokine hormones act by regulating feeding behaviours in response to energy demands (leptin is known as the “satiety hormone”), modulating cellular metabolic rate, and enhancing sensitivity to insulin signalling. A key regulator of healthy adipocyte function is peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor that directs adipocyte maturation, the synthesis and storage of triglycerides, and the production of adipokines such as the insulin-sensitising adiponectin (Guilherme et al., 2008). It has also been shown to negatively regulate inflammatory cytokine production (Siersbaek, Nielsen, & Mandrup, 2010).
Two extremes illustrating the essential role adipose tissue plays in whole-body energy balance are the instances of obesity and lipodystrophy. In obesity, adipocyte function is impaired by the action of pro-inflammatory cytokines, leading to dysregulated insulin signalling, insulin resistance, and systemic inflammation (Guilherme et al., 2008). In lipodystrophy, where a person is unable to maintain adequate stores of adipose tissue, the innately reduced levels of circulating adipokines lead to an excess of circulating triglycerides and fatty acids. This mimics the impaired functionality of obese adipocytes and thus leads to similar inflammatory insult and reduced insulin sensitivity (Guilherme et al., 2008).

1.2.1.2 Macrophage proliferation and recruitment during obesity

During the progression of adipose tissue from lean to obese, the adipokine profile shifts to that of a more inflammatory environment and local macrophage numbers rise (FIG 1.3) (Weisberg et al., 2003). This is believed to result from an increased production of immunomodulatory adipokines such as monocyte chemoattractant protein 1 (MCP-1) in response to stress induced by the swelling cells (Sartipy & Loskutoff, 2003). Early studies using engrafted irradiated mice indicated that macrophage numbers increased due to the recruitment of bone marrow derived macrophages, with little contribution from the tissue-resident population (Weisberg et al., 2003). These data have recently been challenged by Amano et al., who argue that depletion of blood monocytes has little impact on the increasing numbers of adipose tissue macrophages (ATMs) and that proliferation of resident cells is a significant contributor to the ATM population in obesity (Amano et al., 2014). They further report that the obese adipose tissue microenvironment specifically induces ATM proliferation and that this effect is
dependent on MCP-1, an observation replicated in adipose tissue explants treated with this protein. Both groups, however, agree that macrophage numbers in other tissues are not elevated, except where those systems have been invaded by adipose tissue.

**FIGURE 1.3: Polarisation of ATMs over the course of increasing adiposity**

1. In lean individuals, ATMs are polarised toward an M2 state, expressing anti-inflammatory molecules such as the enzyme arginase and cytokine IL-10.  
2. As adiposity progresses, adipocytes undergo hypertrophy and release MCP-1, inducing M1-phenoswitching of ATMs and both recruitment and local proliferation of M1-type macrophages. In this early stage, M2-type ATMs are still present and partially abrogate the inflammatory effects of M1-type ATMs.  
3. In obesity, M1-type ATMs predominate, leading to high levels of inflammatory molecules, the release of FFAs and inflammatory cytokines from adipocytes, and a progressive reduction in insulin sensitivity.  
4. These factors contribute to a state of chronic inflammation and the development of obesity-associated diseases. Abbrev. ATM, adipose tissue macrophage; FFA, free fatty acids; MCP-1, monocyte chemotactic protein-1.

Compared to the sparse numbers seen in lean animal models, ATMs found during obesity appear as large aggregates of classically activated inflammatory cells, reminiscent of chronic inflammatory diseases such as rheumatoid arthritis (Weisberg et al., 2003). ATMs are the primary source of inflammatory cytokines found in adipose tissue, creating a positive feedback loop that maintains a pro-inflammatory state and contributes to impaired insulin signalling (Heilbronn & Campbell, 2008). These data are summarised in
1.2.1.3 Insulin resistance

Obesity is now recognised as a major risk factor for developing insulin resistance and type 2 diabetes, with its underlying inflammatory processes both contributing to and symptomatic of reduced insulin sensitivity (Guilherme et al., 2008). Insulin is a hormone that controls cellular glucose and lipid metabolism by signalling through the insulin receptor, a protein tyrosine kinase, and its associated insulin receptor substrates (Le Marchand-Brustel et al., 2003). Insulin receptors are expressed on the surface of many cells, including adipocytes, hepatocytes, muscle cells, and immune cells (Han, Patterson, Speck, Ehses, & Levings, 2014).

This essential signalling system can be blocked by both inflammatory cytokines and free fatty acids (Le Marchand-Brustel et al., 2003). These molecules manipulate the cycle’s natural negative feedback mechanisms and inactivate insulin receptor substrate proteins by triggering their phosphorylation. The pro-inflammatory cytokine TNFα, produced by both adipocytes and inflammatory ATMs during obesity, delivers a double dose of interference. As its levels rise it prevents PPARγ signalling within adipocytes (Guilherme et al., 2008). This leads to reduced triglyceride storage and increased secretion of free fatty acids, feeding back into the cycle of enhanced insulin resistance. Reduced insulin sensitivity leads to poor glucose and FFA uptake, resulting in altered cellular metabolism that further compounds both adipocyte and immune cell dysregulation (Guilherme et al., 2008; Han et al., 2014).
Thus, obesity is clearly a disease at the crossroads of immune dysfunction and metabolic deregulation, with the intricate interplay of inflammatory immune cells and metabolic tissues resulting in a systemic energy imbalance and altered immune functionality. Macrophages play a distinct role in this cycle and their modulation may be one key to improving patient outcomes (Chawla et al., 2011).

1.2.2 Cancer

Cancer is characterised by the rapid growth and proliferation of transformed cells, often in suboptimal environments. The ability of transformed cells to survive and even thrive in hypoxic, toxic, or low-nutrient conditions is mediated by their ability to remodel their environment to best suit their needs (Chanmee, Ontong, Konno, & Itano, 2014). Central to this ability is the manipulation of local macrophages and exploitation of their various metabolic modes for the benefit of tumour establishment, immune evasion, and metastasis (Chanmee et al., 2014).

1.2.2.1 TAM recruitment and polarisation

The relationship between macrophages and tumours is complex. In the early stages of tumour development, IL-12$^{\text{high}}$IL-10$^{\text{low}}$ M1 macrophages provide an active anti-tumoral defence (Chanmee et al., 2014). However, as cancer develops, tumour-derived factors reprogram infiltrating cells to a more M2-like, IL-12$^{\text{low}}$IL-10$^{\text{high}}$ phenotype. These cells are called tumour-associated macrophages (TAMs).
Unlike the prototypical M1 and M2 macrophages, TAMs have both pro- and anti-inflammatory characteristics that shift in response to the properties of the tumour mass (Chanmee et al., 2014). This phenotype is modulated by a microenvironmental milieu of both tumour-derived and TAM-derived molecules such as the monocyte chemoattractant MCP-1 and high molecular weight hyaluronan (Kobayashi et al., 2010; Qian et al., 2011).

Recruitment of TAMs to tumours occurs through a number of routes. Cancer cells have been observed expressing MCP-1, while the hypoxic centres of tumour masses express high levels of many other known or predicted monocyte/macrophage attracting molecules including vascular endothelial growth factor (VEGF) and endothelin (Murdoch, Giannoudis, & Lewis, 2004; Qian et al., 2011). Synergistically, induction of hypoxia inducible factor-1α (HIF-1α) in TAMs drives the expression of further chemotactic response proteins (Chanmee et al., 2014). Once TAMs reach the tumour, local production of mitogen-activated protein kinase phosphatase-1 inhibits their ability to respond to distal chemokines, resulting in an accumulation of tumour-supportive TAMs (Chanmee et al., 2014; Grimshaw & Balkwill, 2001).

1.2.2.2 Pro-tumoral effects of TAMs

TAMs benefit tumours in a number of ways. The hypoxic tumour environment stimulates HIF-1α-driven production of the potent pro-angiogenic mediator VEGF-A by macrophages (Lewis, Landers, Underwood, Harris, & Lewis, 2000). This leads to further macrophage recruitment, tumour vascularisation, and ultimately cancer growth (Korsisaari et al., 2007). Pro-inflammatory cytokines such as IL-6 stimulate tumour proliferation and suppress apoptosis (Liao et al., 2014; H. Wang et al., 2009). Tumour invasion and metastasis are supported through a wide range of TAM-derived proteases,
such as matrix metalloproteinases (MMPs), and epidermal growth factors that degrade the ECM and weaken endothelial barriers (Chanmee et al., 2014). Finally, and possibly most confoundingly, TAMs also act to dampen the immune response to cancer. Through the production of their own anti-inflammatory cytokines, TAMs assist in the reprogramming of invading macrophages from an anti-tumoral to pro-tumoral phenotype, while also reducing the overall immunostimulatory potential of the tumour (Chanmee et al., 2014; Hao et al., 2012). Some of these cytokines, such as CCL22, attract regulatory T cells, a population of immunosuppressant lymphocytes found in large number in some tumours (Hao et al., 2012). In the instance where cytotoxic T cells migrate to the tumour, association with TAMs has been shown to reduce T cell proliferation, cytotoxic capability, and production of inflammatory cytokines IFN-γ and IL-2, leading to reduced anti-tumour activity (Kuang et al., 2009).

1.2.2.3 Targeting TAMs to treat cancer

Macrophages are thus key determinants in the battle between the immune system and cancer. While tumours exploit macrophage plasticity for their own benefit, current strategies in cancer research believe this process can be manipulated for therapeutic gain (Heusinkveld & van der Burg, 2011). For example, the induction of a pro-inflammatory microenvironment with high levels of M1-polarising cytokines is anticipated to shift TAMs away from their pro-tumoral functions and activate an anti-tumoral phenotype. Cancer vaccines containing TLR agonists or antibodies directed at IL-10 receptor, used during macrophage polarisation to an M2-type, have demonstrated immunostimulatory capacity and a resulting induction of anti-tumoral, M1-type macrophages (Cluff, 2010; Guiducci, Vicari, Sangaletti, Trinchieri, & Colombo, 2005).
1.2.3 Neurodegeneration

Neuroinflammation and neurodegeneration describe a group of disorders that result in the progressive deterioration of neurons, resulting in deleterious effects on cognition and motor control (Cai et al., 2012). Classic examples include Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease. While the connection between these illnesses and immune or metabolic disorders is not intuitively clear, it is becoming apparent that there is a distinct contribution of both immune cell reprogramming and systemic metabolic dysregulation in many, if not all, of these diseases (Cai et al., 2012). This is further illustrated by the correlation between metabolic diseases such as type 2 diabetes and an increased risk of developing neurodegenerative diseases such as Alzheimer’s disease (Cai et al., 2012).

1.2.3.1 Macrophage activation and neuroinflammation

Neuroinflammation may result from a variety of insults, including infection, toxins, or physical trauma (Krause & Müller, 2010). In these instances, infiltrating macrophages and resident microglia are recruited to clear pathogens, dead or dying cells, and toxins. In disease, these macrophages are stimulated by endogenous factors or unknown pathways that do not properly resolve, leading to local damage to the surrounding tissue from the resulting elevation in inflammatory mediators (Krause & Müller, 2010).

Amyloid-β peptides are found naturally in the healthy brain, though their function in health is not clear (Perlmutter, Barron, & Chui, 1990). In Alzheimer’s disease, these peptides aggregate into immunostimulatory plaques (Perlmutter et al., 1990). The schematic in Figure 1.4 describes how microglia, macrophages of the brain, can be
stimulated by inflammatory stress caused by both neuronal loss and the production of amyloid-β plaques in brains affected by Alzheimer’s disease. The resulting microglial activation leads to inflammatory cytokine expression and metabolic reactions producing reactive oxygen species (ROS), both of which have neurotoxic effects leading to the destruction of healthy neurons and further amplification of the inflammatory cycle (Krause & Müller, 2010).

**FIGURE 1.4: Inflammation and neurotoxicity in the Alzheimer’s brain**

1. During the development of Alzheimer’s disease, dying neurons and the formation of amyloid-β plaques contribute to increasing levels of inflammatory stress, which in turn exacerbate amyloid-β production. 2. Inflammatory stress leads to the activation and pro-inflammatory polarisation of resident microglia. 3. Activated microglia produce pro-inflammatory mediators such as cytokines and ROS, creating a neurotoxic environment that leads to neuron death and further amplification of the inflammatory cascade. Abbrev. Aβ, amyloid-β; ROS, reactive oxygen species.

Several studies have observed an increased number of pro-inflammatory macrophages clustered in close proximity to plaques within Alzheimer’s brains (Krause & Müller, 2010). These clusters are believed to primarily consist of peripherally recruited macrophages. It has also been suggested that phenotypic differences exist between recruited and resident microglia, with newly recruited microglia being more highly
phagocytic (Krause & Müller, 2010). While high phagocytic potential facilitates plaque removal, the associated increase in inflammatory mediators is highly damaging, especially when plaques are not efficiently cleared and inflammation is perpetuated. There also appears to be an effect of duration of inflammatory stimulus on microglial function (Schlachetzki & Hull, 2009). While acute stimulation generates highly efficient phagocytes that reduce plaque load in an Alzheimer's disease animal model, sustained stimulation leads to an increase in plaque production. In addition, the ability of inflammatory cytokines to attenuate microglial phagocytic potential has also been described (Koenigsknecht-Talboo & Landreth, 2005). Together, these data suggest that as inflammation persists in Alzheimer's disease, the phagocytic potential of microglia at the site dampens while the neurotoxic microenvironment is maintained, contributing to an inflammatory population with progressively limited capacity to remove a growing number of plaques.

1.2.3.2 Alzheimer's disease as type 3 diabetes?

A surprising connection between Alzheimer's disease and obesity is the repeated observation of dysregulated insulin signalling or reduced insulin availability in the brains of patients with Alzheimer's disease (de la Monte & Wands, 2008). While some literature attempts to attribute this connection to the neurological impairment sometimes symptomatic of type 2 diabetes, others argue that the effects seen in type 2 diabetes do not fully recapitulate the characteristic symptoms of Alzheimer's disease. Similarly, the metabolic impairment seen in Alzheimer's disease is not fully explained by co-morbidity with type 2 diabetes. Instead, these data have led to a proposed classification of Alzheimer's disease itself as “type 3 diabetes” (de la Monte & Wands, 2008).
Support for this classification has been investigated extensively by the research group lead by Suzanne de la Monte. Through the post-mortem study of brains from patients with varying degrees of Alzheimer’s disease severity, they observed a significant reduction in genes coding for insulin, insulin-like growth factors, and insulin-like growth factor receptors, that correlated with increasing disease severity (Steen et al., 2005). They were also able to replicate and validate all key Alzheimer’s disease characteristics, such as brain atrophy and reduced capacity for learning and memory, in a murine model produced through the intracerebral injection of a compound commonly used to induce diabetes (de la Monte & Wands, 2008; Lester-Coll et al., 2006). The type 3 diabetes model was limited to the brain, having normal levels of blood glucose and insulin, and exhibited healthy pancreatic structure and systemic insulin sensitivity outside of the degenerating cranial compartment. These data were recapitulated in their observations of the human brain samples but with the added observation of elevated levels of pro-inflammatory molecules and microglial markers, indicating the site-specific immune response to the induced diabetic state (de la Monte & Wands, 2008).

While having an impaired immune system has been shown to reduce cognitive function, growing evidence now suggests that aberrant immune activation within the brain leads to the progression of neurodegenerative disease (Cai et al., 2012). Given recent developments implicating both innate immune macrophages/microglia and insulin signalling in neurodegenerative diseases, approaching these disorders from the position of metabolic dysfunction may shed new light on their underlying processes and potential routes of treatment. It has already been noted that patients undergoing treatment for diabetes or insulin resistance have reported improvements in the symptoms of co-morbid neuroinflammatory diseases (Aviles-Olmos, Limousin, Lees, & Foltynie, 2013;
Martin et al., 2009; Watson et al., 2005). This suggests that neurodegenerative disorders and metabolic diseases may share a common, metabolism-based macrophage pathology and offers hope that further understanding macrophage activation may reveal new strategies to treat these diseases.

1.3 Metabolic pathways supporting macrophage activation states

The ability of macrophages to respond to microenvironmental cues in both health and disease with such varied functionalities suggests a broad range of metabolic variety and sensitivity. This introduces another aspect of immunometabolism – the effect of intracellular metabolism on immune cell phenotypes. By altering preferences for internal metabolic pathways, a macrophage may direct the production of a broad range of signalling and effector compounds. Through a greater understanding of these pathways and methods of their regulation, it may be possible to manipulate macrophage phenotypes in instances of inappropriate response.

1.3.1 Cytosolic pathways

1.3.1.1 L-arginine

L-arginine metabolism is often used as a key discriminator between M1 and M2 polarisation (Bronte & Zanovello, 2005). Pro-inflammatory stimuli, including IFN-γ and LPS signalling through IFN-R and TLR4 respectively, result in IRF-1 production and NFκB activation in M1 macrophages (Lowenstein & Padalko, 2004). These transcription
factors go on to promote transcription of an inducible form of nitric oxide synthase (iNOS) that catabolises L-arginine in combination with molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to generate the highly reactive and antimicrobial molecule nitric oxide (NO). In contrast, the anti-inflammatory program of M2 macrophages results in the synthesis of arginase 1 (Arg-1) following STAT6-dependent IL-4 signalling (M. J. Gray, Poljakovic, Kepka-Lenhart, & Morris, 2005). Arg-1 acts on L-arginine to produce ornithine, which is then further metabolised into polyamines and proline for use in tissue repair and cell proliferation. These complex interactions are illustrated in **Figure 1.5**.

![Figure 1.5: The competing pathways of L-arginine utilisation in differently polarised macrophages](image)

Pro- and anti-inflammatory stimuli regulate the activity of iNOS and Arg-1, two enzymes that share the substrate L-arginine. These enzymes additionally directly inhibit each other through a number of regulatory pathways. Activity of iNOS primarily results in the production of reactive nitric oxide, while Arg-1 contributes precursors for the biosynthetic demands of growth and repair, including spermidine and spermine. Abbrev. iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; NOHA, N⁶-hydroxy-L-arginine.

Competition for use of L-arginine in each of these systems extends beyond simple enzyme-substrate binding efficiencies, with the iNOS intermediate N⁶-hydroxy-L-
arginine (NOHA) additionally inhibiting Arg-1 activity and the downstream product of Arg-1, spermine, preventing translation of iNOS mRNA (FIG 1.5) (Boucher, Moali, & Tenu, 1999). The pathogen *Helicobacter pylori* has been shown to manipulate this sensitive balance through constitutive expression of its own arginase enzyme, resulting in reduced NO production from activated, attacking host macrophages (Bussiere et al, 2005; Gobert et al, 2001).

1.3.1.2 Glycolysis

Glycolysis is the cytosolic breakdown of glucose into two molecules each of pyruvate, ATP, and reduced nicotinamide adenine dinucleotide (NADH) (Lunt & Vander Heiden, 2011). Pyruvate is typically then translocated into the mitochondria for additional ATP generation through the citric acid cycle (CAC) and oxidative phosphorylation (OXPHOS), though in some circumstances this connection is bypassed as cytosolic energy production takes preference (FIG 1.6).

While M2 macrophage metabolism typically mirrors that of unstimulated macrophages in its moderate rate of glycolysis and high oxygen consumption (indicating high levels of subsequent oxidative phosphorylation), M1 phenotypes exhibit a distinct increase in glycolytic metabolism and a reduction in oxygen consumption, even in the presence of adequate oxygen supply (Palsson-McDermott & O’Neill, 2013). This results in the fermentation of pyruvate to lactate in the cytosol, bypassing the CAC and oxidative phosphorylation in the mitochondria (FIG 1.6). A metabolic skew towards glycolysis may provide energy more rapidly, though it could also be considered wasteful as it produces only two ATP molecules per molecule of glucose, rather than the full 36 ATP possible through oxidative phosphorylation (Lunt & Vander Heiden, 2011). It does,
however, produce a large number of intermediates that are supportive of the high rate of biosynthesis required for growth and proliferation (Lunt & Vander Heiden, 2011). Additionally, due to its lower oxygen requirement, glycolysis provides an obvious benefit in hypoxic conditions such as tumours (Palsson-McDermott & O’Neill, 2013). In fact, M1 macrophage metabolism shares many similarities with the “Warburg effect” described in transformed cells. The Warburg effect was first described by Otto Warburg in 1927 following the observation that cancer cells primarily produce energy through glycolysis, rather than oxidative phosphorylation, even in conditions of normoxia (Warburg, Wind, & Negelein, 1927). Originally explained as being a result of mitochondrial damage, more recent studies have shown that mitochondrial respiration is not necessarily dampened following transformation. Rather, glycolysis is just dramatically promoted by comparison (S. Biswas, Lunec, & Bartlett, 2012; Palsson-McDermott & O’Neill, 2013).

1.3.1.3 Pentose phosphate pathway

The pentose phosphate pathway (PPP) is of key importance to the production of reactive nitrogen species by M1 macrophages (Ghesquière, Wong, Kuchnio, & Carmeliet, 2014). This pathway produces NADPH through the conversion of glucose-6-phosphate into five-carbon sugars. NADPH is then used by iNOS, in combination with L-arginine and molecular oxygen, to produce NO (Aktan, 2004). Meanwhile, M2 macrophages circumvent this pathway through the production of CARKL, a protein that inhibits downstream elements of the PPP (Blagih & Jones, 2012; Haschemi et al., 2012). When responding to pro-inflammatory stimuli such as LPS, downregulation of CARKL expression occurs as the macrophage takes on a more M1-type phenotype (Ghesquiere et al., 2014).
1.3.2 Mitochondrial pathways

1.3.2.1 Oxidative phosphorylation and the citric acid cycle

Mitochondria are frequently labelled as the “powerhouse” of the cell – responding to the cell's energy needs and producing ATP via oxidative phosphorylation (OXPHOS) (X. Li et al., 2013). OXPHOS is the sequence of aerobic reactions that utilise electron donors produced through the citric acid cycle (CAC) in order to establish and maintain a proton gradient across the inner mitochondrial membrane that enables ATP regeneration (Shapiro, Lutaty, & Ariel, 2011). The CAC, also known as the Krebs or tricarboxylic acid cycle, occurs predominantly in the matrix of the mitochondrion and metabolises acetyl coenzyme A, the predominant fate of pyruvate translocated from the cytosol following glycolysis (FIG 1.6). Oxidation of acetyl coenzyme A through the CAC produces the reducing cofactors NADH and reduced flavin adenine dinucleotide (FADH₂), which then feed into OXPHOS at the mitochondrial membrane (Shapiro et al., 2011).

1.3.2.2 Fatty acid catabolism

Another important mode of mitochondrial metabolism in macrophages is fatty acid catabolism. This process provides an alternative source of acetyl coenzyme A from the breakdown of fatty acids (FIG 1.6).
FIGURE 1.6: M1 vs. M2 macrophage metabolism

**M1** macrophages are characterised by increased rates of glycolysis (purple), high production of ROS, NADPH production through the PPP (green) and the metabolism of L-arginine to L-citruline and NO via iNOS. Fatty acid oxidation (orange) also feeds into the CAC (pink) and subsequent OXPHOS in M1 macrophages, but at a lower rate. **M2** macrophages preferentially utilise fatty acid oxidation (orange) and exhibit a reduced rate of glycolysis (purple), while driving L-arginine metabolism towards L-Ornithine production and its subsequent products and inhibiting the PPP through CARKL. Abbrev. Acetyl-CoA, acetyl-coenzyme A; Arg-1, Arginase-1; ATP, adenosine triphosphate; CAC, citric acid cycle; FFA, free fatty acids; iNOS, inducible nitrous oxide synthase; NO, nitrous oxide; OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; mROS, mitochondrial reactive oxygen species.

Over the course of inflammation, as glycolytic, M1 macrophages become less necessary, M2-polarising stimuli increase (Shapiro et al., 2011). Signalling through PPARγ leads to the transcription of genes encoding important elements of β-oxidation and M2 macrophage effector functions, including *carnitine palmitoyltransferase I* (*cpt1*), encoding a key enzyme involved in fatty acid transport into the mitochondria (Mashek, Li, & Coleman, 2007). Fatty acids are first activated in the cytosol by acyl-CoA synthetase before being transferred via carnitine-dependent transporters (including Cpt1) into the mitochondria to be oxidised. Increased fatty acid oxidation fuels the CAC and OXPHOS and the cell shifts, as a whole, to a more mitochondrial metabolic profile (Shapiro et al., 2011). These links between metabolic mode and macrophage phenotype have been strengthened through studies that show inhibition of OXPHOS or β-oxidation can actually block M2-related gene transcription and prevent M2-stimulated macrophages from
taking on the accompanying phenotype (Vats et al., 2006). Similarly, recent data from our laboratory correlated a reduced capacity of macrophages to take up FFAs with decreased β-oxidation and impaired bactericidal activity (Hall et al., 2013). A simplified representation of the key metabolic pathways used by classic M1 and M2 macrophages can be seen in Figure 1.6.

However, somewhat paradoxically, Hall et al. have recently observed that in vivo fatty acid trafficking to the mitochondria and subsequent β-oxidation was distinctly increased in LPS-stimulated, M1-polarised zebrafish macrophages (Hall, Sanderson, Crosier, & Crosier, 2014). This observation required LPS stimulation and was dependent on Cpt1. They linked this metabolic process to an increased production of mitochondrial reactive oxygen species (mROS) and improved bactericidal activity, an effect that was further enhanced by exogenous supply of free fatty acids. This serves as an additional example of the wide range of metabolic fine-tuning at play behind macrophage functionality. This is the first study to examine the metabolic mode of macrophages within a whole animal, live-imaging system, enabling observation of the effects of an intact microenvironment (Hall et al., 2014).

1.3.3 Metabolic basis of macrophage memory

It has long been the belief that one of the defining differences between the innate and adaptive immune branches is that the specificities of innate immunity are genetically predetermined and comparatively non-specific, while adaptive immune cells can “learn” to acquire stronger, directed responses (Medzhitov & Janeway, 2000). A shift in this way of thinking has recently been presented, describing a method of “learned” immune memory in macrophages. Building from the observation that highly specific vaccines
could confer protection against a range pathogens, scientists at the Radbound University Medical Center in the Netherlands sought to investigate instances of improved immunity in the absence of an adaptive immune system (Netea, Quintin, & van der Meer, 2011). Observations of an enhanced response to subsequent infection within invertebrate model systems following a primary challenge are particularly supportive, as these organisms do not have adaptive immune capacity (Kurtz & Franz, 2003; Netea et al., 2011). A similar protective effect was subsequently modelled in mice lacking adaptive immunity and this protection was determined to be monocyte-driven (Quintin et al., 2012).

How then, are these memory traits developed? When studying the innate immune response of lymphocyte-depleted mice to re-infection with *Candida albicans*, Quintin and colleagues investigated the potential role of epigenetic regulation in facilitating the increased transcription of inflammatory genes they observed in re-stimulated animals (Quintin et al., 2012). They noted that inhibition of histone methyltransferases abrogated this phenotype, suggesting a positive correlation between histone methylation and monocyte training. Further study of this connection revealed that epigenetic modifications during monocyte-to-macrophage differentiation lead to increased expression of enzymes involved in the CAC as well the metabolism of amino acid substrates, strongly positioning metabolic adaptation as a mechanism of innate immune training (Saeed et al., 2014). This hypothesis is strongly supported by further work by the same group that identified epigenetic upregulation of genes in the glycolysis pathway of macrophages responding to immune training that lead to a metabolic shift from oxidative phosphorylation to aerobic glycolysis (Cheng et al., 2014). This shift was found to be dependent on mTOR-HIF-1α signalling, an established pathway that directs T cell differentiation in response to environmental cues and is increasingly believed to perform
a similar function in innate immune cells (Chi, 2012). However, it is important to note that, unlike traditional notions of immune memory where target-specific acquired information and ability persist over multiple cell generations, this recently described innate immune memory is not pathogen specific and may more accurately represent an altered baseline reactivity (Netea et al., 2011). As such, the term “trained memory” has been proposed. Nevertheless, these studies strengthen the evidence proposing a role for intracellular metabolism in macrophage function, trained memory, and response to environmental stimuli (Cheng et al., 2014; Netea et al., 2011; Quintin et al., 2012; Saeed et al., 2014).

1.4 mROS and macrophage function

Key effector molecules of pro-inflammatory macrophage functions are the reactive oxygen species produced by mitochondrial metabolism (Hall et al., 2014). During infection, the efficiency of macrophage phagocytosis and subsequent pathogen killing depends on coordinated cross-talk between the mitochondria, cell, and external microenvironment. In addition to direct antimicrobial contributions, mROS act as key messengers relaying these instructions.

1.4.1 Antimicrobial activities

Production of mROS is often described as a “by-product” of OXPHOS. It is estimated that 0.2% - 2.0% of O$_2$ consumed by mitochondria "leaks" out this way (X. Li et al., 2013). The most prevalent species is the short-lived superoxide ion (O$_2^-$), which can be converted to
hydrogen peroxide (H₂O₂) locally by mitochondrial superoxide dismutase (Sena & Chandel, 2012). Their short half-life and the careful scavenging from protective enzymes in the mitochondria result in a very low basal level of mROS (Venditti, Di Stefano, & Di Meo, 2013). Rapid scavenging is crucial for cell health as these molecules can be highly toxic, capable of directly oxidising nucleic acids, proteins, and lipids (X. Li et al., 2013). However, due to their potency, even small changes in their local concentration can have significant effects, as in the case of pathogen clearance (West et al., 2011).

Direct actions of mROS at higher concentrations can enhance destruction of internalised pathogens (Sena & Chandel, 2012). Given the short half-life of these molecules, one would expect that production at a rate fast enough to accumulate a direct effect on pathogens would result in high levels of oxidative stress and damage to the mitochondria (Sena & Chandel, 2012). However, following signalling through TLR pathways generated by infectious stimuli, macrophages have been observed to recruit mitochondria to phagosomes, where local mROS production significantly improves bactericidal phagosome function while limiting the potential for damage to the macrophage (West et al., 2011). This process is depicted in Figure 1.7.
FIGURE 1.7: Recruitment of mitochondria to the phagosome increases intracellular killing efficiency
Following phagocytosis of bacteria by macrophages, TLRs in the membrane of the phagosome are activated by the internalised pathogens. This results in signalling cascades that recruit mitochondria to the phagosome and upregulate production of mROS. The local concentration of mROS is greatly increased and enhances phagosome killing.

West and colleagues observed that mitochondrial recruitment in murine macrophages followed signalling through TLRs recognising bacterial antigen (West et al., 2011). This was mediated through translocation of tumour necrosis factor receptor-associated factor 6 (TRAF6), a TLR adapter molecule, to the mitochondria. This signalling pathway induced mROS production in addition to the mitochondrial recruitment, further contributing to the localised bactericidal impact (West et al., 2011).

1.4.2 Inflammasome activation

Molecular events that lead to increased production of mROS in macrophages can be considered to fall into one of three categories: danger signals (e.g. infection), tissue damage, or metabolic stress (e.g. hypoxia) (Sena & Chandel, 2012). In the instance of an
immune response to infection, pro-inflammatory molecules such as TNFα and IFN-γ have been linked to elevated mROS production (X. Li et al., 2013). The resulting increased levels of mROS act on several signalling pathways within the cell in order to propagate a pro-inflammatory response. These effects are often mediated through redox regulation: the functional modification of target molecules by ROS, frequently through oxidation of reactive cysteine residues (Finkel, 2012). Redox regulation results in the activation, inactivation, or alteration of downstream pathways (Brune et al., 2013). A key example of this mROS-mediated activation of pro-inflammatory responses is activation of the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome (Zhou, Yazdi, Menu, & Tschopp, 2011). The inflammasome is a multi-protein, intracellular structure for the maturation and secretion of inflammatory cytokines in response to “danger” signalling (Schroder & Tschopp, 2010). Instigation of inflammasome activity by mROS leads to activation of caspase-1 and the consequent maturation of inflammatory cytokines pro-IL-1β and pro-IL-18 (Zhou et al., 2011). Of note, the NLRP3 inflammasome has also been associated with the detection of mitochondrial dysfunction resulting in increased oxidative stress (Zhou et al., 2011). When the mitochondrial membrane potential was chemically disturbed, resulting in robust increases in intracellular mROS, a dose-dependent increase in secreted IL-1β was also detected (Zhou et al., 2011). Conversely, when macrophages were treated concomitantly with ROS inhibitors, this stimulatory effect was not seen. These data establish a close relationship between mitochondrial metabolism and inflammasome activation and demonstrate another mechanism by which metabolic modes leading to increased mROS production can contribute to a systemic inflammatory response.
1.4.3 Transcriptional regulation of immune response genes

A third mechanism of mROS-regulated immune function is the mROS-driven transcription of pro-inflammatory mediators, including IL-6, Mmp9, TNFα, and IL-1β, via mitogen-activated protein kinase signalling (Bulua et al., 2011; Kamata et al., 2005; Woo, Lim, & Kim, 2004). Stimulation of a mouse macrophage line with LPS has been directly linked to increased mROS levels, which in turn are necessary for the phosphorylation, and thus activation, of p38 kinase (Woo et al., 2004). This cascade continues with p38 kinase activating the transcription factor AP-1, which then drives transcription of Mmp9 (Woo et al., 2004). Leukocytes are believed to amplify chemokine potency through MMP-mediated cleavage, enhancing migration to sites of inflammation (Khokha, Murthy, & Weiss, 2013).

1.4.4 Targeting mROS to treat inflammatory disease

Through direct cytosolic actions, the promotion of cytokine maturation, and roles in the transcriptional regulation of pro-inflammatory genes, mROS directly impacts both the intra- and extracellular macrophage environment. Having such distinct and directed effects it becomes more apparent that mROS production is not “accidental.” Rather, intentional metabolic regulation to drive or damper mROS production is one method macrophages employ to instigate phenotype switching and fine-tuning of their immune response. As such, when macrophages present undesirable phenotypes, targeting the metabolic pathways that lead to mROS production or clearance may offer a therapeutic option for their retraining (X. Li et al., 2013).
The potential of targeting mROS production as a therapeutic strategy has already been demonstrated in a laboratory setting. For example, excessive ROS production in the vasculature, kidney, and central nervous system is often characteristic of hypertension (Harrison & Gongora, 2009). Transgenic mice overexpressing ROS scavengers have been shown to resist development of this condition, while treatment with a mitochondrial-targeting antioxidant attenuated progression of existing hypertension and vascular oxidative stress (Dikalova et al., 2010; Widder et al., 2009).

### 1.5 Irg1 as an immunometabolic enzyme

A new connection between metabolic regulation and immune function was recently supported by work from our laboratory investigating the function of immunoresponsive gene 1 (irg1), the zebrafish homologue of a bacterial enzyme known to be involved in propionate metabolism (B. Chen, Zhang, & Pollard, 2003; Hall et al., 2013). The bacterial protein, methylcitrate dehydratase (PrpD), specifically dehydrates \((2\text{S},3\text{S})\)-methylcitrate to 2-methyl-\(\text{cis}\)-aconitate as part of the methylcitrate cycle. This sequence of reactions derives pyruvate from propionate to feed into the citric acid and glyoxylate cycles to provide energy and enable growth of the bacterium (Brock, Maerker, Schutz, Volker, & Buckel, 2002). While the metabolic role of IRG1 in vertebrates is less well defined, it does localise to the mitochondria and studies by Hall et al. have indicated it has a role in fatty acid β-oxidation (Degrandi et al., 2009; Hall et al., 2013). Interestingly, the most documented characteristic of IRG1 in vertebrates is its acute induction following infectious stimulation (Basler, Jeckstadt, Valentin-Weigand, & Goethe, 2006; Degrandi et al., 2009; Lee, Jenkins, Gilbert, Copeland, & O’Brien, 1995).
1.5.1 Irg1 and inflammation

Vertebrate Irg1 was first described in the murine monocyte/macrophage cell line RAW 264.7 as a highly, and specifically, LPS-inducible mRNA (Lee et al., 1995). Lee and colleagues observed that while inhibiting LPS signalling with known anti-inflammatory agents prevented Irg1 transcription, the converse chemical stimulation of secondary messenger pathways in the absence of LPS was insufficient to stimulate expression. This suggests that there are a number of regulatory and sensory mechanisms that contribute to irg1 expression. Subsequent studies in mice, zebrafish, chickens, and human have also clearly linked Irg1 expression to macrophage-lineage cells and noted its rapid induction in response to either viral or bacterial infection, as simulated by LPS, Toxoplasma gondii antigen, recombinant lymphocytic choriomeningitis virus nucleoprotein, and live oncogenic alphaherpesvirus (H. Li et al., 2006; Michelucci et al., 2013; Smith et al., 2011; Xiao et al., 2011). Expression has not been seen in non-stimulated immune cells or immune cells that infiltrate sterile wounds.

A small number of exceptions to this apparent need for infectious stimulation currently exist in the literature. One is the observation in mice that Irg1 is expressed at high levels in luminal epithelial cells of the uterus during uterine implantation (Cheon, Xu, Bagchi, & Bagchi, 2003). Blocking translation of IRG1 reduced the number of implanted embryos by 80%. Transcription of Irg1 was linked to progesterone receptor signalling, a critical network regulating uterine receptivity. At present, exactly how IRG1 contributes to this environment is unknown (Cheon et al., 2003). The mouse model has also reported expression of IRG1 in neurons, which conferred an increased resistance to neurotropic viruses (Cho et al., 2013). In human glioma however, the correlation of IRG1 expression with growth and invasiveness of the cancer has led to its proposed classification as an
oncogene (Pan et al., 2014). Expression of *Irg1* has also been observed in the epidermis of a murine model of atopic dermatitis, in the absence of detectable infectious signalling (Hall, Boyle et al., 2014). In this study, Hall and colleagues showed that Irg1 localised to the mitochondria of epidermal cells following sterile induction of cutaneous inflammation.

These studies represent the only descriptions as yet of non-macrophage-restricted *Irg1* expression. It is important to note that the discovery of IRG1 expression in a murine model of atopic dermatitis stemmed from functional studies of the zebrafish paralog of *irg1*, termed *irg1-like*, that is expressed in the zebrafish epidermis following infection (Hall et al., 2014). Numerous paralogs exist in the zebrafish genome due to a genomic duplication event that occurred in the teleost lineage 300 million years ago (Postlethwait, Amores, Cresko, Singer, & Yan, 2004). This has lead to the neo-, non-, or sub-functionalisation of many duplicated genes (Postlethwait et al., 2004). Similarly to *irg1*, the expression of *irg1-like* in larval zebrafish has been connected to increased fatty acid β-oxidation and production of mROS (Hall et al., 2014).

While IRG1 has been associated with both mitochondrial metabolism and immunity, its functions within these domains have only recently begun to be defined. Hall *et al.* have described a link that couples antimicrobial macrophage function to metabolic reprogramming through *irg1* expression (Hall et al., 2013). Following injection with live bacteria, *in vivo* induction of *irg1* expression in zebrafish larval macrophages was reported as early as 30 minutes post-stimulation. Subsequently, a metabolic skew towards increased β-oxidation in these macrophages resulted in increased fatty acid metabolism that directly contributed to elevated mROS production (Hall et al., 2013). As discussed earlier, mROS is a key signalling and effector molecule in activated immune
This is further demonstrated by the reduced bacterial clearance and resulting decreased survival of infected, Irg1-depleted larvae (Hall et al., 2013). A second, more direct antibacterial role has been ascribed by Michelucci et al. in their report that murine IRG1 directly catalyses cis-aconitate, an intermediate in the CAC, to the antimicrobial metabolite itaconic acid within macrophages (Michelucci et al., 2013). Whether, and how, these complementary processes may be linked has not yet been investigated. Adding an additional layer of complexity is the recent suggestion that IRG1 may even function differently during subsequent immune insults, with macrophages re-challenged by LPS presenting a dramatically reduced inflammatory response in an IRG1-driven-ROS-dependent manner (Y. Li et al., 2013). Contrary to studies of its early induction in primary infections, this led to immune suppression and endotoxin tolerance, implicating it in the pathology of late-stage sepsis.

1.5.2 Induction and regulation

The signalling pathways that lead to induction of Irg1 expression are not yet well understood. In zebrafish, irg1 expression has been shown to co-localise with that of cebpβ in macrophages (Hall et al., 2013). When cebpβ transcription was knocked down, irg1 expression was nearly completely ablated following infectious stimulation. The positioning of irg1 downstream of this transcription factor was additionally supported when sequence analysis identified CCAAT/enhancer binding protein beta (C/EBPβ) consensus binding sites in the promoter region of irg1 (Hall et al., 2013). C/EBPβ has been described as a master regulator of the innate immune response due to its specific, infection-induced involvement in a variety of innate immune responses, including immune cell proliferation/differentiation and the production of acute phase proteins (Poli, 1998; Yiangou et al., 1998). The rapid induction of C/EBPβ following infectious
stimulation in these scenarios suggests a role as a primary response gene: a “first responder” whose amplification occurs without the need for de novo protein synthesis. This classification has been supported by studies reporting increased expression of cebpβ mRNA following inhibition of protein synthesis, implying the existence of a regulatory transcriptional repressor (Hall et al., 2013; Matsuno et al., 1996). C/EBPβ also has documented mechanisms for controlling target gene expression through competition dynamics with the related, but functionally opposing, transcription factor C/EBPα (Jakobsen et al., 2013; H. Zhang et al., 2010). C/EBPβ itself is sensitive to regulation via phosphorylation, direct protein degradation, and altered transcription through the production of truncated proteins with autoinhibitory or dramatically reduced activation properties (Poli, 1998; Y. Wang, Singh, Xiang, Greenbaum, & Czaja, 2010). Thus, mechanisms regulating CEBPβ production and function are expected to similarly effect irg1 expression.

While cebpβ expression is necessary for the induction of irg1, it is not sufficient alone, as its ectopic overexpression does not induce irg1 expression in the absence of infection (Hall et al., 2013). Further investigations have implicated involvement of both signal transducer and activator of transcription 3 (STAT3) and glucocorticoid signalling, as inhibiting either of these pathways results in near-complete inhibition of irg1 transcription (FIG 1.8) (Hall et al., 2013). However, the effects of inhibiting glucocorticoid signalling may primarily stem from a required role in cebpβ induction, with specific glucocorticoid pathway stimulation, in the absence of LPS, resulting in amplification of cebpβ but not irg1 expression (Hall et al., 2013).
Transcription of \( \text{irg1} \) in macrophages follows infectious stimulation. Glucocorticoid signalling leads to production of the transcription factor C/EBP\( \beta \), which cooperates with phosphorylated STAT3 to drive \( \text{irg1} \) transcription. Irg1 translocates to the mitochondria where it enhances fatty acid metabolism and associated mROS production. Abbrev. CAC, citric acid cycle; C/EBP\( \beta \), CCAAT/enhancer binding protein \( \beta \); ETC, electron transport chain; FFA, free fatty acids; GR, glucocorticoid receptor; mROS, mitochondrial reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TLR, toll-like receptor.

STAT3 is a transcription factor that has been reported to act as a transactivator by amplifying signalling pathways or complexing with and enhancing the activity of other transcription factors (De Miguel, Lee, Onate, & Gao, 2003). As STAT3 has been previously described to associate with C/EBP\( \beta \) during immune signalling, this may explain the complementary actions of C/EBP\( \beta \) and STAT3 during \( \text{irg1} \) expression (Arambašic et al., 2010; H. Zhang et al., 2010). In the uterus, where \( \text{Irg1} \) expression has been shown to be dependent on progesterone, STAT3 is hypothesised to support its induction by enhancing the transcriptional activity of downstream progesterone receptor signalling (De Miguel et al., 2003). Given the similarity between the progesterone and glucocorticoid receptors and the earlier implication of glucocorticoid
signalling in \textit{Irg1} expression, STAT3 may similarly enhance downstream glucocorticoid receptor signalling (Issar, Sahasranaman, Buchwald, & Hochhaus, 2006).

Regulation of \textit{Irg1} beyond the level of transcription may also exist. Sequence analysis of the predicted murine protein identified multiple C-terminal phosphorylation sites, suggesting that IRG1 activity may be affected by phosphorylation (Lee et al., 1995). Further transcript analysis also identified sequence elements that indicate high mRNA turnover and a possible role of post-transcriptional regulation. This has since been supported by Basler et al., who noted stability differences in \textit{Irg1} mRNA produced by murine macrophage cell lines in response to either LPS or the macrophage-targeting pathogen \textit{Mycobacterium avium} subspecies \textit{paratuberculosis} (MAP) (Basler et al., 2006). The LPS-stimulated transcripts exhibited a half-life of over six hours, while the half-life of the MAP-induced mRNA was only two-three hours (Basler et al., 2006). Either of these mechanisms would aid in the quick attenuation of inflammatory signalling and response, enabling rapid entry into the resolution phase of inflammation.

1.5.3 \textit{Irg1} and disease

The elevated production of IRG1 within pro-inflammatory, macrophage-lineage cells and its ability to enhance their bactericidal functions by way of increased mROS production is intriguing considering the increasing number of disease states where macrophages contribute to disease progression, in part, through mROS production. This leads to the question of what contribution might IRG1 have to the functions of macrophages in these environments and can it be used as a biomarker for certain diseases?
Li et al. identified a population of IRG1-expressing microglia that exhibited enhanced inflammatory activity that lead to neuronal damage after they had been primed and re-challenged with neurotropic pathogens (H. Li et al., 2006). They hypothesised that IRG1+ cells contribute to the pathophysiology of neurodegenerative and neuroinflammatory disorders such as Alzheimer’s disease. These cells were shown to be resistant to apoptosis and were classified as neurotoxic due to their high production of TNFα, while their “neurosupportive” IRG1- counterparts were more sensitive to apoptosis and produced the anti-inflammatory cytokines IL-10 and TGFβ (H. Li et al., 2006). Induction of Irg1 in microglia has also been noted when modelling early stage neurodegenerative diseases and has been implicated as a potential contributor to the severity of blast-induced neural trauma (Kane et al., 2012; Thomas, Francescutti-Verbeem, & Kuhn, 2006).

At the crossroads of metabolic disease and immune disorder, mitochondria are key (Hall et al., 2014). They are responsible for both energy balance through their well-characterised metabolic programs and immune cell efficacy through directing functional plasticity. Given the connections Irg1 has shown to both immune function and mitochondrial metabolism, it presents itself as a model immunometabolic link between the often disparately-viewed fields of immunity and metabolism. Further understanding this connection may reveal new targets for the treatment of diseases mediated by inappropriate macrophage activity.
1.6 The zebrafish as a model organism

The zebrafish, *Danio rerio*, is an established model organism that provides a number of advantages over both *in vitro* and mammalian systems. Their early transparency and small size allow direct observation of *in vivo* cellular events in an intact, whole animal (Deng & Huttenlocher, 2012). When compared with mammalian animal facilities, the zebrafish is relatively inexpensive to maintain. In addition, their high fecundity, external development, and rapid maturity facilitate large-scale studies within relatively short periods of time and often with greater technical ease (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Santoriello & Zon, 2012). Zebrafish are also well-known for their genetic tractability, enabling the development of mutant, knock-out, and transgenic reporter lines for the modelling and investigation of development and disease (Santoriello & Zon, 2012).

1.6.1 Genomic manipulation techniques in zebrafish

Genetic models of human diseases have been made possible due to the zebrafish genome’s accessibility and ease of manipulation. While the phenotype-to-genotype approach of forward genetics allows discovery of unknown genetic roots of disease, the genotype-to-phenotype approach of reverse genetics allows in-depth investigation of the roles of genes of expected or known importance. Transgenesis techniques are also available to provide tools for further gene analysis. The genetic tractability of zebrafish is well-suited for these studies and a number of techniques are available for the generation of transgenic, mutant, or knock-out models.
1.6.1.1 Forward genetic screens in the zebrafish

Forward genetic screens via aqueous $N$-ethyl-$N$-nitrosourea (ENU) gamete mutagenesis provide an unbiased approach to identify genetic aberrations associated with specific phenotypes (Driever et al., 1996). Many mutant zebrafish lines that have contributed to our understanding of zebrafish immunity have been generated this way. For example, the *durif* mutant, lacking neutrophil myeloperoxidase, was discovered in an ENU screen designed to identify mutants with recessive defects in myeloid cell development (Pase, Nowell, & Lieschke, 2012).

While providing useful mutants to study important phenotypes and their underlying mechanisms, forward genetic screens also reveal the high level of conservation between zebrafish and human haematopoiesis, through the identification of evolutionarily conserved genes and pathways. In fact, the amenability of zebrafish to forward genetic screens has even revealed a number of previously unknown genetic origins of human disorders, including the role of Ferroportin1 in iron transport to the blood and Mitoferrin in iron transport into the mitochondria (Donovan et al., 2000; Shaw et al., 2006). Loss-of-function defects in Ferroportin1 leads to haemochromotosis, while mutations in Mitoferrin produce hypochromic anaemia.

1.6.1.2 Reverse genetics techniques

Multiple methods exist for the generation of specific mutant zebrafish lines. Targeting induced local lesions in genomes (TILLING) is a method that uses automated, high throughput sequence analysis to identify mutations in a specific gene of interest following large scale mutagenesis, such as by treatment with ENU (Stemple, 2004). This
technique further improves upon the forward genetics approach to ENU screens, as it is not dependent on an easily visible phenotype. Zinc finger nucleases (ZFNs) are targeted restriction enzymes created by the fusion of an N-terminal zinc finger DNA-binding domain and a C-terminal non-specific DNA cleavage domain, typically from the Fok1 nuclease (Y. G. Kim, Cha, & Chandrasegaran, 1996). Double-stranded DNA breaks are created when the cleavage domains of two ZFNs dimerise, resulting in site-specific mutagenesis via the imperfect process of non-homologous end joining or the introduction of insertions or deletions during homologous recombination (Smith et al., 2000). The necessity for dimerisation for nuclease activity reduces the likelihood of off-target cleavage but also demands a fairly large stretch of unique target sequence, as each ZFN typically recognises 9-18 bp. Transcription activator-like effector nucleases (TALENs) are a more recent genome editing tool that also rely on double-stranded DNA breaks created by dimerised Fok1 cleavage domains (Joung & Sander, 2013; Y. Kim et al., 2013). These nucleases are also directed by target-specific recognition sequences but are less expensive and less technically challenging to synthesise, as evidenced by the recent generation of an entire TALEN library directed at the human genome. TALENs have been reported as being much more efficient at generating somatic and germ-line mutations in zebrafish than ZFNs, with a mutagenicity rate 10-fold greater, though they are also more sensitive to target site methylation (S. Chen et al., 2013). The CRISPR-Cas9 system (Clustered, Regularly-Interspaced, Short Palindromic Repeats and CRISPR-associated 9) further improves on the principle of site-directed mutagenesis by separating the nuclease from the targeted guide sequence (Sander & Joung, 2014). This means that only simple RNA guides need to be generated for each new application, rather than an entire unique enzyme. An additional benefit of CRISPR-Cas9 over ZFNs and TALENs is that this system is readily and efficiently multiplexed through the injection of one nuclease and multiple guide RNAs (H. Wang et al., 2013). Each of these
techniques is easily applied to the zebrafish system through simple injection of its constituents into eggs at the single-cell stage.

### 1.6.1.3 Transgenesis

Transgenesis in the zebrafish is accomplished through the injection of DNA into single cell embryos. Since its first report in 1988, using naked DNA (Stuart, McMurray, & Westerfield, 1988), the efficiency of this technique has been dramatically improved through the incorporation of transposable elements, the most successful of which is the Tol2 element (Suster, Kikuta, Urasaki, Asakawa, & Kawakami, 2009). When co-injected with transposase mRNA, DNA flanked by Tol2 recognition sites is integrated into the host genome in a site-nonspecific manner. Benefits of Tol2 over similar systems, such as Sleeping Beauty, include a higher rate of transgenesis, less restriction on transgene size, and stronger expression in resulting transgenics (Clark, Urban, Skuster, & Ekker, 2011). High zebrafish fecundity and ease of egg manipulation enable injection of hundreds of eggs in each sitting, contributing to the relatively simple and rapid generation of transgenic animals.

### 1.6.1.4 Transient expression modification

Transient modification of gene expression is an alternative to creating heritable genetic changes. This approach enables more rapid investigation of gene effects and the potential to analyse lethal phenotypes. The simplest form of expression manipulation is the injection of mRNA into zebrafish eggs, leading to transient expression of the gene of interest (Yuan & Sun, 2009). Depending on the activity of the protein generated, this
may produce a gain- or loss-of-function phenotype. Additionally, mRNA injection may be used to validate loss-of-function models, whereby transient expression of an ablated gene “rescues” the phenotype under observation.

Gene expression can also be transiently impaired. The most well-recognised technique of this type used in zebrafish is the use of morpholinos to knock-down translation of targeted transcripts. Morpholinos are stable, antisense oligonucleotides that bind targeted transcripts at a 1:1 ratio (J. Summerton & Weller, 1997). This means that doseable abrogation of protein production is possible, allowing titration of gene activity. An advantage of morpholinos over expression abrogation systems like RNAi is that morpholinos act passively, without the necessity for cell machinery. They are also believed to be much more target-specific than RNAi technology, given their longer binding sequence (J. E. Summerton, 2007). Morpholinos act through one of two different mechanisms. Splice-blocking morpholinos bind to the splice acceptor or splice donor site on a pre-mRNA, preventing accurate mRNA processing and often leading to truncated, non-functional protein (J. E. Summerton, 2007). ATG-blocking morpholinos bind in close proximity to the starting codon, preventing binding of the translation initiation complex and thus preventing the production of any protein.

Despite the strengths of morpholinos in studying gene function, some researchers have questioned the validity of morpholino work in zebrafish in light of the advances being made in targeted genome editing. Schulte-Merker and Stainier argue that, while some morpholinos do faithfully recapitulate phenotypes later confirmed by knock-out models, a number have produced off-target effects that have led to misinterpretations of gene function (Schulte-Merker & Stainier, 2014). Additionally, off-target effects are difficult to assess as there is no way to effectively differentiate them from the results of a true target
knock-down without comparing them to an appropriate mutant model. Given the increasing feasibility of generating these mutants, Schulte-Merker and Stainier therefore suggest that this comparison be made prior to undertaking any extensive studies using morpholinos and that morpholinos are insufficient on their own to describe loss-of-function phenotypes.

1.7  **Zebrasfish models in inflammation and immunity**

The zebrafish boasts a full array of blood cell lineages, in both the innate and adaptive branches of the immune system, that are developmentally and functionally regulated through genetic drivers homologous to those described in mammals (Lieschke & Currie, 2007). This high level of conservation supports the zebrafish as an elegant and relevant model for examining both immune development and response in vertebrates. In addition, its early transparency and rapid, oviparous development allow for the quantification and tracking of fluorescently-tagged immune cells in real time as they migrate and interact with other components of the immune system (Deng & Huttenlocher, 2012). Novel insights using the zebrafish animal model have been achieved by coupling its impressive genetic tractability with the ability to observe biological events, at single cell resolution, within a living, whole-animal environment.

1.7.1  **Haematopoiesis and the immune response**

Zebrafish, like mammals, experience both primitive and definitive waves of blood cell development. Primitive haematopoiesis is initiated in the intermediate cell mass and
rostral blood island, the zebrafish equivalent to the mammalian extraembryonic yolk sac (Davidson & Zon, 2004; Warga, Kane, & Ho, 2009). These sites give rise to primarily erythroblasts and early macrophages, respectively, just prior to the onset of circulation at 24 hours post-fertilisation (hpf). Differentiation of these primitive macrophages begins from approximately 18 hpf and cephalic invasion starts at approximately 22 hpf, with distinct, substantial population of the head by 30 hpf (Deng and Huttenlocher 2012). The mode of this extravascular migration has not been fully examined, though studies indicate that it occurs primarily through the epithelium (Herbomel, Thisse, & Thisse, 2001). Figure 1.9 depicts the sites of haematopoiesis in the developing zebrafish at several stages.

**FIGURE 1.9: Haematopoietic sites in the zebrafish embryo and larva**

The primitive wave of haematopoiesis in the zebrafish begins in the intermediate cell mass and rostral blood island near 12 hpf. This is followed by the first definitive progenitors from the posterior blood island and haematopoietic stem cells arising from haemogenic endothelial cells in the ventral wall of the dorsal aorta at 24 and 28 hpf. Haematopoietic stem cells progressively seed the aorta-gonad-mesonephros-equivalent region and caudal haematopoietic tissue by 48 hpf, and the thymus and kidney by 96 hpf.
Definitive haematopoiesis is defined by the production of multipotent haematopoietic stem and progenitor cells (Davidson & Zon, 2004). In zebrafish, the first definitive progenitor cells arise from the posterior blood island at 24 hpf and consist of a transient population with erythroid and myeloid potential, termed erythromyeloid progenitors (EMPs) (Bertrand et al., 2007). EMPs do not appear to seed other haematopoietic tissues and are maintained within the posterior blood island and then caudal haematopoietic tissue until approximately 48 hpf (Bertrand et al., 2007). The first definitive HSCs emerge from haemogenic endothelial cells lining the ventral wall of the dorsal aorta between 28 and 48 hpf (Bertrand, Kim, Teng, & Traver, 2008; Herbomel et al., 2001; E. Y. Lam, Hall, Crosier, Crosier, & Flores, 2010). These cells then transiently occupy the aorta-gonad-mesonephros-equivalent region, located between the dorsal aorta and posterior cardinal vein, where they undergo limited cell division prior to entering the circulation via the posterior cardinal vein. Endothelially-derived HSCs then home to the caudal haematopoietic tissue, the zebrafish equivalent to the mammalian fetal liver, where they expand and differentiate before finally seeding the zebrafish thymus and bone marrow analogue, the kidney (Davidson & Zon, 2004). These haematopoietic stages are outlined in Figure 1.10 in parallel with those described in the mouse and frog, giving a sense of the strong conservation of this essential process across species.
FIGURE 1.10: Conservation of embryonic haematopoiesis across mice, frogs, and zebrafish

The progression of haematopoietic differentiation is highly conserved between mice, frogs, and zebrafish. These systems exhibit comparable waves of haematopoiesis, haematopoietic niches, and seeding events. Coloured bars indicate relative timing of haematopoietic stages. Arrows represent seeding events and sources. Abbrev. AGM, aorta-gonad-mesonephros-equivalent region; BM, bone marrow; CHT, caudal haematopoietic tissue; dpf, days post-fertilisation; E, embryonic days post-conception; FL, fetal liver; HSCs, haematopoietic stem cells. Modified from (Ciau-Uitz, Monteiro, Kirmizitas, & Patient, 2014)

While expression of genetic markers indicative of adaptive immune cell populations are detectable from the onset of thymopoiesis at 3 dpf, the zebrafish adaptive immune compartment does not fully mature until 4-6 weeks of age (S. H. Lam, Chua, Gong, Lam, & Sin, 2004). It is not until this later timepoint that the production of secreted immunoglobulin and the observation of humoral response against introduced pathogens have been detected (S. H. Lam et al., 2004). The temporally disparate development of the innate and adaptive immune branches in zebrafish, when combined with their many unique strengths described earlier, enables powerful investigations into the development, functions, and regulation of vertebrate innate immunity in isolation from the potential involvements of adaptive responses.
The genetic drivers behind haematopoiesis are also highly conserved across species and zebrafish are no exception. Homologues for key haematopoietic transcription factors such as runx1, gata1, and spi1/pu.1 have been discovered within zebrafish, that possess similar functional roles to those in mammals (Bertrand et al., 2007; E. Y. Lam et al., 2009; S. H. Lam et al., 2004). Their identification and functional characterisation in zebrafish both supports the expected evolutionary conservation of these important genetic elements and has led to new insights into our understanding of vertebrate haematopoiesis. Taking advantage of the genetic tractability of the zebrafish system has further enabled the observation in reporter lines of haematopoietic lineages directed by these genes, giving us access to directly observe the in vivo emergence, development, and migration of primitive erythromyeloid progenitors and definitive HSCs in real time (TABLE 1.2).

**TABLE 1.2: Examples of zebrafish reporter lines marking specific blood cell populations**

<table>
<thead>
<tr>
<th>Labelled Population</th>
<th>Transgenic line</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial/Haematopoietic Progenitors</td>
<td>Tg(PAC-tal1:GFP), Tg(S.0tal1:EGFP)</td>
<td>(H. Jin et al., 2006; X. Y. Zhang &amp; Rodaway, 2007)</td>
</tr>
<tr>
<td></td>
<td>Tg(lmo2:EGFP), Tg[lmo2:DsRed]</td>
<td>(H. Zhu et al., 2005)</td>
</tr>
<tr>
<td>Erythroid/Myeloid Progenitors</td>
<td>Tg(runx1P1:EGFP)</td>
<td>(E. Y. Lam et al., 2010)</td>
</tr>
<tr>
<td>Haematopoietic Stem Cells</td>
<td>Tg(runx1P2:EGFP)</td>
<td>(E. Y. Lam et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Tg(CD41:GFP)</td>
<td>(Ma, Zhang, Lin, Italiano, &amp; Handin, 2011)</td>
</tr>
<tr>
<td></td>
<td>Tg(cmyb:EGFP)</td>
<td>(Bertrand et al., 2010)</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Tg(gata1a:GFP), Tg(gata1a:DsRed)</td>
<td>(Long et al., 1997; Traver et al., 2003)</td>
</tr>
<tr>
<td>Thrombocytes</td>
<td>Tg(CD41:GFP)</td>
<td>(Lin et al., 2005)</td>
</tr>
<tr>
<td>Granulocyte/Macrophage Progenitors</td>
<td>Tg(spi1:EGFP), Tg(zpu.1:EGFP)</td>
<td>(Hsu et al., 2004; Ward et al., 2003)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Tg(mp1:GFP)</td>
<td>(Renshaw et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Tg(lyz:EGFP)</td>
<td>(Hall, Flores, Storm, Crosier, &amp; Crosier, 2007)</td>
</tr>
<tr>
<td>Monocytes/Macrophages</td>
<td>Tg(mpeg1:EGFP), Tg(mpeg1:mCherry)</td>
<td>(Ellett, Pase, Hayman, Andrianopoulos, &amp; Lieschke, 2011)</td>
</tr>
<tr>
<td></td>
<td>Tg(fms:GAL4.VP16)</td>
<td>(C. Gray et al., 2011)</td>
</tr>
<tr>
<td>Microglia</td>
<td>Tg(Apo-E:GFP)</td>
<td>(Peri &amp; Nusslein-Volhard, 2008)</td>
</tr>
</tbody>
</table>
For example, Lam et al. have used the \( Tg(\text{runx1P1:EGFP})_{\text{zf187}} \) and \( Tg(\text{runx1P2:EGFP})_{\text{zf188}} \) zebrafish reporter lines, driving the fluorescent protein EGFP from two distinct promoter regions of the essential haematopoietic transcription factor \( \text{runx1} \), to reveal discrete sites and timings for the emergence of different haematopoietic progenitors (E. Y. Lam et al., 2009). While the existence of multiple haemogenic tissues was already accepted, these studies clarified uncertainties concerning the differentiation potential of cells that arose from each. Lam et al. demonstrated that \( \text{runx1} \) expression driven by the runx1P1 promoter specifically marked erythryomyeloid progenitors in the posterior blood island, while the runx1P2 promoter drove expression in HSCs that occupy the aorta-gonad-mesonephros-equivalent region (E. Y. Lam et al., 2009). These studies also provided strong evidence for the transcriptional regulation of \( \text{runx1} \) isoforms through the use of distinct promoters.

### 1.7.1.1 Notable contributions from the zebrafish to the field of immunology

Beyond studies into immune cell ontogeny, zebrafish have contributed considerably to our understanding of the functional activities and applications of both mature innate and adaptive immune cell populations. For example, previous work by our laboratory on the innate immune response has led to the development of the transgenic zebrafish reporter lines \( Tg(\text{lyz:EGFP})_{\text{nz117}} \) and \( Tg(\text{lyz:DsRED2})_{\text{nz101}} \), in which neutrophils are fluorescently marked with either EGFP or DsRED2 (Hall et al., 2007). These marked cells have been observed during infection, disease, and wound healing, revealing unique insight into
mechanisms of neutrophilic defence, propagation, and inflammation resolution. In early responses, neutrophils are rapidly recruited to the inflammatory site and demonstrate substantial phagocytosis of invading bacteria within just two hours of infection (Brannon et al., 2009). This is followed by a period of neutropenia and demand-driven propagation, termed ‘emergency’ granulopoiesis, mirroring the mammalian response but visualised for the first time in zebrafish by our research group (Hall et al., 2012). Over the course of inflammation induction through to resolution, multiple modes of migration are observed (Hall et al., 2007). One of particular interest is retrograde chemotaxis, the unexpected observation of many neutrophils migrating to and then away from a site of injury rather than apoptosing at the site (Mathias et al., 2006). This unique behaviour has been suggested to contribute to wound healing by complementing apoptosis as a means of resolving inflammation. This implies that defects in neutrophil migration or chemotactic signalling gradients, leading to neutrophil retention at inflammatory sites, may significantly contribute to chronic inflammatory diseases.

More recent developments have shed light on the contributions of the monocyte/macrophage compartment of innate host defence as well, through the identification and isolation of the mpeg1 (Ellett et al., 2011) and fms/csf1r (C. Gray et al., 2011) promoters. Using the fms transgenic reporter, Gray et al. demonstrated the variable kinetics of macrophage versus neutrophil recruitment during the inflammatory response (C. Gray et al., 2011). Macrophages were seen gradually and continuously migrating to the wound site for a minimum of 48 hours post-injury, while neutrophil numbers peaked at just six hours. Interestingly, macrophage migration accelerated when the cells were in contact with the endothelium (C. Gray et al., 2011). Ellett and colleagues further expanded on the nature of macrophage-neutrophil interactions with the mpeg1 transgenic reporter lines Tg(mpeg1:EGFP)$^{0122}$, Tg(mpeg1:mCherry)$^{0123}$, and
Tg(mpeg1:Gal4-VP16)gl24, describing macrophage phagocytosis of neutrophils at the site of injury and indicating that neutrophil death was not always required prior to engulfment (Ellett et al., 2011). Additionally, non-phagocytic interactions with live neutrophils occasionally led to the transfer of a portion of neutrophil cytoplasm to the macrophage, though reasoning as to the purpose of this unidirectional communication is, as yet, uncertain (Ellett et al., 2011).

Further innate immune subdivision was made possible with the development of the Tg(Apo-E:GFP) transgenic reporter that specifically labels the resident macrophages of the brain, microglia (Peri & Nusslein-Volhard, 2008). This transgenic allows analysis of the phenotype and behaviours of a resident macrophage population separately from those of invading macrophages. Initially, the Tg(Apo-E:GFP) line was developed in order to observe the well-known role of microglia in phagocytosing of apoptotic neurons, as an increased understanding of this process may offer insight into of the mechanisms behind enhanced neuronal degradation in disease (Peri & Nusslein-Volhard, 2008). In their studies, Peri et al. discovered that the vacuolar ATPase subunit Atp6v0a1 was required for digestion of internalised neuronal material by way of its role in phagosome-lysosome fusion. Li et al. continued the investigation into neuron-microglia relationships by using the Tg(Apo-E:GFP) reporter line to analyse the effects of neuronal activity on microglial responses in vivo (Y. Li, Du, & Du, 2013). This revealed that microglia associated with more highly active neurons and that this association resulted in the microglia losing their dynamic migratory behaviour and taking on a more static and rounded shape, with blunted dendritic projections suggestive of direct microglia-neuron contact. This potential direct connection was seen in response to both healthy neuron activity and during injury, where responding microglia appeared to induce increased activity in the neurons surrounding the injury site (Y. Li et al., 2013). These data suggest that
communication channels and co-regulation may exist between neurons and resident microglia in both health and illness.

Examination of intracellular immune processes has also been made possible following the development of an NFκB zebrafish reporter line. Kanther et al. generated the transgenic line \textit{Tg(NFκB:EGFP)} to assess the spatiotemporal expression profile of this transcription factor during immune responses (Kanther et al., 2011). They observed these fish during the process of intestinal colonisation with commensal bacteria, starting from a germ-free state, and noted that NFκB expression was strongly induced by this non-infectious bacterial colonisation. These observations were complemented by work from Gratacap et al., where development of a swim bladder model of candidiasis enabled observation of NFκB activation in the epithelial cell interface during fungal infections (Gratacap, Rawls, & Wheeler, 2013). The level of NFκB activation observed was found to correspond with the severity of infection. In turn, the resulting induction of downstream inflammatory mediators was dependent on high levels of NFκB. Of interest, increased phagocytosis of the yeast by immigrating immune cells reduced NFκB expression in the epithelium, suggesting a level of direct epithelial sampling and response to external pathogens (Gratacap et al., 2013).

Zebrafish and mammals share a comparable array of lymphocyte populations, though there are some differences regarding their lymphoid organs. While both models possess a thymus for T cell development, the zebrafish bone marrow tissue equivalent is the kidney, and no lymph nodes have yet been identified in the fish (Davidson & Zon, 2004). Boehm and colleagues have examined the evolutionary conservation of thymopoiesis in this model by investigating known mammalian regulators of thymus. Their studies revealed the involvement of \textit{foxn1} (thymus development) (Boehm, Bleul, & Schorpp,
complementing the previously characterised roles of zebrafish *ikaros* (lymphocyte development) (Willett, Kawasaki, Amemiya, Lin, & Steiner, 2001), and *rag1* (lymphocyte differentiation) (Trede, Zapata, & Zon, 2001). The subsequent development of the *TgBAC(foxn1:mcherry)fr103* transgenic line has allowed *in vivo* observation of early thymopoiesis, from development of the thymic microenvironment to lymphocyte colonisation, maturation, and egress (Hess & Boehm, 2012). Recessive mutations pointing to regulators of thymus development have been identified by the Boehm laboratory through a forward genetic screen selecting mutations that affected *rag1* patterning at 5 dpf in a forward genetic screen (Boehm et al., 2003). Further characterisation of these lines has since revealed a previously unattributed, tissue-specific requirement for topoisomerase IIIa in T cell development, acting through an as yet unidentified pathway unrelated to somatic recombination (Monnich et al., 2010).

Zebrafish B lymphocyte biology has also been recently advanced through the use of transgenic lines. While early WMISH studies had misidentified the pancreas as the site of B cell development in the zebrafish, subsequent examination of fluorescently-labelled immature lymphocytes from the *Tg(rag2:DsRed2)* transgenic line on a *Tg(insulin:GFP)* transgenic background (which fluorescently labels β cells in the developing pancreas) has dismissed this possibility (Danilova & Steiner, 2002; Page et al., 2013). B cell development in the zebrafish has since been examined in much greater detail with the development of the *Tg(IgM1:eGFP)* transgenic line by the Traver group. This transgenic line allows tracing of the IgM-expressing subset of B cells (the more prevalent of the two subsets produced in zebrafish), which proved useful in the identification of the kidney as the site of B-cell development (Page et al., 2013). When the *Tg(IgM1:eGFP)* transgenic was further crossed with a *Tg(rag2:DsRed2)* transgenic labelling immature lymphocytes, three phenotypes were observed: Rag2*–*IgM⁺, Rag2*⁺*IgM⁺, and Rag2*⁺*IgM−, corresponding
to pro-B, pre-B, and mature B cells respectively, allowing analysis of B cell development at distinct stages (Page et al., 2013). This transgenic tool also enabled the discovery that, contrary to prior reports in zebrafish and other teleosts, only very low numbers of the B cell population in zebrafish are phagocytic (J. Li et al., 2006; Page et al., 2013). While this observation holds true to the long-standing belief that mammalian B cells are also non-phagocytic, it may actually be a point of difference in light of more recent studies in mice describing subsets of highly phagocytic B cells (Nakashima et al., 2012; Parra et al., 2012). The Tg(IgM1:eGFP) transgenic reporter line provides an excellent visual tool for further probing of these populations to dissect their similarities and differences and assess their unique role in immunity.

Despite the considerable array of fluorescent reporter lines available in the zebrafish toolbox, labelling innate and adaptive immune populations, subpopulations, and even cellular processes, a system in which specific activation states can be easily observed is distinctly lacking. This is of particular significance for the study of macrophages, given the increasing awareness of their range of plasticity and the importance these different phenotypes have on disease progression and overall immune function.

1.7.1.2 Contributions of zebrafish to the study of inflammation and disease

There are several methods for modelling sterile and infectious inflammation in zebrafish. Sterile injury to study leukocyte behaviour and wound healing can be accomplished through a simple tail transection (Lieschke, Oates, Crowhurst, Ward, & Layton, 2001; Mathias et al., 2006; Redd, Kelly, Dunn, Way, & Martin, 2006; Renshaw et al., 2006). Bacterial infection responses can be induced through injection of bacteria or bacterial products, such as LPS, into one of a number of tissues or organ systems (Davis et al., 2006).
2002; Hall et al., 2012; Herbomel, Thisse, & Thisse, 1999; Meijer et al., 2005). Sites such as the hindbrain ventricle, otic vesicle, tail muscle, and notochord can be used to simulate local infection, while yolk, blood island, and intravenous injections result in a systemic spread and response (Benard et al., 2012). When coupled with in vivo live imaging, such pathogen infection strategies have provided new insights into host-pathogen interactions. To illustrate, the zebrafish is an established model for tuberculosis research, where injection of the natural fish pathogen Mycobacterium marinum results in granuloma formation and exhibits characteristics similar to the human disease (Pozos & Ramakrishnan, 2004). Studies with this model have revealed the disease-specific in vivo transcriptome response to M. marinum, highlighting such genes as cholesterol 24-hydroxylase, a metabolic enzyme believed to play a role in mycobacterial invasion and survival in macrophages (Meijer et al., 2005). This lipid connection has been further discussed by Tobin and Ramakrishnan, who noted deleterious effects on the host resulting from an overproduction of either pro- or anti-inflammatory lipid mediators. Compounding a forward genetic screen with the zebrafish tuberculosis model enabled the identification of leukotriene A4 hydrolase (LTA4H) as being a key mediator of these varied states of lipid balance (Tobin & Ramakrishnan, 2013). They suggest that approaching disease treatment with reference to an individual patients’ LTA4H activity may lead to improved patient outcomes. Select zebrafish models for the study of immune and metabolic diseases are described in Table 1.3.
### Infectious Inflammation

<table>
<thead>
<tr>
<th>Zebrafish model</th>
<th>Application or Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubercular</td>
<td>Infect embryos or adults with <em>Mycobacterium marinum</em></td>
<td>Fluorescent pathogens are available for tracing. Assess pathogenesis and virulence factors. Observe interactions with and reaction of immune compartments when combined with appropriate reporter lines. Embryonic infection allows specific analysis of innate immune properties. Multiple routes for systemic and local infections.</td>
</tr>
<tr>
<td>Gram-positive</td>
<td>Infect embryos with <em>Bacillus subtilis</em></td>
<td></td>
</tr>
<tr>
<td>Gram-negative</td>
<td>Infect embryos with <em>Escherichia coli</em>, <em>Salmonella arizonae</em>, <em>Salmonella typhimurium</em></td>
<td></td>
</tr>
</tbody>
</table>

### Sterile Inflammation

<table>
<thead>
<tr>
<th>Zebrafish model</th>
<th>Application or Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Sterile' wounding</td>
<td>Transect embryonic tail, or wound fin</td>
<td>Observe and quantify leukocyte behaviour.</td>
</tr>
</tbody>
</table>

### Wound Healing

<table>
<thead>
<tr>
<th>Zebrafish model</th>
<th>Application or Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regeneration</td>
<td>Fin transection</td>
<td>Observe and quantify regrowth.</td>
</tr>
<tr>
<td></td>
<td>Removal of cardiac ventricular muscle</td>
<td>Observe and quantify regrowth.</td>
</tr>
</tbody>
</table>

### Inflammatory disease

<table>
<thead>
<tr>
<th>Zebrafish model</th>
<th>Application or Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterocolitis</td>
<td>Intestinal disruption through exposure to trinitrobenzene sulfonic acid</td>
<td>Intestinal inflammation akin to intestinal bowel disease.</td>
</tr>
<tr>
<td>Cutaneous inflammation</td>
<td>Fin transection/<em>Salmonella typhimurium</em> injection</td>
<td>Examine epidermal cell response.</td>
</tr>
</tbody>
</table>

### Metabolic Disease

<table>
<thead>
<tr>
<th>Zebrafish model</th>
<th>Application or Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td>Overfeeding, high fat diet</td>
<td>Study the pathophysiology and treatment of diet-induced obesity.</td>
</tr>
<tr>
<td><em>Tg</em>(β-actin-zAgRP) transgenic</td>
<td>Genetic model of obesity for the study of energy imbalance. Ectopic expression of zAgRP leads to adipocyte hypertrophy.</td>
<td>(Y. Song &amp; Cone, 2007)</td>
</tr>
<tr>
<td>Embryo staining with Nile Red</td>
<td>Stains adipocytes. Assay for obesogenic/anti-obesogenic compounds.</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>Treatment with known anti-diabetic compounds</td>
<td>Study glucose metabolism.</td>
</tr>
<tr>
<td><em>Tg</em>(pck1:Luc2) transgenic</td>
<td>Monitor gluconeogenesis during fasting. Application in drug discovery for modulation of metabolic dysregulation.</td>
<td>(Gut et al., 2013)</td>
</tr>
</tbody>
</table>
An effective immune response to injury begins with the rapid recruitment of pro-inflammatory effector cells. Zebrafish have contributed significantly to our understanding of the molecular processes of inflammation through a series of notable discoveries. In 2009, Niethammer et al. observed that wounded tissue releases H$_2$O$_2$ and, using a zebrafish transgenic carrying a H$_2$O$_2$ sensor, reported that this molecule diffused locally to produce a concentration gradient outward from the wound (Niethammer, Grabher, Look, & Mitchison, 2009). This gradient was shown to be necessary for neutrophil recruitment to the damaged tissue. Further study by Yoo et al. in 2011 revealed that neutrophils sense extracellular H$_2$O$_2$ by way of the Src-family kinase, Lyn (Yoo, Starnes, Deng, & Huttenlocher, 2011). As neutrophils respond, the H$_2$O$_2$ gradient fades and recruitment is reduced. The resulting gradual reduction in neutrophil numbers contributes to the resolution of inflammation. However, the mechanism by which H$_2$O$_2$ is cleared was not revealed until, following the observation that neutrophils were required for H$_2$O$_2$ reduction, Pase et al. decided to investigate the potential role of neutrophil myeloperoxidase (Pase et al., 2012). Myeloperoxidase is produced abundantly in responding neutrophils and catalyses the conversion of H$_2$O$_2$ to the antimicrobial compound hypochlorous acid (Gaut et al., 2001). Through the use of durif, a myeloperoxidase-deficient zebrafish mutant, it was confirmed that neutrophil-derived myeloperoxidase was necessary for H$_2$O$_2$ clearance (Pase et al., 2012). This study concluded the journey of H$_2$O$_2$ from induction in injury to clearance as a means of progressing resolution. It also introduced durif as a valuable tool for the further study of H$_2$O$_2$ signalling and myeloperoxidase-deficiency, one of the most common congenital neutrophil disorders (Pase et al., 2012).
1.7.2 Zebrafish in drug discovery

Due to its aqueous environment, small size, and potential for visible phenotypic or cellular readout, the zebrafish is an excellent platform for small molecule drug screens. Zebrafish can be exposed to micromolar concentrations of test compounds for precisely controlled lengths of time through the use of simple immersion and wash-out protocols, providing a highly cost-effective alternative to mammalian approaches (Zon & Peterson, 2005). As zebrafish are a whole-animal system, off-target effects and toxicities can also be observed and considered during first-pass triaging. This focuses results more rapidly, reducing subsequent workload. Importantly, the metabolic processes involved in drug metabolism have also been identified as highly conserved between zebrafish and mammals (Hung et al., 2012). When used in the context of medium-throughput drug screening, these allow the decoding of molecular pathways through known drug interactions or the repurposing of approved drugs upon the discovery of novel activities. A generalised schematic of the process of drug screening in zebrafish is shown in Figure 1.11.

The strength and speed of the zebrafish drug discovery platform is exemplified by work from the Zon lab at Boston Children’s Hospital. A library of biologically active compounds was screened for effects on haematopoietic stem cell numbers in wild type fish using in situ hybridisation analysis of runx1 and cmyb expression (North et al., 2007). Following rigorous genetic, visual, and chemical validations, they identified key involvement of prostaglandin E2 signalling in haematopoietic stem cell formation during homeostasis, post-radiation recovery, and murine bone marrow transplant establishment (North et al., 2007). Notably, they described a role for the small molecule 16,16-dimethyl-PGE2 – a stabilised, long-acting derivative of prostaglandin E2 – in the ex
**vivo** expansion of haematopoietic stem cells for therapeutic applications. The activity of this molecule is currently being explored in phase II clinical trials for the expansion of human cord blood stem cells prior to transplant for bone marrow reconstitution (Goessling et al., 2011).

**FIGURE 1.11: Generalised drug-screening protocol in zebrafish**
1. Adult fish are paired the evening before spawning.  
2. Adults mate in the morning, producing up to 300 embryos per female.  
3. Larvae are grown to the appropriate age for screening and arrayed in microtitre plates with compounds from a drug library diluted in fish-water.  
4. Larvae are monitored for a phenotypic readout. This may be the inhibition or induction of fluorescence in transgenic tissues, delayed or altered larva development, or the abrogation of a disease phenotype.  
5. Positive hits are identified and validated in further analyses.

Benefits of the whole-animal approach to drug screening extend well beyond toxicity analyses. As epitomised by a screen performed by Rihel *et al.*, complex phenomena such as behaviour responses can be analysed as well (Rihel *et al.*, 2010). Through their study, Rihel *et al.* were able to identify and cluster large groups of drugs according to similar
induced behavioural effects on the zebrafish rest/wake cycle. Subsequent analyses of these clusters revealed similarities in annotated targets or therapeutic use, suggesting mechanisms of action for lesser-characterised members. This study demonstrated an unbiased approach to dissecting multifaceted drug pathways and directing further drug annotation, observations that would have been impossible to assess in vitro and challenging on a large scale in other vertebrate models (Rihel et al., 2010).

Chemical screens have been performed using transgenic zebrafish lines. Using fluorescent reporters or genetic disease models in zebrafish drug screens enables more detailed analyses of drug effects and facilitates the interrogation of specific conditions. Hall et al. used a tail transection model of sterile inflammation in Tg(lyz:EGFP)nz117 larvae, a transgenic reporter labelling neutrophils with green fluorescence, to identify drugs that affected neutrophil migration to the wound (Hall, Wicker et al., 2014). By counting fluorescent cells at the site of injury four hours post-wounding, they were able to identify 251 compounds from an initial panel of 1280 drugs with existing uses that had a statistically significant effect on neutrophil recruitment. Confocal live-imaging of larvae treated with top hits from this screen revealed that most of these drugs exerted effects through reduced neutrophil migration speed and displacement rate. Additional validation in a murine model of allergic dermatitis confirmed the previously unrecognised anti-inflammatory action of these compounds and potential therapeutic use (Hall et al., 2014). The Renshaw group addressed a different aspect of the inflammatory process in their recent zebrafish screen, assessing a panel of 2000 drugs for their potential use in enhancing inflammation resolution, as measured by increasing the rate of neutrophil reverse migration and/or apoptosis (Robertson et al., 2014). A similar style of screen by Ridges et al. probed a collection of 26,400 molecules for specific effects on T cell development using the Tg(lck:EGFP) reporter line, a fluorescent reporter
labelling T cells, and scoring for reduced thymus fluorescence (Ridges et al., 2012). The rationale for this study was that drugs that inhibit T cell development might be beneficial in the treatment of T-cell acute lymphoblastic leukemia (T-ALL). This was tested by applying the strongest hits from the initial screen to a genetic zebrafish model of T-ALL generated by crossing Tg(lck:EGFP) to Tg(rag2:cMYC-ER), a line driving the conditionally-activated, oncogenic EGFP-myc-ER transgene from the T-cell-restricted rag2 promoter. The transgene, comprised of human MYC fused to a modified estrogen receptor, drives the development of T-ALL in fish treated with the estrogen receptor modulator 4-hydroxytamoxifen (Gutierrez et al., 2011). Crossing these two lines allowed tracking of T cell activities at all times during larval development and disease progression. These combined studies revealed a strong inhibitory effect of the drug Lenaldekar on the development of T-ALL while demonstrating the power of zebrafish transgenics (Ridges et al., 2012).

Meanwhile, advances in technology are further broadening the potential of zebrafish drug screens by exploring the possibility of a high-throughput, fully automated screening protocol. Systems have been developed incorporating robotic embryo dispensation and orientation, drug delivery and circulation, and microscopic imaging and data collection. Of particular interest to the investigation of immune responses is the development of protocols that expedite embryo stimulation. Researchers at the Center for Genome Regulation in Chile have described a method for specifically damaging lateral line neuromasts by immersion in a solution of copper sulphate, termed the chemically induced inflammation assay (ChIn) (d’Alencon et al., 2010). This treatment can be performed rapidly on large number of embryos and results in the recruitment of leukocytes to the damaged neuromasts. When complexed with a chemical screen, transgenic embryos expressing fluorescent proteins in leukocyte populations, and
automatic imaging and fluorescence quantification, d’Alencon et al. were able to efficiently assess drug affects on leukocyte recruitment in a high-throughput manner (d’Alencon et al., 2010). In addition to quantifying fluorescent cells, observations that can be automated include embryo viability in toxicity studies, as measured by the increasing opacity of necrotic tissues, and a range of swimming behaviours (Akagi et al., 2014; Letamendia et al., 2012; Richendrfer & Creton, 2013).

This variety of tools and applications available for the zebrafish model present it as an ideal system in which to further investigate the role of Irg1 in macrophage metabolism and immune effector functions. The current availability of fluorescent macrophage reporter lines simplifies the visual analyses of these cells while the genetic tractability of this model offers multiple means of specifically assessing the contributions of irg1 in physiologically relevant, in vivo environments.

### 1.8 Thesis outline

Irg1 transcription is highly specific and short-lived. Data suggest this enzyme has a distinct role in directing macrophage function through effects on macrophage metabolic program selection. The stringent regulation and spatio-temporal specificity of irg1 expression suggests that its presence in non-infectious compartments is undesirable and of significance to the propagation of immunometabolic disease. The zebrafish system offers a number of powerful tools that will enable the direct, in vivo observation and quantification of irg1 expression or inhibition in a variety of environments, as well as genetic techniques for the analysis of up and downstream signalling pathways.
The work presented in this thesis has aimed to manipulate the advantages of the zebrafish system to investigate the role and impact of irg1 in the innate immune response. This has been achieved through

1) Generation of the Tg(irg1:EGFP)nz4 zebrafish transgenic line to recapitulate irg1 expression in an accessible, high resolution, in vivo system, as described in Chapter 3.

2) Presentation of Tg(irg1:EGFP)nz4 as a tool for live imaging inflammatory macrophage activation and function in immunogenic circumstances such as infection and tumour engraftment, as discussed in Chapter 4.

3) Analysis of the transcriptome of stimulated, irg1-depleted macrophages to identify Irg1-dependent macrophage processes, detailed in Chapter 5.

4) Completion of a small molecule screen using a drug-repurposing approach to identify compounds that abrogate irg1 expression as potential therapeutics for the treatment of immunometabolic disease, presented in Chapter 6.

Taken together, through the analyses of Irg1 function and expression in zebrafish, these studies have improved current understanding of the fields of immunometabolism and macrophage plasticity and suggest how each of these may be manipulated for the benefit of human health in instances of disease.
2 Materials and Methods

2.1 Materials and Reagents

2.1.1 Primers

The sequences and targets of primers used in this study are listed in Table 2.1. Primers for use in whole-mount in situ hybridisation (WMISH) and promoter cloning were designed using MacVector v 13.0.5. Promoter cloning required the addition of restriction enzyme recognition sites as indicated in blue on the relevant sequence. Quantitative PCR (qPCR) primers were designed using Primer Express v 3.0 unless previously described in the literature, as mentioned. All primers were purchased from Life Technologies. Upon receipt, lyophilised primers were reconstituted with nuclease-free water to a stock concentration of 100 μM and stored at -20 °C.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Use</th>
<th>Sequence (5' - 3')</th>
<th>Ensembl Target I.D.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>irg1UP1</td>
<td>WMISH probe template</td>
<td>AAGCGGATGATTCTGGACAC</td>
<td>ENSDARG00000069844</td>
<td>(Hall et al., 2013)</td>
</tr>
<tr>
<td>irg1DO1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRG1 prom F2</td>
<td>promoter cloning</td>
<td>TACGGTCACTGGAGGAAACC</td>
<td>ENSDARG00000069844</td>
<td></td>
</tr>
<tr>
<td>IRG1 prom R4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>irg1 Do2</td>
<td>RT-PCR</td>
<td>TACGGTCAGTTGAGGAAACC</td>
<td>ENSDARG00000069844</td>
<td></td>
</tr>
<tr>
<td>irg1 Up6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRG1 F</td>
<td>qPCR</td>
<td>TTTAAAGAGGACACAACATT</td>
<td>ENSDARG00000069844</td>
<td></td>
</tr>
<tr>
<td>IRG1 R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF1a qPCR F</td>
<td></td>
<td>TGCCCTTCCTCCAATTTCAG</td>
<td>ENSDARG00000020850</td>
<td>(Hall et al., 2013)</td>
</tr>
<tr>
<td>EF1a qPCR R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.2 Morpholinos

The sequences and targets of antisense morpholino oligonucleotides required for this study are listed in Table 2.2. Morpholinos were designed by and purchased from Gene Tools LLC. Upon receipt, lyophilised morpholinos were reconstituted with nuclease-free water to a stock concentration of 5 mM and stored at -20 °C.

### TABLE 2.2: Morpholinos required by this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Sequence (5’ - 3’)</th>
<th>Target</th>
<th>Ensembl Target I.D.</th>
<th>Junction</th>
<th>Dose (pmol)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRG1SBM01</td>
<td>splice blocking</td>
<td>TGCTGCCTCTAATTCATAA</td>
<td>irg1</td>
<td>ENSDART0000147471</td>
<td>intron2-exon3</td>
<td>1</td>
<td>Gene Tools, LLC</td>
</tr>
<tr>
<td>IRG1SBM03</td>
<td>splice blocking</td>
<td>TGAAAATCTGTTTTACCT</td>
<td>irg1</td>
<td>ENSDART0000147471</td>
<td>exon4-intron4</td>
<td>1.5</td>
<td>Gene Tools, LLC</td>
</tr>
<tr>
<td>CTRLMO</td>
<td>control</td>
<td>CCTCTTACCTCAGTACAAATTTATA</td>
<td>none</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>Gene Tools, LLC</td>
</tr>
</tbody>
</table>

2.1.3 Bacterial Strains

Routine cloning and plasmid propagation was performed using the chemically-competent *Escherichia coli* strain DH5α, prepared in house and stored at -80 °C in CCMB80 buffer containing 10% (v/v) glycerol. Cloning of the Tol2-*irg1* promoter construct used Life Technologies One Shot Top10 Chemically Competent *Escherichia coli*, also stored at -80 °C.

2.1.4 Bacterial Growth Media and Supplements

The composition of bacterial growth media and associated supplements are described in Table 2.3.
TABLE 2.3: Media and supplements required for bacterial growth and plasmid selection

| Media and supplements required for bacterial growth and plasmid selection |
|-----------------------------|-----------------------------|
| **Media**                   | **Composition**             |
| Luria Broth (LB)            | 1% (w/v) Peptone 140        |
|                             | 0.5% (w/v) Yeast extract    |
|                             | 171.11 mM NaCl              |
| LB Agar                     | 1% (w/v) Peptone 140        |
|                             | 0.5% (w/v) Yeast extract    |
|                             | 85.56 mM NaCl               |
|                             | 1.2% (w/v) Agar             |
| Super Optimal Broth (SOB)   | 2% w/v tryptone             |
|                             | 0.5% w/v yeast extract      |
|                             | 10 mM NaCl                  |
|                             | 2.5 mM KCl                  |
|                             | pH to 7.0                   |
| SOB with catabolite suppression (SOC) supplement | 10 mM MgCl\(_2\)•6H\(_2\)O |
|                             | 20 mM glucose               |
|                             | 10 mM MgSO\(_4\)•7H\(_2\)O |
|                             | sterile filter, add to autoclaved SOB |
| Ampicillin (1000X)          | 100 mg/mL in 50% EtOH      |
| Chloramphenicol (1000X)     | 12.5 mg/mL in 100% EtOH    |
| X-Gal                      | 20 mg/mL in dimethylformamide |
| IPTG                        | 25 mg/mL in dH\(_2\)O      |

2.1.5 DNA Vectors

DNA vectors used in this study are described in Table 2.4. Bacterial artificial chromosomes (BACs) were obtained from ImaGenes GmbH in order to clone relevant promoter regions for transgenic zebrafish production. Plasmids were used for subcloning, transgenesis, and riboprobe synthesis as indicated. The Tol2 plasmid, pTol2linkerswitchGFP, was a generous gift from Richard Naylor in the Davidson lab, while pCS-TP was donated by Koichi Kawakami (Kawakami et al., 2004).

TABLE 2.4: DNA Vectors used in this study

<table>
<thead>
<tr>
<th>Vector</th>
<th>Type</th>
<th>Selection</th>
<th>Use</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIndigoBAC-536, clone DKEY-57A22</td>
<td>BAC</td>
<td>Chloramphenicol</td>
<td>irg1 promoter isolation</td>
<td>ImaGenes GmbH</td>
</tr>
<tr>
<td>pTol2linkerswitch GFP</td>
<td>Plasmid</td>
<td>Ampicillin</td>
<td>Tol2 vector for transgenesis</td>
<td>Davidson Lab (University of Auckland)</td>
</tr>
<tr>
<td>pCS-TP</td>
<td>Plasmid</td>
<td>Ampicillin</td>
<td>Transposase mRNA for transgenesis</td>
<td>Kawakami Lab (National Institute of Genetics, Japan)</td>
</tr>
<tr>
<td>pBSKS</td>
<td>Plasmid</td>
<td>Ampicillin</td>
<td>Cloning with blue-white selection</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pGEM-T/EASY</td>
<td>Plasmid</td>
<td>Ampicillin</td>
<td>Cloning RT-PCR fragments</td>
<td>Promega</td>
</tr>
<tr>
<td>pBSKS (-) egfp</td>
<td>Plasmid</td>
<td>Ampicillin</td>
<td>anti-egfp riboprobe synthesis</td>
<td>In house</td>
</tr>
<tr>
<td>pGEM-T/EASY irg1</td>
<td>Plasmid</td>
<td>Ampicillin</td>
<td>anti-irg1 riboprobe synthesis</td>
<td>In house</td>
</tr>
</tbody>
</table>
2.1.6 Buffers and Solutions

Please refer to Table 2.5 for detailed information concerning the composition and source of common solutions.

### Table 2.5: Common buffers and solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Use</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-phenyl-2-thiourea (PTU), 100X</td>
<td>Inhibition of melanocyte development</td>
<td>0.3% (w/v) 1-phenyl-2-thiourea 1X E3 Dissolve overnight with heating, let sit at least 24 h before use</td>
</tr>
<tr>
<td>1kb+ DNA ladder</td>
<td>Gel electrophoresis</td>
<td>6% (v/v) Fermentas loading dye 30% (v/v) Fermentas GeneRuler™ 1 kb Plus DNA Ladder, 75-20,000bp dH₂O</td>
</tr>
<tr>
<td>Agarose gel</td>
<td>Drug screen</td>
<td>1.2% (w/v) Agarose 1X E3 0.2% Methylene blue</td>
</tr>
<tr>
<td>Agarose gel</td>
<td>Gel electrophoresis</td>
<td>1% (w/v) Agarose 1X TAE buffer</td>
</tr>
<tr>
<td>Block solution</td>
<td>Fluorescent WMISH</td>
<td>10% (v/v) Western blocking reagent (Roche) PBST</td>
</tr>
<tr>
<td>CCMB80 Buffer</td>
<td>Chemically competent <em>E. coli</em></td>
<td>10 mM KOAc pH7 80 mM CaCl₂•2H₂O 20 mM MnCl₂•4H₂O 10 mM MgCl₂•6H₂O 10% glycerol</td>
</tr>
<tr>
<td>Cell Culture Medium</td>
<td>Cell culture</td>
<td>10% (v/v) FBS 1% (v/v) PSF Minimum Essential Medium α (Life Technologies)</td>
</tr>
<tr>
<td>Cracking Buffer</td>
<td>Cloning</td>
<td>50% (v/v) Buffer F1 (Qiagen, no LyseBlue) 50% (v/v) Buffer F2 (Qiagen)</td>
</tr>
<tr>
<td>Danieau’s buffer</td>
<td>Working stock morpholino dilution</td>
<td>58 mM NaCl 0.4 mM MgSO₄ 0.6 mM Ca(NO₃)₂ 0.5 mM HEPES pH adjusted to 7.6</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>RNase-sensitive applications</td>
<td>0.1% (v/v) diethyl pyrocarbonate dH₂O autoclave before use</td>
</tr>
<tr>
<td>DIG labelling mix, 10X (Roche)</td>
<td>WMISH probe synthesis</td>
<td>10 mM ATP 10 mM CTP 10 mM GTP 6.5 mM UTP 3.5 mM DIG-11-UTP</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>cDNA synthesis</td>
<td>10 mM ATP 10 mM CTP 10 mM GTP 10 mM TTP 10 mM Tris-HCl</td>
</tr>
<tr>
<td>Dissociation medium</td>
<td>FACS/Flow cytometry</td>
<td>0.25% Trypsin-EDTA (Gibco) PBS</td>
</tr>
<tr>
<td>Dissociation stop solution</td>
<td>FACS/Flow cytometry</td>
<td>1 mM CaCl₂ 5% FBS dH₂O</td>
</tr>
<tr>
<td>E3 medium (50X)</td>
<td>Raising embryos</td>
<td>250 mM NaCl 8.5 mM KCl 16.5 mM CaCl₂ 16.5 mM MgSO₄ dH₂O</td>
</tr>
<tr>
<td>E3 medium (0-24 hpf)</td>
<td>Raising embryos</td>
<td>1X E3 0.2% Methylene blue</td>
</tr>
<tr>
<td>E3 medium (&gt;24 hpf)</td>
<td>Raising embryos</td>
<td>1X E3, sterile</td>
</tr>
<tr>
<td>Ethidium Bromide Wash</td>
<td>Gel electrophoresis</td>
<td>0.005% Ethidium Bromide (v/v)</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>FLU labelling mix, 10X (Roche)</td>
<td>WMISH probe synthesis</td>
<td>10 mM ATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM GTP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5 mM Fluorescin-12-UTP</td>
</tr>
<tr>
<td>Hybridisation buffer (HYB-)</td>
<td>WMISH</td>
<td>50% Formamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 μg/mL heparin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1% Tween20</td>
</tr>
<tr>
<td>Loading dye (6X)</td>
<td>Gel electrophoresis</td>
<td>0.0125% (w/v) bromophenol blue</td>
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<tr>
<td></td>
<td></td>
<td>10 mM ATP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM GTP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5 mM Fluorescin-12-UTP</td>
</tr>
<tr>
<td>Low melting-point agarose gel</td>
<td>Confocal imaging fluorescent WMISH</td>
<td>1% (w/v) UltraPure™ LMP Agarose (Life Technologies)</td>
</tr>
<tr>
<td>Low melting-point agarose gel</td>
<td>Confocal live imaging</td>
<td>0.75% (w/v) UltraPure™ LMP Agarose (Life Technologies)</td>
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<tr>
<td>Maleic acid blocking buffer</td>
<td>WMISH</td>
<td>100 mM maleic acid</td>
</tr>
<tr>
<td>Methyl cellulose (3%)</td>
<td>Mounting larvae</td>
<td>3% (w/v) methyl cellulose</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>WMISH</td>
<td>4% (w/v) Paraformaldehyde</td>
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<tr>
<td>Phenol Red</td>
<td>Injection</td>
<td>0.5% (w/v) phenol red</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS)</td>
<td>Solvent for various solutions</td>
<td>4 mM KH₂PO₄</td>
</tr>
<tr>
<td>Phosphate-buffered saline with Tween (PBST)</td>
<td>Solvent, wash solution</td>
<td>10 mM Na₂HPO₄</td>
</tr>
<tr>
<td>Post-hybridisation buffer (HYB-)</td>
<td>WMISH</td>
<td>50% Formamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 μg/mL heparin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEPC-treated water</td>
</tr>
<tr>
<td>Pronase</td>
<td>Embryo dechorionation</td>
<td>30 mg/mL Pronase</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>WMISH</td>
<td>100 mg/mL Proteinase K</td>
</tr>
<tr>
<td>Resuspension medium</td>
<td>FACS/Flow cytometry</td>
<td>0.9X PBS</td>
</tr>
<tr>
<td>Ringer’s (10X)</td>
<td>FACS/Flow cytometry</td>
<td>20 mM KCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM MgSO₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 M NaCl</td>
</tr>
<tr>
<td>Ringer’s Solution</td>
<td>FACS/Flow cytometry</td>
<td>1X Ringer’s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM MgCl₂</td>
</tr>
<tr>
<td>Saline sodium citrate (SSC) buffer, 20X</td>
<td>WMISH</td>
<td>3 M NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dH₂O</td>
</tr>
<tr>
<td>Screening Medium</td>
<td>Drug screen</td>
<td>1X PTU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05 U/mL Penicillin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM Tris pH 7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1X E3 medium</td>
</tr>
<tr>
<td>Sheep anti-DIG-POD</td>
<td>Fluorescent WMISH</td>
<td>1:500 in Block Solution (Roche)</td>
</tr>
<tr>
<td>Sheep anti-FLU-POD</td>
<td>Fluorescent WMISH</td>
<td>1:500 in Block Solution (Roche)</td>
</tr>
<tr>
<td>Staining buffer</td>
<td>WMISH</td>
<td>20 mM Tris pH 9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DEPC-treated water</td>
</tr>
</tbody>
</table>
2.1.7 Antibodies

Antibodies were used during WMISH. **Table 2.6** describes their targets, conjugations, and sources.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reconstitution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep anti-DIG-POD</td>
<td>150 U/mL</td>
<td>Roche</td>
</tr>
<tr>
<td>Sheep anti-FLU-POD</td>
<td>150 U/mL</td>
<td>Roche</td>
</tr>
<tr>
<td>Chicken anti-GFP</td>
<td>10 mg/mL</td>
<td>Abcam</td>
</tr>
<tr>
<td>Goat anti-chicken-Alexa Fluor 488</td>
<td>2 mg/mL</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>

2.1.8 Fluorescent Probes

Fluorescent probes were used in confocal live imaging analyses. Their excitation/emission parameters and suppliers are listed in **Table 2.7**.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Use</th>
<th>Excitation/ Emission (nm)</th>
<th>Notes</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMPSense 645 FAST</td>
<td>Observing MMP production/activity</td>
<td>649/666</td>
<td>Fluorescence activation requires cleavage by matrix metalloproteinases</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>Nuclear labelling of injected cancer cells</td>
<td>350/461</td>
<td>Fluorescence requires binding to dsDNA</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CellTracker Green BODIPY Dye</td>
<td>Labelling of injected cancer cells</td>
<td>522/529</td>
<td></td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>
2.1.9 Cell Lines

The human metastatic breast cancer cell line MDA-MB-231 was obtained from ATCC for use in zebrafish larva immune challenge by tumour cell injection.

2.1.10 Chemicals and Restriction Enzymes

Standard lab chemicals were purchased from Merck, Scharlau, BD, Bio-Rad, BDH, GibcoBRL, Boehringer Mannheim, Roche, and Sigma-Aldrich. Refer to Table 2.5 in section 2.1.6 for details of their specific use. Suppliers of speciality solutions are indicated. Restriction enzymes were purchased from Roche, New England BioLabs, Life Technologies, and Fermentas.

2.1.11 Drug Library and Additional Drug Amounts

The Prestwick Chemical Library® was chosen for use in the drug repurposing screen. It was purchased from Prestwick Chemical and provided 1120 unique, FDA-approved compounds at 2 mg/mL in 100% dimethyl sulfoxide (DMSO) in plate format. Additional amounts of top candidates were also purchased from Prestwick Chemical and supplied in 10 mg vials of lyophilised compound. Upon receipt, drugs were reconstituted in 1 mL of 100% DMSO and stored at -20 °C.
2.1.12 Zebrafish Stock

Zebrafish lines used in this study are described in Table 2.8.

<table>
<thead>
<tr>
<th>Zebrafish Line</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (AB)</td>
<td>ZIRC, University of Oregon, United States of America</td>
<td></td>
</tr>
<tr>
<td>Tg(irg1:EGFP)nez4</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Tg(mpeg1:EGFP)gl22</td>
<td>Lieschke laboratory, Monash University, Australia (Ellett et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>Tg(mpeg1:mCherry)gl23</td>
<td>Lieschke laboratory, Monash University, Australia (Ellett et al., 2011)</td>
<td></td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Zebrafish Husbandry

All zebrafish husbandry and manipulations were conducted in accordance with approvals granted by the University of Auckland Animal Ethics Committee (protocol 001343).

2.2.1.1 Stock and Maintenance

Adult fish were maintained in a custom zebrafish facility on an automated 14 hour day/10 hour night light cycle with water conditions between 25.5-29.5 °C, 250-500 μS conductivity, and pH 7.2-7.6. Juvenile and adult fish (>30 dpf) were fed Artemia Shell Free (INVE Aquaculture Nutrition) supplemented with powdered TetraMin Tropical Flakes (Tetra) three times daily on weekdays and once daily on weekends. Larval fish (<30 dpf) were fed Hatchfry Encapsulon Grade III (Argent Chemical Laboratories) on the same schedule.
2.2.1.2  Spawning

Adult fish were paired in separate breeding tanks in the late afternoon of the day prior to spawning. The following morning, the water in the breeding tanks was exchanged within the first hour of light and then as necessary in order to collect embryos as spawning continued. Spawnings were staggered when necessary through the use of dividers in the breeding tanks that segregated the female and male fish. Adults were spawned at a maximum frequency of once every four days for males and once every seven days for females.

2.2.1.3  Embryo Collection

Eggs were collected from breeding tanks with the aid of a tea strainer then rinsed in tap water and transferred to Petri dishes with E3 medium supplemented with methylene blue. Petri dishes were maintained in incubators at 28.5 °C. Clutches were cleaned at 8 hpf and 24 hpf, removing all dead and unfertilised embryos.

2.2.1.4  Rearing

Following cleaning at 24 hpf, embryos were transferred into fresh, sterile E3 medium. If subsequent studies required transparency for imaging, media was supplemented with 1X 1-phenyl-2-thiourea (PTU) (0.003%) to inhibit melanocyte formation. Larvae selected for rearing were moved to Techniplast tanks in a nursery system at 6 dpf. At 30 dpf, fish were transferred from the nursery to the main adult systems.
2.2.2  Egg and Embryo Manipulation

2.2.2.1  Microinjection of Morpholinos

Morpholinos were diluted in Danieau's buffer and supplemented with 0.2% phenol red in order to visualise injection volume and placement. Injection doses were optimized for maximum effect and minimal toxicity on a per-morpholino basis and typically ranged between 0.2 and 1.5 pmol. Microinjection needles were made from borosilicate glass capillaries (Warner Instruments) loaded into a P-80 micropipette puller (Sutter Instruments). Needles were back-loaded with injection solution using Eppendorf microloader tips prior to cutting off the needle tip with a razor to produce a narrow, angled bore. Injection volumes were standardised to 1 nL by measuring the diameter of droplets injected into mineral oil on top of a haemocytometer. Injection pulse length on an MPPI-2 Pressure Injector (Applied Scientific Instrumentation) was adjusted until a droplet diameter of 2.5 units was consistently produced. Eggs were arrayed on a plastic egg tray and morpholinos were injected by hand into the cell-yolk interphase up until the late two-cell stage.

2.2.2.2  Generating Transgenic Lines

Transgene DNA housed in a Tol2 vector (gifted from Richard Naylor in the Davidson lab, University of Auckland) was co-injected with transposase mRNA and phenol red at a dose of 30 pg plasmid DNA and 50 pg mRNA per egg. Plasmid details are described in Table 2.4 in section 2.1.5. Eggs were arrayed on a plastic egg tray and injected by hand
into the cell of single cell-staged eggs. For detailed descriptions of plasmid vector generation and screening for transgenic embryos, please see Chapter 3.

Transposase mRNA was produced by linearising pCS-TP with Not1 restriction enzyme and running a capped transcription reaction using the Ambion SP6 mMESSAGE mMACHINE kit. RNA was recovered by standard lithium-chloride precipitation and resuspended in UltraPure water (Life Technologies), as per manufacturer instructions.

2.2.2.3 Immune Stimulation

Hindbrain injections were used to stimulate an immune response in 2 dpf larvae. Standard injection solutions were diluted in phosphate buffered saline (PBS) and supplemented with 1% phenol red. Working concentrations were 0.8 mg/mL LPS and 20 μM MMPSense for a 1 nL injection. Larvae were anaesthetised in E3 Medium supplemented with 4.2% (v/v) Tricaine Solution and mounted in 3% methyl cellulose. Injection was facilitated with the use of a Narashige micromanipulator model M-152. Larvae prepared for confocal microscopy were mounted in Low Melting Point Agarose for live imaging (0.75% w/v agarose) prior to hindbrain injection. For a detailed description of the injection system used for drug repurposing, please see Chapter 6.

Immune response was also assessed following injection of MDA-MB-231 human metastatic breast cancer cells into the perivitelline space over the yolk, immediately posterior to the CCV. Cells were grown in 75cm² flasks in 20 mL of cell culture medium at 37 °C and 5% CO₂, passaging at 85-95% confluency. To passage, media was removed from the flask and cells were rinsed with 5 mL PBS. PBS was then removed and cells were incubated with 2 mL 0.25% Trypsin for 1 min to dissociate them from the floor of
the flask. Once all cells had lifted off, 8 mL of cell culture medium was added to neutralise the enzyme and 1 mL of the resulting suspension was carried over to a new flask to maintain the line.

To prepare cells for injection, the remaining suspension was pelleted at 1800 rpm in a Sorvall RT-7 Plus benchtop centrifuge for 2 min at 10 °C, the supernatant was discarded, and cells were resuspended in 2 mL of 5 µg/mL Hoechst 33342 or 25 µM CellTracker Green diluted in Minimum Essential Medium α (Life Technologies). Cells were incubated at 37 °C and 5% CO₂ for 1 h with gentle mixing every 15 min. This suspension was centrifuged again at 1800 rpm for 2 min at 10 °C, the supernatant was removed, and cells were resuspended in 1V of matrigel (50% final concentration). Larvae were mounted in 2% methyl cellulose as described above and injected with 1.5 nL of cell suspension, corresponding to 100-150 cells.

2.2.3 Cloning

2.2.3.1 Competent Cells

Chemically competent DH5α *Escherichia coli* were prepared by first streaking a fresh Luria-Bertani (LB) agar plate (no selection) with bacteria from a glycerol stock. After overnight incubation at 37 °C, one colony was inoculated into 2 mL of Luria Broth and grown overnight at 37 °C at 200 rpm. The following morning, 100 µL of the broth culture was used to inoculate 100 mL of super optimal broth (SOB) (a 1:100 dilution), which was then grown for 3-4 hours at 28 °C and 200 rpm until an optical density of approximately 0.2 was achieved. Bacteria were pelleted at 4000 rpm for 10 min at 4 °C the resuspended
in 32 mL ice-cold CCMB80 buffer and incubated on ice for 20 min. Pelleting was repeated as stated earlier and cells were resuspended in 4 mL ice-cold CCMB80 followed by another incubation on ice for 20 min. Aliquots were stored at -80 °C.

2.2.3.2 Restriction Enzyme Digest

Restriction digests were carried out using enzyme to a maximum contribution of 10% of the reaction volume in reaction conditions (reaction buffer and temperature) as described by the enzyme provider. Double digests were carried out with consultation to the New England BioLabs online Double Digest Finder tool. If no star activity was documented for enzymes used, large volume digests (50-120 µL) were run overnight.

For an in-depth description of identification and isolation of the irg1 promoter region and subsequent Tol2 vector construction, please refer to Chapter 3.

2.2.3.3 Gel Electrophoresis

Nucleic acids were electrophoretically separated by size using agarose gels prepared using TAE buffer. Gel composition was varied according to expected band size, with 0.8% agarose gels used for separating bands >10 kilobases (kb) and 1% agarose gels for separating bands <10kb. Gels used in Crystal Violet Gel Extraction were prepared as suggested by the kit provider (Life Technologies), supplementing cooled liquid agarose with 20 µL of 2 mg/mL Crystal Violet solution per 50 mL of agarose before pouring. Samples were mixed with loading dye and dH2O for standard gels or Crystal Violet Loading Buffer for Crystal Violet gels before being loaded into wells. Gels were run,
submerged in TAE buffer, at 110V in a Bio-Rad Mini-Sub Cell GT system. Nucleic acids in standard gels were imaged under ultraviolet light on a Bio-Rad Gel Doc Imager with Bio-Rad Quantity One Software (v4.4.1) after soaking the gel in ethidium bromide wash solution for 10 min. Crystal Violet gels were visualised on a light box. Band sizes were determined in each instance by comparison to a reference lane containing 1 kb Plus DNA Ladder (Fermentas).

2.2.3.4 Gel Extraction

Standard gel extractions were performed using the QIAquick Gel Extraction Kit (Qiagen) as described by the manufacturer. Gel slices were extracted with the use of a razor and a transilluminator following ethidium bromide staining of the gel, with effort taken to minimize the length of time the gel was exposed to the ultraviolet light. Purified DNA was eluted with dH₂O and stored at -20 °C until future use.

Crystal Violet Gel extractions were carried out using the Crystal Violet gel purification reagents and protocol included in the Life Technologies TOPO® XL PCR Cloning Kit. Gel slices were extracted with the use of a razor and light box. Purified DNA was eluted with dH₂O and stored at -20 °C until future use.

2.2.3.5 RT-PCR

Routine RT-PCR amplification from linearised plasmid used Platinum PCR Supermix from Life Technologies. Working dilutions of forward and reverse primers were prepared at 10µM. Of this, 1 µL of each was added to 1 µL of template (10-200 ng/µL)
and 42 µL of Supermix. A hot-start reaction was run on a Bio-Rad C1000 thermal cycler, with a 3 min enzyme activation at 94 °C followed by 35 cycles of 30s at 94 °C for template denaturing, 30s at the annealing temperature, and 1-3 min of extension at 72 °C. Once all cycles were run, the protocol was finished with 5 min at 72 °C and an indefinite hold at 4 °C until removed for further analyses or storage. Annealing temperature was determined by MacVector for each primer pair and extension time at 72 °C was calculated as 1 min per kb of template amplified. RT-PCR products were purified using the PureLink PCR Purification Kit from Life Technologies according to the manufacturer’s instructions.

2.2.3.6 Long-Template RT-PCR

Amplification of long targets (>4kb) was carried out using the Expand Long Template PCR System by Roche using 150 ng of BAC template. Reactions were trialled with both Buffer 1 and Buffer 2 as previous use of the system sometimes displayed better amplification from buffers optimised to different fragment lengths. The amplification protocol was run on a Bio-Rad C1000 thermal cycler and consisted of a 2 min enzyme activation at 94 °C followed by a first round of ten cycles of 10s at 94 °C for template denaturing, 30s at the annealing temperature, and 8 min of extension at 68 °C, and a second round of 25 cycles of 15s at 94 °C, 30s at the annealing temperature, and 8min+20s/cycle of extension at 68 °C. Once all cycles were run, the protocol was finished with 7 min at 68 °C and an indefinite hold at 4 °C until removed for further analyses or storage.
2.2.3.7 Nucleic Acid Quantification

Routine quantification of RNA and DNA was carried out using the Nanodrop ND-1000-UV-Vis Spectrophotometer. Absorbance at 260nm was used to determine nucleic acid concentration and the ratios of $A_{260}/A_{280}$ and $A_{260}/A_{230}$ were used as qualitative analyses of sample purity. When determining the concentration of vector and insert fragments for use in ligation reactions, equal volumes of each were run on an agarose gel with a known quantity of DNA ladder. Comparisons with both the ladder and each other enabled determination of proper insert:vector ratios.

Nucleic acid quantification for RNA-Seq was performed on an Agilent Bioanalyzer 2100. The RNA 6000 Pico kit and High Sensitivity DNA kit were used for RNA and DNA analysis respectively, following manufacturer instructions (Agilent Technologies).

2.2.3.8 Ligation

For all ligations, an insert:vector molar ratio of 3:1 was used. For ligations using Long-Template PCR amplified inserts or restriction digest fragments substantially larger than the respective plasmid vector, the larger DNA fragment was treated as the “vector” for molar ratio calculations.

Products generated through standard RT-PCR reactions were cloned into the pGEM-T/EASY vector in a total volume of 10 µL as directed by the supplier (Promega). Following addition of the T4 ligase, the reaction was gently mixed and incubated at room temperature for one hour before advancing to transformation.
Ligation of products generated through restriction digests used 1 U of T4 DNA ligase per 10 µL reaction in 1X T4 ligase buffer (Life Technologies). Sticky-end ligations were incubated at room temperature for 1-2 hours before transformation. Blunt-end ligations were incubated at 4 °C overnight.

Promoter isolation generated very large fragments through Long-Template PCR amplification. These fragments were purified via Crystal Violet Gel extraction prior to restriction enzyme digest. Ligation efficiency was enhanced by calf intestinal phosphatase treatment of digested vector. Following the restriction digest, enzymes were heat-inactivated by incubating at 65 °C for 20 min. Reaction volumes were then doubled with dH2O, the appropriate buffer to 10% of the final volume, and calf intestinal phosphatase to 5% of the final volume (ensuring no more than 10% glycerol in the final reaction volume once inactive enzymes from the prior digestion are taken into consideration). CIP-treated reaction mixtures were purified using the PureLink PCR Purification Kit from Life Technologies and eluted in dH2O.

2.2.3.9 Transformation

Transformation of chemically competent E. coli with plasmid constructs followed a basic heat-shock protocol. Bacteria were thawed on ice in 50 µL aliquots and 1 µL of ligation mixture or purified plasmid was added at a concentration of 10-50 ng/µL. Cells were gently stirred and then incubated on ice for 30 min. Heat shock was applied for exactly 60 seconds at 42 °C, followed by the addition of 250 µL room temperature SOC and a one-hour incubation at 37 °C and 200 rpm. The bacterial solution was divided onto two agar plates of appropriate selection and grown overnight at 37 °C.
2.2.3.10  Plasmid Preparation and Colony Cracking

Transformed colonies that met selection criteria were used to inoculate 3 mL Luria Broth cultures, supplemented with appropriate selective antibiotics, and grown overnight at 37 °C and 200 rpm for additional analyses. Plasmid was isolated from overnight cultures using the Qiagen Plasmid Mini Kit according to the manufacturer's standard protocol.

When screening large numbers of colonies, colony cracking was performed prior to broth inoculation. Single colonies were picked from a transformation plate with a sterile pipette tip and used to inoculate a second agar plate marked with a grid to identify colonies. The pipette tip was then mixed in 6 µL of cracking buffer. The resulting suspension was incubated for 1 min at 100 °C, vortexed vigorously for 10s, combined with gel loading dye, and run on an agarose gel to determine plasmid sizes. This method extracts both genomic and plasmid DNA. The agar plate originally spotted with colonies was grown overnight at 37 °C and used to inoculate broth cultures the following day, using colonies identified from the gel as containing plasmids of the expected size.

2.2.3.11  Glycerol Stocks

All plasmids generated were stored in bacterial glycerol stocks at -80 °C. Stocks were generated by combining 500 µL of fresh, overnight bacterial culture with 500 µL of sterile-filtered 50% glycerol/PBS in a 1.6 mL cryotube, resulting in a final glycerol concentration of 25%.
2.2.3.12 Sequencing

Sequencing of plasmid inserts was carried out by the School of Biological Sciences, Centre for Genomics and Proteomics (The University of Auckland). Returned sequence files were analysed using MacVector v 13.0.5.

2.2.4 RNA Analysis

To avoid RNA degradation, all consumables were certified nuclease-free and solutions were prepared using nuclease-free water (either diethyl pyrocarbonate (DEPC)-treated or UltraPure). Isolated RNA was stored at -80 °C.

2.2.4.1 Total RNA Extraction

Total RNA was extracted from whole larvae using Trizol Reagent (Life Technologies) and subsequent chloroform-isopropanol precipitation. Up to 25 larvae were homogenised in 500 μL Trizol by repeated passage through a 22.5G needle. Following a 5 min incubation at room temperature, homogenate was either processed immediately or stored at -20 °C. Processing involved the addition of 200 μL chloroform followed by 15s of vigorous shaking by hand and a 3 min incubation at room temperature. Centrifugation for 15 min at 12,000 rpm and 4 °C resulted in phase separation, with the RNA contained in the upper, aqueous layer. The aqueous layer was transferred to a new microfuge tube and 250 μL of 100% isopropanol was added to initiate precipitation. Following a 10 min incubation at room temperature, RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was washed once with 500 μL 70% ethanol and the briefly
with 100 µL of 100% ethanol. The final pellet was resuspended immediately in 20 µL of UltraPure water (Life Technologies).

Extraction from cells isolated by fluorescence-activated cell sorting (FACS) followed a similar protocol to that of larval extraction but used Trizol LS Reagent (Life Technologies). Cells were sorted directly into 300 µL Trizol LS and the solution was brought to a final volume of 400 µL with UltraPure water. Cells were homogenised by passing the solution through a 26G needle three times. A 5 min incubation at room temperature was followed by the addition of 200 µL chloroform, 15s of vigorous shaking by hand, a 5 min incubation at room temperature, and centrifugation at 12,000 rpm and 4 °C as before. The aqueous phase was transferred to a new microfuge tube and 0.53V of 100% ethanol was added, followed by mixing by pipetting and a 5 min incubation at room temperature. Subsequent RNA isolation was carried out using reagents and protocols from the Macherey Nagel NucleoSpin RNA XS kit. The solution was bound to the column and washed with 400 µL of provided buffer RA3 for 30s at 11,000 rpm. Eluate was discarded and the column was washed once more with 200 µL buffer RA3 for 2 min at 11,000 rpm. The column was transferred into a microfuge tube and RNA was eluted in 30 µL UltraPure water (Life Technologies) and stored at -80 °C.

2.2.4.2 Reverse Transcription

Reverse transcription of isolated RNA used the Bio-Rad iScript cDNA Synthesis Kit and recommended protocol. In a total volume of 20 µL, 1 µg of RNA was combined with 4 µL of the 5X iScript reaction mix, 1 µL of iScript reverse transcriptase, and UltraPure water (Life Technologies). The incubation protocol of 5 min at 25 °C, 50 min at 42 °C, and 5 min
at 85 °C was carried out in a Bio-Rad C1000 thermal cycler followed by indefinite holding at 4 °C until moved to storage at -20 °C or further downstream processing.

2.2.4.3 qPCR

Transcript levels were quantified using PerfeCTa SYBR Green FastMix with ROX (Quanta Biosciences) in an ABI PRISM 7900HT fast sequence detection system (Life Technologies). UltraPure water (Life Technologies) was used to dilute 20 µL cDNA reactions to a final volume of 400 µL. Forward and reverse primers were diluted with UltraPure water and combined to a final concentration of 1.5µM each. Each primer pair was combined with PerfeCTa SYBR Green FastMix with ROX at a ratio of 3:5 in order to reduce viscosity of the FastMix and facilitate more accurate dispensation. Reactions were prepared in 384-well plates using an epMotion 5070 liquid handling robot (Eppendorf), dispensing 8 µL of FastMix-primer solution and 4 µL of diluted cDNA to a total of 12 µL with four technical replicated per primer pair, per sample. PCR cycling, fluorescence detection, critical threshold (CT) calculations, and dissociation curve calculations were carried out using an ABI PRISM 7900HT Fast Sequence Detection System (Life Technologies) and SDS software v2.3 (Life Technologies). Cycle parameters were 50 °C for 2 min followed by 95 °C for 2 min and 40 cycles of 95 °C for 15s and 60 °C for 30s. Resulting data were analysed in Microsoft Excel using the comparative CT method as described by Schmittgen and Livak (Schmittgen & Livak, 2008). Eukaryotic translation elongation factor 1 alpha 1 (ef1-α) was used as an endogenous control (McCurley & Callard, 2008).
RNA-Seq was carried out on RNA samples purified from FACS-collected cells using the combined Trizol LS and Machery Nagel NucleoSpin RNA XS kit protocol as described in 2.2.4.1. Sample quality and quantity was assessed on an Agilent Bioanalyzer 2100 (see section 2.2.3.7) before being processed into cDNA libraries using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs). Library quality was confirmed on the Agilent Bioanalyzer 2100 before being sent to the Otago branch of New Zealand Genomics Limited for Next Generation Sequencing on an Illumina HiSeq 2500. Detailed descriptions of sample processing and analyses are found in Chapter 5. Sequence reads were mapped using TopHat v2.0.12 against the zebrafish reference genome Zv9, obtained from Ensembl (D. Kim et al., 2013). Statistical comparisons were made using Cuffdiff, part of the open source software program, Cufflinks (C. Trapnell et al., 2010). Transcript reads were compared between control and treatment groups and assessed for both significantly different normalised expression values (taken as a $P$-value < 0.05) and low probabilities of false discovery (taken as a $q$-value of <0.05). Gene set enrichment analysis utilised the online, searchable meta-database GeneSetDB (Araki, Knapp, Tsai, & Print, 2012).

2.2.4.5 Preparation of WMISH riboprobes

Probes used for transcript detection in WMISH were produced by applying the Roche RNA Labelling Kit to plasmid template that had been linearised either 5’ or 3’ to the insert, depending on the polymerase binding site to be used for transcription of either antisense or sense probe. Dioxigenin (DIG)- or fluorescein (FLU)-labelled riboprobes were synthesised according to kit protocol, using 1 µg of linearised template, 2 µL 10X
NTP stock, 2 µL 10X transcription buffer, 1 µL RNase inhibitor, 1 µL polymerase, and 
dH2O to bring the reaction to a 20 µL total volume. Reactions were incubated at 37 °C for 
2 h then treated with 2 µL DNase for 10 min at 37 °C. Precipitation proceeded by adding 
0.7 µL 0.5M ethylenediaminetetraacetic acid (EDTA), 2.5 µL 4M LiCl and 75 µL 100% 
EtOH to each reaction and storing for 30 min at -80 °C. Pellets were collected by 
centrifuging at 14,000 rpm for 20 min at 4 °C followed by a wash with 70% EtOH. 
Riboprobes were resuspended in 100 µL UltraPure water supplemented with 1% (v/v) 
RNase inhibitor and concentration was estimated by running a small volume on an 
agarose gel. Probes were stored at -20 °C.

2.2.4.6 WMISH

Following 24 h fixation in 4% paraformaldehyde (PFA), larvae were dehydrated by 
stepwise passage through 25, 50, and 75% solutions of methanol prepared in phosphate 
buffered saline with Tween20 (PBST), ending in 100% methanol. At this point, samples 
were stored at -20 °C for a minimum of 1hr. Dehydrated larvae were then rehydrated by 
5 min washes in methanol solutions in reverse (75, 50, and 25% methanol in PBST, 
ending in 100% PBST). Larvae were permeabilised by digestion with proteinase K 
diluted to 1:1000 from a stock of 10 mg/mL. An incubation length of 25 min was used 
for larvae over 2 dpf and 10 min for embryos younger than 2 dpf. The digest reaction 
was stopped by washing twice in PBST for 5 min. Larvae were re-fixed in 4% PFA for 20 
min at room temperature then rinsed five times in PBST. Pre-hybridisation was carried 
out in 750 µL hybridisation buffer with tRNA (HYB+) at 65 °C for 3 h to overnight with 
gentle rocking. Probes were diluted in HYB+ at a dilution determined by band intensity 
on an agarose gel – typically between 1:200 and 1:500 – and denatured by incubation at 
90 °C for 10 min. Probes used in this study targeted transcripts for egfp (1:200), mpeg1
Pre-hybridisation solution was exchanged for HYB+ containing denatured probe and samples were incubated overnight at 65 °C with gentle rocking. Hot washes were carried out in the same heated, rocking oven and consisted of 15 min each of 75% HYB-/2X saline sodium citrate (SSC), 50% HYB-/2X SSC, 25% HYB-/2X SSC, and 100% 2X SSC followed by two 30 min washed with 0.2X SSC. Room temperature washes consisted of passage through 5 min washes with 75% 0.2X SSC/PBST, 50% 0.2X SSC/PBST, 25% 0.2X SSC/PBST, and 100% PBST. Larvae were then soaked in maleic acid blocking buffer for a minimum of 1h at room temperature. Roche anti-DIG (or anti-FLU) antibody was diluted 1:8000 into maleic acid blocking buffer and larvae were incubated in this solution overnight at 4 °C with gentle rocking. The following day, samples were washed with a minimum of eight changes of PBST over 2 h and three changes of staining buffer over 15 min. All washes were done at room temperature. After the final wash, larvae were treated with 1 mL of staining solution and incubated in the dark. Staining progress was monitored closely and required between 30 min and 2days, with overnight incubations carried out at 4 °C to slow progress. Staining was stopped by three washes in PBST and one in 1 mM EDTA/PBST. Samples were cleared by incubation in 50% glycerol/PBST followed by final storage in 100% glycerol at 4 °C. Staining larvae using the Intavis InsituPro VSi robot, as for the drug repurposing study described in Chapter 6, followed a similar protocol optimised for automated buffer exchange. Further details can be found in Chapter 6 and the exact robot protocols can be found in Appendix 1.

2.2.4.7 Fluorescent WMISH

In order to obtain approximately equal fluorescence intensity during double fluorescent WMISH, the more strongly-expressed gene was stained using a FLU-labelled riboprobe
and Alexa Fluor 555, and the more weakly expressed gene was stained using a DIG-labelled riboprobe and Alexa Fluor 488. Protocol was performed as for WMISH until blocking, at which point larvae were incubated in 1 mL Block Solution (Roche, 10% v/v in PBST) for 2 hours at room temperature before overnight incubation in 500 µL primary antibody (sheep anti-DIG-POD 1:500 in Block Solution, Roche) at 4 °C with gentle rocking. Unbound antibody was removed with six washes in PBST for 10 min each at room temperature. Staining proceeded in the dark for 1 hour with 200 µL Alexa Fluor 488 as provided in the Life Technologies TSA kit #2 and prepared 1:50 in fresh 0.0015% H₂O₂. All subsequent steps were carried out in the dark. Stain solution was removed by five 1 min washes in PBST and the reaction was stopped by the addition of 500 µL 6% H₂O₂ for 30 min. After six 10 min washes in PBST, larvae were transferred to 12-well plates and staining was confirmed before returning to tubes for an additional 2 h incubation in 1 mL Block Solution (Roche, 10% v/v in PBST) at room temperature followed by overnight incubation in 500 µL secondary antibody (sheep anti-FLU-POD 1:500 in Block Solution, Roche) at 4 °C with gentle agitation. Again, unbound antibody was removed with six washes in PBST for 10 min each at room temperature before staining in the dark for 1 hour with 200 µL Alexa Fluor 555 as provided in the Life Technologies TSA kit #4 prepared 1:50 in fresh 0.0015% H₂O₂. Stain solution was removed by five 1 min washes in PBST and the reaction was stopped by the addition of 500 µL 6% H₂O₂ for 30 min. Following four 15 min washes in PBST, larvae were once again transferred to 12-well plates to check staining before being re-fixed in 4% PFA for 2 h at room temperature. Larvae were cleared through two 10 min PBST washes and stored at 4 °C in PBST.
2.2.5 Drug screen

Compounds from the Prestwick chemical library were applied to anaesthetised larvae at a concentration of 5 μg/mL, diluted in screening medium. Larvae were pre-treated with drug for three hours prior to hindbrain stimulation with LPS. For a detailed description of the drug screening methodology, see Chapter 6.

Structure-activity relationships of top candidates were assessed using SYBYL-X2.1.1 from Certara. Compound clustering was performed using the SARMAP function within the Molecular Data Explorer (Certara).

2.2.6 Flow Cytometry and FACS

Zebrafish larvae were rinsed in ice-cold Ringer’s solution for 15 min. Yolks were removed by repeated passage through a 200 μL micropipette tip. Larvae were then transferred to 6-well dishes and dissociated in 3 mL dissociation medium for two hours on a 28 °C hotplate. Dissociation was mechanically assisted by passage through a 1000 μL micropipette tip every 10 min. Digestion was stopped by the addition of 1 mL dissociation stop solution. Cell suspensions were transferred into two 2 mL microfuge tubes each and pelleted at 3000 rpm for 5 min at 4 °C. Pellets were resuspended in 500 μL ice-cold resuspension medium and passed through a 40μm cell strainer (BD Falcon). Cell suspensions were stored on ice prior to analysis or sorting.

Flow cytometry was performed on a BD LSRII (Becton Dickinson) and FACS was carried out using the BD FACSARia II with the sample chamber chilled to 4 °C. Cells were analysed based on fluorescence expression and forward and side scatter characteristics.
Cells isolated for further RNA analysis were sorted directly into Trizol LS and processed as described in section 2.2.4.1.

2.2.7 Microscopy

For fluorescence and light microscopy, live larvae were anaesthetised in 4.2% (v/v) Tricaine Solution and mounted in 3% (w/v) methyl cellulose in 1X E3. Larvae processed through WMISH were mounted in 100% glycerol. Larvae were imaged on either the Nikon SMZ1500 or Leica MZ16FA fluorescence stereomicroscopes fitted with a DS-U2/L2 or DFC490 camera respectively.

2.2.8 Confocal Imaging

Live imaging of larvae required mounting in 0.75% (w/v) UltraPure LMP agarose (Life Technologies) in E3 medium supplemented with 3.15% (v/v) Tricaine Solution and 1X PTU. Once set, agarose was immersed in liquid E3 containing the same supplements. Larvae were imaged on an Olympus FV1000 confocal microscope fitted with a Solent incubation system (Solent Scientific). Temperature was maintained between 27 and 29 °C. A 20X water immersion objective was used. Images were taken as Z-stacks of 12-25 slices at a 5µm interval. Time-lapse imaging frequency varied from 45s to 10 min depending on experiment design. Confocal imaging of fluorescent WMISH larvae utilised a Nikon D-Eclipse C1 confocal microscope and larvae were mounted in 1% (w/v) UltraPure LMP agarose prepared in PBS. Images and image sequences were further assessed using Volocity v6.3 (Perkin Elmer) and Fiji (Schindelin et al., 2012) software.
2.2.9 Statistics

Statistical analyses and data plotting were performed using GraphPad Prism version 6.0 (GraphPad Software). Comparisons were made by one-way analysis of variance and interpreted with Dunnett’s multiple comparisons test or by Student’s t-test, as indicated. Scatter plot data are presented with mean and SD values. Statistical significance was taken as a $P$-value of less than 0.05. For statistical analysis of RNA-Seq data, see 2.2.4.4.
3 Generation of the \textit{Tg(irg1:EGFP)nz4} transgenic zebrafish line

Macrophages are a highly heterogeneous population of innate immune cells that are known to alter their phenotype in response to a variety of different stimuli (Mosser & Edwards, 2008; Murray & Wynn, 2011). While many of these triggers, signalling pathways, and resulting effector responses are well known, direct observation of cells undergoing these phenotypic shifts has proven difficult. Furthermore, \textit{in vivo} models for live imaging these specific subpopulations are lacking. IRG1 has been shown to be a specific marker for a subpopulation of pro-inflammatory, stimulated macrophages, that is rapidly induced following infection-induced inflammation (Hall et al., 2013; H. Li et al., 2006). To date, observation of \textit{Irg1} expression in living cells has only been possible \textit{ex vivo} (H. Li et al., 2006). While transgenic zebrafish with fluorescently labelled macrophages and microglia do exist, these lines do not allow differentiation of macrophage activation states (Ellett et al., 2011; Peri & Nusslein-Volhard, 2008). Developing a transgenic reporter line that fluorescently labels \textit{irg1}-expressing cells offers the opportunity for unprecedented, \textit{in vivo} access to the process of macrophage activation while enabling further investigations into the functions of Irg1 in this highly disease-relevant immune cell population. These studies include observation of \textit{irg1} expression in macrophages responding to different inflammatory stimuli, as described in \textbf{Chapter 4}, and analysis of the macrophage transcriptome to determine elements that are dependent on Irg1 function, as described in \textbf{Chapter 5}. 

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3.1 Temporal expression profile of LPS-induced irg1 transcription

Previous reports have indicated that the expression of irg1 in zebrafish embryos is infection-driven (Hall et al., 2013). Thus, it was anticipated that embryos carrying a fluorescent reporter driven by the irg1 promoter would require immune stimulation in order to induce transgene expression. Establishing the spatio-temporal expression profile of irg1 by WMISH following infection would also be required to ascertain the regions and timelines of expected maximum reporter expression. This would help inform when and where to expect to observe reporter expression in infected embryos injected with the transgene construct. In addition to infection with live bacteria, hindbrain injection with LPS has been shown to reliably induce irg1 expression in zebrafish macrophages (Hall et al., 2013).

The infection-induced nature of irg1 was confirmed by comparing larvae injected at 2 dpf in the hindbrain with 1 nL sterile PBS with those injected at the same site with 1 nL 0.8 mg/mL LPS. Consistent with previous studies, WMISH revealed rapid irg1 expression in response to LPS injection, with distinct, punctate staining visible as early as 1 hpi (FIG 3.1) (Hall et al., 2013). Staining was observed throughout the larva, albeit at increased density in the hindbrain and the caudal haematopoietic tissue, a site of definitive haematopoiesis in the larval zebrafish, which suggested the LPS acted systemically. No irg1 expression was observed in PBS-injected larvae (FIG 3.1 A). Over a four-hour time course, LPS-induced irg1 expression peaked at 2 hpi and returned to steady state levels by 4 hpi (FIG 3.1 B-E).
As injection of LPS into the hindbrain of potential transgene founder larvae would be time consuming, we also investigated whether immersion in a solution of LPS was sufficient to induce *irg1* expression. Larvae immersed in 500 μg/mL LPS for 1 to 48 hours did not show any evidence of *irg1* expression, as detected by WMISH (FIG 3.1 F, representative). Thus, injection of an immunogenic agent such as LPS was chosen as the means of inducing *irg1* transcription.
3.2 Transgenesis strategy

With hindbrain injection of LPS chosen as the most practical means of inducing expression of *irg1*, this treatment needed to be incorporated into the strategy used to develop a transgenic zebrafish line that fluorescently marks *irg1*-expressing cells. This strategy is described in Figure 3.2.

**FIGURE 3.2: Strategy for the generation and selection of Tg(*irg1*:EGFP) transgenic zebrafish founders**

1. A Tol2 construct is cloned carrying a section of the *irg1* promoter region upstream of an EGFP reporter gene. In parallel, transposase mRNA is generated.  
2. Zebrafish embryos are injected at the one-cell stage with the transgene construct and transposase mRNA.  
3. Embryos are grown to 48 hpf and injected with LPS into the hindbrain compartment to stimulate transgene expression.  
4a,b. Stimulated larvae are monitored from 8 hpi to identify those with transgene expression in expected compartments. These larvae are isolated and raised to adulthood. If transgene expression is not detected, or not expressed in anticipated regions, a new transgene construct is generated.  
5. Adult F0 founders are outcrossed to WT adults to screen for stable germline transgenesis.  
6. Progeny are stimulated at 48 hpf with injection of LPS into the hindbrain to stimulate transgene expression.  
7. Transgene-expressing F1 larvae are grown to adulthood as stable transgenic lines. The line that faithfully recapitulates expected spatio-temporal expression with the brightest reporter expression is maintained and used for all subsequent analyses.  

Abbrev. WT, wild type.
3.3 Cloning and characterisation of the *irg1* promoter sequence

The zebrafish *irg1* gene has been previously annotated (Ensembl I.D. ENSDARG00000069844). This genetic element and its upstream sequence are contained within pIndigoBAC-536 clone DKEY-57A22 (ImaGenes GmbH). Previous analysis of the promoter region of *irg1* identified multiple recognition sites for binding of the transcription factor C/EBPβ, an infection-responsive regulator of the early immune response that has been shown to be necessary for infection-induced *irg1* expression in zebrafish macrophages (Hall et al., 2013). To further characterise potential transcription factors acting on *irg1*, 5kb of the promoter region immediately upstream of the *irg1* transcription start site was analysed with the TFSearch online tool using the default threshold score of 85.0 and the TRANSFAC MATRIX TABLE release 3.3 (Yukata Akiyama, Kyoto University, Kyoto). Results were compared with similar regions upstream of the mouse and human homologues of *irg1* to identify potentially conserved regulatory elements (**FIG 3.3**). In addition to conserved C/EBPβ recognition sequences, multiple binding sites for the transcription factors upstream stimulatory factor (USF), runt-related transcription factor 1 (Runx-1), sterol regulatory element-binding protein (SREBP), and cAMP response element-binding protein (CRE-BP) were also identified in each sequence.

The observation of putative USF binding sites is interesting, as increased levels of this transcription factor have been observed in M1-type macrophages in inflammatory environments (Gobin et al., 2001). Runx1 binding sites may also indicate a role for Irg1 in macrophage function, given recent data from Zusso *et al.* suggesting involvement of Runx1 in modulating the phenotype of microglia (Zusso *et al.*, 2012).
FIGURE 3.3: Analysis of predicted transcription factor binding sites in the 5kb sequence upstream of the zebrafish, mouse, and human irg1 orthologs

Predicted binding sites are indicated for the following: C/EBPβ (blue), USF (purple), Runx-1 (orange), SREBP (red), and CRE-BP (green). Abbrev. CRE-BP, cAMP response element-binding protein; Runx-1, Runt-related transcription factor 1; SREBP, Sterol Regulatory Element-Binding Protein; USF, Upstream Stimulatory Factor.
SREBP1s are key regulators of lipid homeostasis, with well-characterised functions in governing the expression of enzymes involved in fatty acid synthesis, lipogenesis, and cholesterol synthesis (Eberlé, Hegarty, Bossard, Ferré, & Foufelle, 2004) and the transcription factor CRE-BP is known to regulate the expression of pro-inflammatory cytokines such as IL-6 and TNFα (Eberlé et al., 2004; Wen, Sakamoto, & Miller, 2010).

Taken together, the observation of putative binding sites for transcription factors USF, Runx-1, SREBP, and CRE-BP supports current data positioning *irg1* as a metabolic driver of inflammatory macrophage effector function.

### 3.4 Construction of the -4.7*irg1*:EGFP transgene

The Tol2 plasmid pTol2linkerswitch was chosen to facilitate transfer of the *irg1*:EGFP transgene into the zebrafish genome. This plasmid was developed by Richard Naylor in the laboratory of Alan Davidson, University of Auckland, through modification of the pT2AL200R150G plasmid originally developed and described by the Kawakami lab (Urasaki, Morvan, & Kawakami, 2006). The modified construct carries shorter Tol2 arms and incorporates unique restriction sites 5’ to the EGFP element to simplify promoter integration ([Appendix 2; Richard Naylor, unpublished](#)).

Primers were designed to amplify -1 to -4663, relative to the ATG start codon, of the genomic sequence upstream of the zebrafish Irg1 coding sequence. Promoter sequence analysis, as described above, indicated that the majority of potentially relevant transcription factor binding sites were contained within this region ([FIG 3.3](#)). Recognition sites for the restriction enzymes FseI and Xmal were engineered into the 3’
and 5’ primers respectively to correspond with restriction sites in the pTol2linkerswitch vector. The cloning strategy is summarised in Figure 3.4.

**FIGURE 3.4:** Cloning workflow for the generation of the pTol2linkerswitch-irg1 transgene construct

Sequence upstream of *irg1* was isolated via PCR from the DKEY-57A22 BAC template and purified by Crystal Violet Gel extraction. Both PCR insert and plasmid backbone were digested appropriately and remaining enzyme was removed or inactivated prior to ligation. The ligation reaction was checked via gel electrophoresis prior to transformation. Abbrev. BAC, bacterial artificial chromosome; CIP, calf intestinal phosphatase; PCR, polymerase chain reaction.
The DKEY-57A22 BAC containing zebrafish *irg1* and its upstream sequence was obtained from ImaGenes GmbH and used as a template for PCR amplification of the desired 4.7kb *irg1* promoter segment. The amplified fragment was purified via Crystal Violet Gel extraction and digested, along with the pTol2linkerswitch vector DNA, with FseI and Xmal restriction enzymes. Ligation was performed overnight at 4 °C using the T4 ligase and buffer solution from the pGEM-T/EASY kit (Promega). Ligation success was analysed by running a small sample of the reaction on an agarose gel stained with ethidium bromide and noting a larger band at the expected size (10kb). The resulting construct, pTol2linkerswitch-irg1, contains 4.7kb of *irg1* promoter sequence upstream from *EGFP*. Plasmid identity was supported by restriction digesting with NotI, producing two bands at 6.3kb and 3.9kb as expected. Ligation sites on the final construct were then sequenced to ensure map accuracy and insert identity prior to injection for transgenesis. See Appendix 3 for a map of pTol2linkerswitch-irg1.

### 3.5 Expression analysis of the -4.7*irg1*:EGFP transgene within transient embryos

Observation of embryo development following transgene and mRNA co-injection noted fluorescent expression in the caudal haematopoietic tissue, aorta-gonad-mesonephros-equivalent region and over the yolk at 25 hpf (FIG 3.5 A). This expression occurred in the absence of LPS injection and diminished to undetectable levels by 2 dpf. To determine whether this was reflective of early *irg1* expression induced by exposure to components of the injection solution, rather than ectopic transgene expression, WMISH was performed on -4.7*irg*:EGFP-injected embryos that demonstrated transgene expression at 25 hpf. The resulting strong *irg1* expression throughout the embryo body,
confirmed that transgene expression was faithfully recapitulating *irg1* expression and indicated that injection of nucleic acids was sufficient to induce *irg1* expression (FIG 3.5 B). As microbial dsDNA has previously been shown to be a potent inducer of innate immune response, expression of *irg1* in this instance is not unexpected (Stetson & Medzhitov, 2006).

![FIGURE 3.5: Expression of *irg1* in 25 hpf embryos injected with the -4.7*irg1*:EGFP transgene construct](image)

**A.** Embryos were observed expressing systemic, punctate EGFP at 25 hpf following injection of the -4.7*irg1*:EGFP transgene construct and transposase mRNA. **B.** WMISH analysis of 25 hpf -4.7*irg1*:EGFP-injected fluorescing embryos revealed punctate *irg1* expression throughout the larvae. Scale bar 100 μm in **A**. Numbers indicate the proportion of embryos that displayed the presented phenotype. Abbrev. hpf, hours post-fertilisation; WMISH, whole mount *in situ* hybridisation. Embryos oriented anterior to left.

At 2 dpf, the infection-inducible nature of the injected -4.7*irg1*:EGFP transgene construct was assessed by injecting potential transgenic larvae with LPS. EGFP was observed as distinct points throughout the larva as rapidly as 30 min post-injection (FIG 3.6). However, larvae were screened at 8 hpi to identify potential transgenic founder larvae.
FIGURE 3.6: EGFP expression in an F0 larva 30 min post-hindbrain injection with LPS

**A.** As early as 30 min following stimulation with LPS, larvae that had been injected with the -4.7irg1:EGFP transgene construct demonstrated bright, punctate expression throughout the body and an indistinct glow from the yolk sac. **B.** Larvae that had not been injected with the transgene construct displayed very low levels of autofluorescence by comparison. Arrow indicates hindbrain injection site. Scale bar 100 μm.

Abbrev. CHT, caudal haematopoietic tissue; dpf, days post-fertilisation; pi, post-injection. Larva oriented anterior to left.

In agreement with irg1 expression as detected by WMISH following LPS injection (see section 3.1), punctate fluorescence was distinctly and consistently present in the caudal haematopoietic tissue and cranial regions. There also appeared to be some EGFP expression within the yolks of founder larvae, though this has not been seen reproducibly in WMISH assays and was variable between clutches of potential founders. The inconsistency of the yolk fluorescence suggests it may be an artefact of the embryo environment rather than being representative of true irg1 stimulation. For example, bright autofluorescence of the yolk is sporadically seen in uninjected zebrafish embryos and often attributed to the inclusion of methylene blue, an antifungal agent, in embryo-rearing media (Conn, 2012).
3.6 Selecting stable \( Tg(\text{irg1:EGFP}) \) F1 progeny

Approximately 40 fluorescing larvae were successfully reared to maturity. These adults were outcrossed to wild type zebrafish to screen for germline incorporation of the \(-4.7\text{irg1:EGFP}\) transgene. Of note, non LPS-injected embryos generated from transgenic adults did not express EGFP at 1 dpf as had been observed in those injected with transgene DNA and transposase mRNA. In addition, the variable yolk expression that had been seen in some F0 larvae was consistently absent in F1 larvae. However, an unanticipated weak fluorescence in the forebrain region at 2 dpf was observed in 50% of the outcrossed F1 progeny (FIG 3.7). It was present from 36 hpf and persisted to at least 3 dpf, though expression became less distinct during this time. This non-induced expression of the \(-4.7\text{irg1:EGFP}\) transgene provided a useful mechanism to identify \( Tg(\text{irg1:EGFP}) \) larvae without the need for hindbrain injection of LPS (FIG 3.7). Unless otherwise stated, this dim forebrain expression was used to pre-select transgenic embryos for subsequent analyses.
FIGURE 3.7: Fluorescence in the forebrain of non-injected transgenic Tg(irg1:EGFP) larvae at 2 dpf

A,B. Breeding a heterozygous Tg(irg1:EGFP) parent to a wild type parent produced clutches containing 50% transgenic larvae. At 2 dpf, transgenic larvae were identified by a weak fluorescence in the forebrain region (white arrows).

C,D. Confocal imaging of the forebrain region of a transgenic larva viewed ventrally and laterally respectively. Scale bars 100 μm in each frame. Larvae oriented anterior to left. Ventral side presented in C.
Outcrossed F1 progeny were injected with LPS at 48 hpf to stimulate expression of the -4.7irg1:EGFP transgene. Following the identification of four founders, progeny from these lines were compared using fluorescence microscopy to determine which founder expressed the fluorescent reporter most intensely (FIG 3.8).

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<thead>
<tr>
<th>8hpi PBS</th>
<th>8hpi LPS</th>
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<td>![Image](96x332 to 547x656)</td>
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**FIGURE 3.8: Comparison of transgene expression intensity in four Tg(irg1:EGFP) founder lines A-D.** F1 larvae were injected into the hindbrain with LPS at 48 hpf and monitored for transgene expression. A'-D'. A control group from each clutch was injected with PBS into the hindbrain. Scale bar 100 μm in A. Numbers indicate the proportion of larvae that displayed the presented phenotype. Larvae oriented anterior to left.

Visual analysis using fluorescence microscopy did not identify any distinct EGFP intensity differences between LPS-injected progeny from the four founder lines. Of note, PBS-injected controls did not demonstrate transgene induction, as expected (FIG 3.8 A-D). As such, fluorescence intensity of the expressed transgene was then quantified by flow cytometry using 50 larvae per founder line. Heterozygous clutches used in this analysis were not sorted for transgene expression prior to treatment and dissociation.
Larvae were stimulated at 48 hpf by hindbrain injection with LPS and dissociated at 2 hpi. Mean fluorescence intensity was calculated for both the EGFP+ and EGFP- populations. These values were compared as a ratio to determine which line had the greatest separation between positive and negative populations. Calculated values and raw histogram data can be seen in Figure 3.9.

**FIGURE 3.9:** Comparison of fluorescence intensity in four Tg(irg1:EGFP) founder lines as detected by flow cytometry

The mean fluorescence intensities of four founder lines were compared to determine the line carrying the most intense and distinct transgene expression. **A.** Live cells were selected by gating for cells of appropriate size using forward (FSC) and side (SSC) scatter. Very small and very large events, predominantly representing debris and cell clumps, were excluded. The live cell population was further refined by selecting events with consistent side scatter and forward scatter, indicating single cells. **B.** Single cell populations were assessed for EGFP+ events, determined via histogram analysis. The average fluorescence intensity of EGFP+ events was also compared to the signal from the EGFP- events in the same channel. Each line had comparable proportions of EGFP+ cells.

Founder 1 demonstrated both the brightest transgene expression and greatest separation between EGFP+ and EGFP- cell populations. This line was designated Tg(irg1:EGFP)$^{nz4}$ and used for all subsequent analyses.
3.7 Transgene expression in Tg(irg1:EGFP)nz4 faithfully recapitulates endogenous irg1 expression

The accuracy of the -4.7irg1:EGFP transgene in recapitulating endogenous irg1 expression was next assessed by performing a double fluorescent WMISH staining for both irg1 and EGFP transcripts. To ensure that expression was also infection-specific, as previously observed, Tg(irg1:EGFP)nz4 larvae were first treated with either hindbrain PBS or LPS injection (FIG 3.10).

**FIGURE 3.10:** Double fluorescent WMISH confirmed specific, LPS-induced colocalisation of endogenous irg1 and transgene EGFP transcript in the hindbrain of Tg(irg1:EGFP)nz4 larvae
No expression of either gene was detectable two hours post-injection of PBS into the hindbrain. Expression of both *irg1* and *EGFP* was detected in overlapping compartments two hours following stimulation with LPS. *A, B*. *EGFP*-FLU probe and Alexa Fluor 488 staining. *A’, B’.* *irg1*-DIG probe and Alexa Fluor 555 staining. *A’’, B’’.* Merged Alexa Fluor 488 and 555 signals. Scale bar 100 μm in *A*. Numbers indicate the proportion of larvae that displayed the presented phenotype. Abbrev. hpi, hours post-injection; DIG, Dioxigenin; FLU, Fluorescein. Larvae oriented anterior to left.

Double fluorescent WMISH analysis revealed that both endogenous *irg1* and transgene *EGFP* expression were specifically induced by inflammatory stimulation with injected LPS but not by sterile PBS, demonstrating that LPS-stimulated *Tg(irg1:EGFP)* larvae faithfully report endogenous *irg1* expression.

### 3.8 Induced transgene expression in *Tg(irg1:EGFP)* is restricted to macrophage-lineage cells

Outcrossing heterozygous *Tg(irg1:EGFP)* to homozygous *Tg(mpeg1:mCherry)* transgenic zebrafish, labelling macrophage-lineage cells with mCherry fluorescent protein (Ellett et al., 2011), confirmed that *-4.7irg1:EGFP* transgene expression was specific to this population following LPS injection. This is in agreement with previous studies examining the localisation of induced *irg1* expression (Hall et al., 2013). Both confocal imaging (*FIG 3.11*) and flow cytometry (*FIG 3.12*) revealed that *-4.7irg1:EGFP* transgene expression was exclusive to *mpeg1:mCherry*-expressing cells following LPS injection. Comparing hindbrain injection of LPS to PBS further confirmed the infection-driven nature of the *-4.7irg1:EGFP* transgene (*FIG 3.11*).
FIGURE 3.11: Expression of the -4.7irg1:EGFP transgene in Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 double transgenic larvae following injection with LPS is restricted to mCherry+ macrophage-lineage cells

A,A′,A″. Hindbrain injection of PBS into double transgenic embryos at 48 hpf did not induce EGFP expression when assessed by confocal imaging at 8 hpi. B,B′,B″. Hindbrain stimulation with LPS induced bright EGFP expression that overlapped with mCherry+ macrophage-lineage cells, as detected by confocal microscopy at 8 hpi. Scale bar 100 μm in A. Numbers indicate the proportion of larvae that displayed the presented phenotype. Larvae oriented anterior to left.

Supporting the confocal microscopy analysis, flow cytometry of similarly injected double transgenic Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae at 6 and 27 hpi revealed that EGFP+ cells were also mCherry+ (FIG 3.12). As this experiment was performed using clutches from an outcrossed heterozygous Tg(irg1:EGFP)nz4 parent, it was expected that only 50% of the macrophage-lineage cells would carry the -4.7irg1:EGFP transgene. As double positive cells were present at approximately 50-80% of the frequency of mCherry single positive cells, this implied that a similar proportion of the total macrophage-
lineage cell population at this stage responded to the injected LPS by stimulating the *irg1* promoter.

![Diagram](image)

**FIGURE 3.12: Dissociated Tg(*irg1:EGFP*)\textsuperscript{nz4}/Tg(*mpeg1:mCherry*)\textsuperscript{gl23} larvae show that EGFP driven by the -4.7*irg1* promoter is coexpressed with mCherry driven by the *mpeg1* promoter when assessed by flow cytometry**

Larvae from a Tg(*irg1:EGFP*)\textsuperscript{nz4} heterozygote outcross to a Tg(*mpeg1:mCherry*)\textsuperscript{gl23} homozygote were injected with either PBS or LPS at 2 dpf and dissociated at 2 and 23 hpi. As the dissociation process takes 4h, this resulted in analyses at 6 and 27 hpi respectively. **A,C.** While PBS-injected embryos showed an EGFP fluorescence shift at both timepoints, this expression was at a reduced intensity when compared with the LPS-stimulated groups **(B,D).**

While confocal imaging of double transgenic larvae did not detect EGFP expression following PBS stimulation, mCherry\textsuperscript{+}EGFP\textsuperscript{+} populations were present in the equivalent flow cytometry samples **(FIG 3.12 A,C).** This suggested that the dissociation process itself might induce transcription of *irg1*. To assess whether or not *irg1* was expressed by the EGFP\textsuperscript{+} populations found in both the PBS- and LPS- injected samples, mCherry\textsuperscript{+}EGFP\textsuperscript{+} cells were collected at 6 hpi and 27 hpi and compared using qPCR analysis **(FIG 3.13).**
FIGURE 3.13: Comparison of *irg1* expression in macrophage-lineage populations from PBS- or LPS-injected *Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23* larvae at 6 and 27 hpi

EGFP++mCherry++ populations were collected by FACS at 6 and 27 hpi from both PBS and LPS-injected *Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23* larvae. Expression of *irg1* in each population was quantified by qPCR and reported relative to the 6 hpi PBS-treated sample. All four populations were found to express *irg1*, however macrophage-lineage cells from LPS-injected larvae expressed *irg1* more strongly than their PBS-injected counterparts at both 6 and 27 hpi.

When comparing *irg1* transcript levels between EGFP-expressing macrophage-lineage populations from PBS- and LPS-injected *Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23* larvae, *irg1* transcript was detected in all four populations tested; however, the LPS-stimulated samples appeared to demonstrate enhanced expression at both 6 and 27 hpi when compared to their PBS-injected counterparts. While further study is required to determine the extent and significance of the enhanced expression seen here, detection of both *irg1* and EGFP within mCherry++ cells from PBS-injected *Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23* larvae supports the possibility that the process of tissue dissociation in preparation for flow cytometry may be immunostimulatory, as PBS injection alone has not previously been observed to stimulate expression of either *irg1* or the *irg1:EGFP* transgene in larvae assessed by WMISH or confocal live imaging.
3.9 Conclusions

Analysis of the \textit{irg1} promoter region during design of the \textit{-4.7irg1:EGFP} transgene identified a number of conserved recognition sequences between zebrafish, mouse, and human for transcription factors associated with aspects of metabolism and immune response. These transcription factors included USF, Runx1, CRE-BP, SREBP, and C/EBPβ, potentially linking regulation of \textit{irg1} to the M1-type macrophage phenotype, microglial behaviour, pro-inflammatory cytokine production, lipid metabolism, and the early innate immune response, respectively (Eberlé et al., 2004; Gobin et al., 2001; Hall et al., 2013; Wen et al., 2010; Zusso et al., 2012). Further understanding the extent to which these transcription factors, and others, contribute to the regulation of \textit{irg1} expression may provide information about both the functions of this gene and the pathways that may be targeted for potential therapeutic benefit.

Germ-line integration of the \textit{-4.7irg1:EGFP} transgene produced the \textit{Tg(irg1:EGFP)}\textit{nz4} reporter line. Transgenic embryos were found to express a low level of transgene in the forebrain from 36 hpf, providing an easy method for their rapid identification that avoids the anticipated need for immune stimulation. The \textit{Tg(irg1:EGFP)}\textit{nz4} reporter line displays strong, rapid, LPS-responsive EGFP expression within larval macrophage-lineage cells that accurately recapitulates the endogenous \textit{irg1} expression profile as detected by WMISH. Transgene expression is readily detectable by confocal and standard fluorescent microscopy and can be used to isolate \textit{irg1}-expressing macrophage-lineage cells from dissociated larvae via FACS. Importantly, live imaging \textit{Tg(irg1:EGFP)}\textit{nz4} larvae via confocal microscopy following injection with sterile PBS did not induce expression of the \textit{-4.7irg1:EGFP} transgene, supporting prior observations of the infection-responsive nature of \textit{irg1} expression (Hall et al., 2013). The \textit{Tg(irg1:EGFP)}\textit{nz4} reporter line addresses
the need for a tool enabling *in vivo* differentiation and observation of activated macrophages while also facilitating their isolation for further analyses.
4 Live confocal imaging of macrophage activation during the inflammatory response using the Tg(irg1:EGFP)\textsuperscript{nz4} reporter line

The Tg(irg1:EGFP)\textsuperscript{nz4} reporter line developed during this thesis provides the unique opportunity to live image the process of macrophage activation within zebrafish larva in response to immunogenic stimuli. Application of this transgenic reporter line to models of infection and disease will enable assessment of the contribution of irg1-expressing activated macrophage-lineage cells in these different microenvironments and how Irg1 contributes to macrophage functionality. For example, IRG1-expressing microglia have been linked to the progression of neurodegeneration (H. Li et al., 2006), while activated anti-tumour macrophage phenotypes have been described that are suggestive of irg1 expression (Chanmee et al., 2014). Live imaging irg1-expressing, activated macrophage-lineage cells could lend insight into differing macrophage functions and provide a tool for further investigation of appropriate treatments to modulate these behaviours for therapeutic benefit.

This chapter highlights methods of assessing Irg1\textsuperscript{+}, activated macrophage-lineage cells in larval zebrafish by using the Tg(irg1:EGFP)\textsuperscript{nz4} and Tg(mpeg1:mCherry)\textsuperscript{gl23} reporter lines to live image the process of activation during the immune response to either LPS or a tumour xenograft. In all instances, transgenic Tg(irg1:EGFP)\textsuperscript{nz4} larvae were identified prior to stimulation by selecting embryos demonstrating weak expression of the -4.7irg1:EGFP transgene in the forebrain prior to stimulation, as described in 3.6.
4.1 Live confocal imaging of the Tg(irg1:EGFP)nz4 reporter line reveals irg1 expression in activated macrophage-lineage cells responding to hindbrain LPS injection

Infectious stimulation of the hindbrain is a technically simple procedure that enables observation of immune responses to induced neuroinflammation (Benard et al., 2012; Hall et al., 2012; Hall et al., 2013). This site prevents passive dissemination of many injected molecules, due to the presence of the blood brain barrier, while allowing directed recruitment and invasion of immune cells (Xie, Farage, Sugimoto, & Anand-Apte, 2010). Live imaging activated macrophage-lineage cells in the hindbrain, as fluorescently labelled with the -4.7irg1:EGFP transgene, also enables interpretation of the immune responses of microglia, specialised tissue-resident macrophages of the brain that have been specifically implicated as drivers of neurotoxicity and shown to express irg1 following immune challenge (Hall et al., 2013; H. Li et al., 2006).

The response of early macrophage-lineage cells from Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae to hindbrain injection of LPS or PBS from 2 dpf was assessed using confocal time-lapse microscopy in order to determine the proportion of macrophage-lineage cells that responded with expression of the -4.7irg1:EGFP transgene, indicating activation. Microglia, the tissue resident macrophages of the brain, are not fully established in the zebrafish larva until at least 60 hpf (Herbomel et al., 2001). Thus, cells labelled by mpeg1:mCherry transgene expression at 2 dpf are expected to be a combination of early macrophages and macrophage-lineage cells that will go on to contribute to the tissue-resident microglia population. For this reason, the mpeg1:mCherry-expressing cells observed in these studies have been referred to as macrophage-lineage cells. Use of the Tg(mpeg1:mCherry)gl23 transgenic reporter line, which marked all macrophage-lineage cells with mCherry, allowed observation of the
transition from resting, mCherry$^{-}$EGFP$^{-}$ macrophage-lineage cells to activated, mCherry$^{+}$EGFP$^{+}$ macrophage-lineage cells (FIG 4.1).

FIGURE 4.1: Activation of macrophage-lineage cells as detected by induction of the -4.7irg1:EGFP transgene in Tg(irg1:EGFP)$^{nz4}$/Tg(mpeg1:mCherry)$^{gl23}$ double transgenic larvae

A. The Tg(mpeg1:mCherry)$^{gl23}$ transgenic zebrafish line labels all macrophage-lineage cells with red fluorescent mCherry protein. When crossed with Tg(irg1:EGFP)$^{nz4}$ transgenic zebrafish, larval macrophage-lineage cells do not express EGFP prior to stimulation. B. Following stimulation with hindbrain injection of LPS, the -4.7irg1:EGFP transgene is expressed within activated macrophage-lineage cells, producing yellow fluorescence due to co-expression with the mpeg1:mCherry transgene.

4.1.1 Expression of EGFP in macrophage-lineage cells within the hindbrain of Tg(irg1:EGFP)$^{nz4}$/Tg(mpeg1:mCherry)$^{gl23}$ larvae is LPS-dependent

Monitoring the hindbrain of 2 dpf Tg(irg1:EGFP)$^{nz4}$/Tg(mpeg1:mCherry)$^{gl23}$ larvae over a seven-hour period following LPS injection, using confocal time-lapse microscopy, revealed distinct EGFP expression within mCherry$^{+}$ macrophage-lineage cells (FIG 4.2). Of note, similar expression of EGFP was not observed with the PBS-injected Tg(irg1:EGFP)$^{nz4}$/Tg(mpeg1:mCherry)$^{gl23}$ larvae.
FIGURE 4.2: Time-lapse confocal imaging of -4.7irg1:EGFP expression in macrophage-lineage cells of Tg(irg1:EGFP)na4/Tg(mpeg1:mCherry)gl23 larvae following PBS or LPS injection

A-A"". Expression of the -4.7irg1:EGFP transgene was absent in PBS-treated larvae between 1 and 7 hpi. B-B"". Hindbrain injection with LPS generated strong -4.7irg1:EGFP expression by 7 hpi. White arrows indicate injection site. Scale bar 100µm in A. Numbers indicate the proportion of larvae that displayed the presented phenotype. Abbrev. dpf, days post-fertilisation; hpi, hours post-injection. Larvae oriented anterior to left.

Volocity 6.3 imaging analysis software from PerkinElmer was next used to determine the temporal kinetics of -4.7irg1:EGFP transgene expression within 2 dpf Tg(irg1:EGFP)na4/Tg(mpeg1:mCherry)gl23 larvae in response to LPS injection, as approximated through observation of EGFP fluorescence intensity. By selecting single macrophage-lineage cells based on mCherry expression driven from the mpeg1:mCherry
transgene, the development of EGFP fluorescence in these cells could be assessed over 7 and 17 hours following hindbrain injection with PBS or LPS respectively (FIG 4.3).

Assessing the fluorescence intensities of macrophage-lineage cells within the hindbrain of 2 dpf Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)β23 larvae over time revealed invariable,
low levels of EGFP fluorescence within the macrophage-lineage cells from PBS-injected larvae (FIG 4.3 A). In contrast, injection with LPS induced robust EGFP fluorescence in macrophage-lineage cells at an increasing rate from 3-9 hpi, indicating expression of the
-4.7irg1:EGFP transgene (FIG 4.3 B,C). A baseline EGFP fluorescence intensity of 125 fluorescence units, calculated as three standard deviations from the mean as seen in PBS-injected Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae, was determined as the threshold to surpass in order to identify a cell as activated and expressing the
-4.7irg1:EGFP transgene (FIG 4.3, blue line). Consistent laser power and microscope settings were maintained in all subsequent imaging experiments to ensure comparable collected data.

4.1.2 The majority of macrophage-lineage cells within the hindbrain of Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae become activated within six hours following LPS-injection

Calculation of the number of total macrophage-lineage cells and activated macrophage-lineage cells within the hindbrain of 2 dpf PBS- or LPS-injected Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae over time was performed using Volocity 6.3 imaging analysis software (FIG 4.4). Macrophage-lineage cells were identified by expression of mCherry fluorescent protein and activated macrophage-lineage cells were identified through their expression of both EGFP, at an intensity above 125 fluorescent units, and mCherry fluorescent protein.
FIGURE 4.4: Comparison of total macrophage-lineage and activated macrophage-lineage cell population sizes in the hindbrain of 2 dpf Tg(irg1:EGFP)$^{ns4}$/Tg(mpeg1:mCherry)$^{nl23}$ larvae following PBS or LPS injection

A. Total macrophage numbers (red line) remained relatively constant from 1-7 hpi following PBS injection. Very few macrophage-lineage cells within this population were activated.  
B. The proportion of activated macrophage-lineage cells (yellow line) increased from 4-6 hpi in response to LPS stimulation, as indicated by expression of the -4.7irg1:EGFP transgene, by which time nearly all macrophage-lineage cells present in the hindbrain were activated. Data generated from one experiment each but representative of 3/3 PBS replicates and 6/6 LPS replicates. Measurements taken through 29 Z-stack slices 5 μm apart at an interval of 7 min for the PBS experiment and 10 min for the LPS experiment. Abbrev. hpi, hours post-injection.

Very few activated macrophage-lineage cells, measured through expression of EGFP from the -4.7irg1:EGFP transgene at a fluorescent intensity above 125 fluorescence units, were detected by Volocity in the hindbrain of 2 dpf PBS-injected Tg(irg1:EGFP)$^{ns4}$/Tg(mpeg1:mCherry)$^{nl23}$ larvae in the seven hours following injection (FIG 4.4 A). Over the same time period, larvae injected with LPS displayed a sharp increase in activated macrophage-lineage cell numbers within the hindbrain from 4 hpi (FIG 4.4 B). Observing LPS-injected larvae over a longer time course revealed that the majority of macrophage-lineage cells within this region become activated by 6 hpi (FIG
4.4 C). The consistently high proportion of macrophage-lineage cells exhibiting activation within the hindbrain from 6-17 hpi suggested that any macrophage-lineage cells that may have migrated to the site during this time had been activated prior to arrival. This is supported by the literature (Hall et al., 2013) and data in Chapter 3, where WMISH analysis of irg1 expression following hindbrain injection with bacteria or LPS resulted in punctate expression throughout the body, suggesting a systemic signalling response. Additionally, all irg1:EGFP-expressing cells viewed in the hindbrain of Tg(irg1:EGFP)nas4/Tg(mpeg1:mCherry)gl23 larvae co-expressed mpeg1:mCherry. However, due to variability in the fluorescence intensity of the mCherry reporter, observed to be characteristic of the Tg(mpeg1:mCherry)gl23 transgenic line (Crosier lab, unpublished observation), combined with the dramatically higher EGFP fluorescence intensity seen at later time points (FIG 4.3), double-positive cells were often found to appear predominantly green, rather than the anticipated yellow, in overlayed images.

High EGFP fluorescence driven by the -4.7irg1:EGFP transgene did not appear to segregate macrophage-lineage cells from Tg(irg1:EGFP)nas4/Tg(mpeg1:mCherry)gl23 larvae by morphology at 2 dpf, though these cells presented a range of appearances during time lapse confocal live imaging of the 16-hour period following hindbrain injection with LPS (FIG 4.5).
Concordant with fluorescent intensity trends, confocal time-lapse observation revealed robust macrophage activation that increased in proportion from 3-9 hours, at which time nearly all macrophage-lineage cells present were activated. Arrow indicates injection site. Scale bar 100µm. Data generated from one experiment but representative of 6/6 LPS replicates. Images represent extended view through 29 Z-stack slices taken 5 µm apart. Abbrev. hpi, hours post-injection. Larva oriented anterior to left.

Two predominant macrophage-lineage cell morphologies were identified in the hindbrain of LPS-injected *Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23* larvae, which were further categorised as high or low EGFP-expressing cells. Resulting categorisation produced groups of EGFP^high^ and EGFP^lo^ spherical cells, and EGFP^high^ and EGFP^lo^ branched cells (FIG 4.6).
Macrophage-lineage cells with a branched phenotype appeared to migrate within the hindbrain at greater velocity than cells with a more spherical morphology. The depth of these cells relative to the epidermis was calculated along with their velocities along their migration paths (FIG 4.7).

Branched cells were found to be significantly more superficial than those with more spherical morphologies, with the majority present along the epithelial surface or within the first 20µm of hindbrain tissue, while more spherical cells averaged 40µm below the epithelial surface (FIG 4.7 B). There was no significant difference in the velocity of \( \text{EGFP}^{\text{high}} \) versus \( \text{EGFP}^{\text{lo}} \) branched or spherical macrophage-lineage cells within the hindbrain following LPS-injection, though branched cells moved significantly faster than more spherical ones (FIG 4.7 C).
A. Cell depth measurements were made manually relative to the epithelial surface. B. Macrophage-lineage cells with a branched appearance tended to be found more superficially than more spherical cells. C. Branched cells were found to move more rapidly than those with a spherical morphology, though little difference existed between different EGFP expression-level groups. Measurements taken from five experiments at the 8 hpi timepoint. Velocity measurements were made by manually tracking cells along migration paths that could be followed for a minimum of one hour. Significance was determined by Student’s t-test (B) or one-way ANOVA with multiple comparisons (C). Bars indicate average and standard deviation. Abbrev. *, $P<0.05$; ****, $P<0.0001$.
4.1.3 MMP activity associates with activated, -4.7irg1:EGFP-expressing macrophage-lineage cells following hindbrain injection with LPS

The MMPSense 645 FAST reagent (MMPSense), a compound that releases fluorescence after cleavage by one of a broad range of MMPs (PerkinElmer), was next exploited to determine whether MMP activity corresponded with induction of irg1 expression in macrophage-lineage cells. The MMPSense reagent has previously been used in zebrafish to successfully detect MMP activity during the inflammatory response (Hall et al., 2014). As the fluorescent emission of this reagent is in the far-red end of the spectrum, it can be distinctly observed within macrophage lineage cells expressing either the mpeg1:mCherry transgene or the -4.7irg1:EGFP transgene. Expression of matrix metalloproteinases within macrophage-lineage cells has been reported to be indicative of an inflammatory phenotype (Newby, 2008), suggesting that assessing the expression of these enzymes in relation to irg1 expression may aid in further differentiating activated, macrophage-lineage cell subtypes within 2 dpf LPS-injected Tg(irg1:EGFP).nz4/Tg(mpeg1:mCherry)gl23 larvae, while indicating whether irg1-expressing cells also express pro-inflammatory MMPs.

Following a 1 nL co-injection of 20 μM MMPSense and 0.8 mg/mL LPS into the hindbrain of Tg(irg1:EGFP).nz4/Tg(mpeg1:mCherry)gl23 larvae at 2 dpf, MMP activity was rapidly detected within macrophage-lineage cells (FIG 4.8) (MOVIE 4.1).
FIGURE 4.8: MMP activity in the hindbrain of \( Tg(irg1:EGFP)^{nt4}/Tg(mpeg1:mCherry)^{gl23} \) larvae following injection with LPS

A-A". Punctate MMP activity was detected within \( mpeg1:mCherry \)-expressing macrophage-lineage cells at 3 hpi. B-B". By 6.5 hpi, MMP activity was seen in both \( irg1:EGFP \)-expressing and non-expressing macrophage-lineage cells. C. XYZ view of the inset from A" supports colocalisation of the MMPSense probe within \( mpeg1:mCherry \)-expressing macrophage-lineage cells. Scale bar 100\( \mu \)m in A. Data representative of 5/5 LPS+MMPSense replicates. Abbrev. dpf, days post-fertilisation; hpi, hours post-injection. Larva oriented anterior to left.

MMP activity was noted within 30 min following hindbrain injection with MMPSense and LPS, occurring within macrophage-lineage cells of 2 dpf \( Tg(irg1:EGFP)^{nt4}/\newline\newline Tg(mpeg1:mCherry)^{gl23} \) larvae before expression of the -4.7\( irg1:EGFP \) transgene was
readily detected by confocal imaging (FIG 4.8 A, 4.10 B). By 6.5 hpi, the fluorescent signal that marked MMP activity was observed within both activated EGFP\(^{+}\)mCherry\(^{+}\) and EGFP\(^{-}\)mCherry\(^{+}\) macrophage-lineage cells, but was not present in all cells of these populations (FIG 4.8 B-B\(^{+}\)).

Closer examination of the relationship between the fluorescence intensity values of macrophage-lineage cells at 7 hpi, the point by which the population of -4.7irg1:EGFP-expressing, activated macrophage-lineage cells had mostly stabilised, indicated that a subpopulation of macrophage-lineage cells expressing high levels of the -4.7irg1:EGFP transgene also had high MMP activity (FIG 4.9).

![Graph showing correlation between mean EGFP and MMP intensity within individual macrophage-lineage cells at 7 hpi](image)

**FIGURE 4.9: High rates of MMP activity tend to correlate with high expression of the -4.7irg1EGFP transgene by 7 hpi within LPS-injected Tg(irg1:EGFP)\(^{+/-}\)/Tg(mpeg1:mCherry)\(^{+/-}\) larvae**

Assessing the mean EGFP and MMPSense intensities (x and y axis respectively) within individual macrophage-lineage cells 7 h following injection with LPS revealed a subpopulation of cells (indicated by black box) that expressed high levels of the -4.7irg1:EGFP transgene and displayed high MMP activity. Linear regression analysis over all cells at this timepoint (black line) revealed a coefficient of determination (R\(^{2}\)) value of 0.35, suggesting that expression of irg1 may be a moderately reliable predictor of MMP expression. n = 35 cells. Data representative of 5/5 LPS+MMPSense replicates. Abbrev. hpi, hours post-injection.

An association between high MMP activity and high EGFP intensity suggested that sustained irg1 expression, leading to increased expression of the -4.7irg1:EGFP transgene and thus higher EGFP fluorescence within macrophage-lineage cells of 2 dpf
Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae, may lead to, or is correlated with, increased levels of MMP activity following injection with LPS.

Overlaying the mean per-cell fluorescent intensities of MMPSense, mCherry, and EGFP within macrophage-lineage cells from Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae following injection with either PBS or LPS revealed very little MMP activity in the PBS-injected larvae (FIG 4.10 A).

**FIGURE 4.10: Intensity of MMPSense cleavage and -4.7irg1:EGFP transgene expression within macrophage-lineage cells of the hindbrain following LPS or PBS injection of Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae**

A. Both MMP activity and -4.7irg1:EGFP transgene expression within macrophage-lineage cells in PBS-treated larvae was minimal between 1 and 7 hpi. B. Macrophage-lineage cells from LPS-injected larvae exhibited robust MMP activity between 1 and 7 hpi and increasing expression of -4.7irg1:EGFP from 4 hpi. C. Assessing the fluorescence intensity of MMPSense within macrophage-lineage cells between 1 and 17 h following LPS injection indicated that the mean MMP activity per macrophage-lineage cell peaked near four hours following LPS injection. Blue line indicates fluorescence intensity of 125, which was calculated as the threshold EGFP fluorescent intensity to determine macrophage activation. Black lines indicate mean per-cell fluorescence intensities and shaded regions indicate +/- 1 standard deviation. Data generated from one experiment each but representative of 3/3 PBS+MMPSense replicates and 5/5 LPS+MMPSense replicates, measuring approximately 30 cells per timepoint. Measurements taken through 29 Z-stack slices 5 μm apart at an interval of 7 min for the PBS experiment and 10 min for the LPS experiment. Abbrev. hpi, hours post-injection.
Peak MMP activity was noted within macrophage-lineage cells within the first six hours following stimulation with LPS (FIG 4.10 B,C). This period occurred prior to peak expression of the -4.7irg1:EGFP transgene (FIG 4.10 C). The total number of macrophage-lineage cells exhibiting MMP activity follows a similar pattern (FIG 4.11).

**FIGURE 4.11: Comparison of the population sizes of total macrophage-lineage cells, -4.7irg1:EGFP transgene-expressing (activated) macrophage-lineage cells, and macrophage-lineage cells demonstrating MMP activity in the hindbrain of 2 dpf Tg(irg1:EGFP)^hze/Tg(mpeg1:mCherry)^gl23 larvae following PBS or LPS injection**

A. Macrophage-lineage cells from PBS-injected larvae did not exhibit MMP activity. B. Coincident with peak per-cell MMPSense fluorescence intensity levels, the number of macrophage-lineage cells exhibiting MMP activity was greatest near 4 hpi. C. From 4 hpi, the number of macrophage-lineage cells that exhibit MMP activity slowly decreases until reaching steady state near 12 hpi. Data generated from one experiment each but representative of 3/3 PBS+MMPSense replicates and 5/5 LPS+MMPSense replicates. Measurements taken through 29 Z-stack slices 5 μm apart at an interval of 7 min for the PBS experiment and 10 min for the LPS experiment. Abbrev. hpi, hours post-injection.
4.2 Alternate routes of stimulation

While the hindbrain offers advantages such as ease of access and large injection volume capacity, this site creates a relatively large inflammatory “target.” This may contribute to larger initial signalling events resulting in greater activation of macrophage-lineage cells than would be seen with a more localised stimulation. In addition, it may be advantageous to compare the responses of primarily non-resident macrophages, such as the peripheral macrophages anticipated to respond to an injury or insult to the otic vesicle, with those of tissue-resident populations, such as the microglia that predominate in the brain. With these considerations in mind, the activation kinetics of macrophages within Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 double transgenic larvae, determined by expression of the -4.7irg1:EGFP transgene, were assessed in response to an injection of LPS into the otic vesicle.

Additionally, macrophages displaying inflammatory phenotypes have been found in disease states such as cancer and obesity, where no infectious agent has been detected. To assess whether irg1 is expressed in macrophages responding to model of non-infectious disease, Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 double transgenic larvae were also observed following injection of a human breast cancer xenograft into the perivitelline space.

4.2.1 Expression of the -4.7irg1:EGFP transgene in response to LPS injection into the otic vesicle of Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae, as detected by live confocal imaging

In the zebrafish, the otic vesicle offers another specific migratory target site with limited injection dissemination (Benard et al., 2012). When compared with the hindbrain, these
injections and the mounting they require are more technically challenging. The otic vesicle is more difficult to pierce and there is a greater risk of damaging the larva if pressure or volume settings are too great on the microinjector. Following LPS injection into this site in \( Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{\alpha23} \) larvae, macrophages demonstrated similar transgene induction kinetics to that seen in response to LPS injected into the hindbrain. Activated macrophages, expressing the \(-4.7\text{irg1:EGFP}\) transgene, were observed as early as 2 hpi, with the majority of the total macrophage population exhibiting activation by 7 hpi (FIG 4.12) (MOVIE 4.2).

**FIGURE 4.12: Macrophage activation in response to LPS injection into the otic vesicle of \( Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{\alpha23} \) larvae**

A. Activated, \(-4.7\text{irg1:EGFP}\) transgene-expressing macrophages were present from 2 hpi. B. By 7 hpi, the majority of responding macrophages displayed the \(-4.7\text{irg1:EGFP}\) transgene expression indicative of activation. C. PBS injection into the otic vesicle did not stimulate expression of the \(-4.7\text{irg1:EGFP}\) transgene by 7 hpi. Scale bar 100µm in A. Numbers indicate the proportion of larvae that displayed the presented phenotype. Measurements taken through 24 Z-stack slices 5 μm apart. Larva oriented laterally, anterior to left. Abbrev. dpf, days post-fertilisation; hpi, hours post-injection.

Despite the smaller injection site, LPS injection into the otic vesicle of 2 dpf \( Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{\alpha23} \) larvae did not appear to produce a slowed or dampened activation response in macrophages as compared to the response seen to LPS injection into the hindbrain. By 7 hpi, the majority of macrophages found in close proximity to the otic vesicle were expressing the \(-4.7\text{irg1:EGFP}\) transgene and consequent EGFP (FIG 4.12 B).
Observation of MMP production from macrophage-lineage cells attracted to the otic vesicle proved difficult. No fluorescence was observed when the MMPSense reagent was co-injected with LPS into the otic vesicle or injected alone intravenously just prior to otic vesicle injection with LPS. This indicated that the reagent was likely unable to effectively diffuse out from the otic vesicle or vasculature at concentrations high enough to detect cleavage.

4.2.2 Expression of the -4.7irg1:EGFP transgene during macrophage response to cancer xenograft in Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)g123 larvae, as detected by live confocal imaging

To assess whether irg1 expression is induced in macrophages within the tumour microenvironment, Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)g123 larvae were injected with ~150 cells of the human metastatic breast cancer cell line MDA-MB-231 suspended in 50% matrigel. This cell line has been previously used in zebrafish studies to assess cancer growth and immune response (Astin et al., 2014; Drabsch, He, Zhang, Snaar-Jagalska, & ten Dijke, 2013; Teng et al., 2013).

Unpublished data from our research group has indicated that expression of irg1 is induced within macrophages in response to xenografted MDA-MB-231 cells suspended in matrigel, but not in response to the injection of matrigel alone, as detected by WMISH (Appendix 4, Christopher Hall, unpublished).

Based on the observation by fluorescent WMISH of irg1-expressing macrophages surrounding tumour cells, it was anticipated that the Tg(irg1:EGFP)nz4 transgenic zebrafish line may be useful to live-image the process of activation of macrophages in
response to a tumour xenograft while observing the interactions of these macrophages with the tumour cells.

Live-imaging the MDA-MB-231 breast cancer cells in 2 dpf Tg(irg1:EGFP)$^{+/-}$/Tg(mpeg1:mCherry)$^{0/2}$ larvae was facilitated by the use of the Hoechst 33342 nuclear stain (Hoechst) prior to injection into the perivitelline space. This allowed observation of tumour cells within the initial injection bolus as well as those that had migrated to other regions of the body through the circulation (FIG 4.13).

FIGURE 4.13: Macrophage response to a breast cancer xenograft into the perivitelline space of Tg(irg1:EGFP)$^{+/-}$/Tg(mpeg1:mCherry)$^{0/2}$ larvae at 2 dpf

A,A'. Confocal imaging of a whole Tg(irg1:EGFP)$^{+/-}$/Tg(mpeg1:mCherry)$^{0/2}$ larva 12 h post-injection with a breast cancer xenograft revealed activated macrophages over the yolk, in the head, and in the caudal
haematopoietic tissue (CHT). Cancer cells were present in the head, perivitelline space, and CHT. See panel B for yolk (*) inset. See panel C for CHT (x) inset. B,B’. Macrophages within the tumour mass displayed activation by 12 hpi, as detected by expression of the -4.7irg1:EGFP transgene. C-C”. Activated macrophages in the CHT associated with cancer cells at 14 hpi, indicating phagocytosis of the tumour cell by the activated macrophage. Arrow in A indicates cancer cell injection site (through the yolk into the perivitelline space). Scale bars 100 μm in A,B,C. Numbers indicate the proportion of embryos injected with the cancer xenograft that display expression of the -4.7irg1:EGFP transgene within macrophages. Measurements taken through 22 Z-stack slices for imaging the tumour within the perivitelline space and 12 slices for imaging the CHT region, with slices taken 5 μm apart. Abbrev. dpf, days post-fertilisation; hpi, hours post-injection. Larvae oriented anterior to left.

Injection of the Hoechst-labelled breast cancer cells into the perivitelline space of 2 dpf Tg(irc1:EGFP)izz4/Tg(mpeg1:mCherry)gl23 larvae resulted in moderate expression of the -4.7irg1:EGFP transgene from 5-14 hpi in macrophages over the yolk and in the caudal haematopoietic tissue, where cancer cells were frequently found to travel (FIG 4.13). In addition, a number of phagocytosis events, where macrophages were observed surrounding tumour cells, were noted in both the caudal haematopoietic tissue (FIG 4.13 C-C”) and the tumour mass within the perivitelline space (FIG 4.14).
FIGURE 4.14: Human breast cancer cells are engulfed by macrophages expressing the -4.7irg1:EGFP transgene within Tg(irg1:EGFP)nst/Tg(mpeg1:mCherry)gl23 larvae

A-C. Macrophages with weak (white arrow) and intermediate (green arrow) -4.7irg1:EGFP transgene expression were found colocalised with tumour cells at 5 hpi within the tumour mass injected into the perivitelline space. A’-C’. Magnified XYZ view of the -4.7irg1:EGFPnst macrophage indicated by the green arrow in A-C shows engulfment of a tumour cell. Scale bar 100 μm in A, 50 μm in A’. Numbers indicate the proportion of embryos injected with the cancer xenograft that displayed phagocytosis of tumour cells by macrophages that had induced expression of the -4.7irg1:EGFP transgene. Measurements taken through 22 Z-stack slices 5 μm apart. Abbrev. dpf, days post-fertilisation; hpi, hours post-injection. Larvae oriented laterally, anterior to left.

As phagocytosis of cancer cells was seen by macrophages expressing varying levels of the -4.7irg1:EGFP transgene, it was not apparent what effects irg1 expression may have had on either macrophage behaviour or tumour survival in this environment. However, the induction of irg1 expression in macrophages in response to a cancer xenograft is
encouraging as it demonstrates another potential tool for the further dissection of macrophage-tumour relationships.

### 4.3 Conclusions

The expression kinetics of the \(-4.7 \text{irg1:EGFP}\) transgene have been described. Activation of macrophage-lineage cells in 2 dpf \(Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{gl23}\) larvae in response to hindbrain injection with LPS occurred rapidly within nearly all macrophage-lineage cells, i.e. microglia and recruited macrophages, in the hindbrain region between 3 and 7 hpi, as detected by EGFP fluorescence produced by expression of the \(-4.7 \text{irg1:EGFP}\) transgene. A similar induction was not seen in PBS-injected larvae. These data were supported by similar studies investigating the use of the otic vesicle as an injection site to observe the response of macrophage-lineage cells outside of the tightly regulated hindbrain cavity, i.e. peripheral macrophages. Live imaging analysis also revealed two distinct morphologies in cells responding to hindbrain injection of LPS. One, a more branched, superficial macrophage-lineage cell type, tended to migrate more rapidly than the second, more spherical cell type that penetrated the hindbrain more deeply. These cells may represent early Langerhans cells and microglia respectively, though both appeared to display similar ranges of induced transgene expression.

Applying a tumour xenograft model to \(Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{gl23}\) larvae by injecting human breast cancer cells into the perivitelline space has been described as an alternate method of \(\text{irg1}\) induction to explore macrophage behaviour in different immune environments. This model revealed that macrophages in close association with cancer cells also express \(\text{irg1}\). Macrophages were observed phagocytosing cancer cells at both the initial tumour cell injection site as well as at distal locations. While application
of the tumour xenograft model to understanding of tumour-associated macrophages is somewhat limited as it uses foreign tissue rather than host-derived disease, it may prove useful in future to further define Irg1-regulated pathways and to study different macrophage subtypes.

Importantly, the studies described in this chapter have demonstrated novel utilities of the $Tg(\text{irg}1:\text{EGFP})^{nz4}$ transgenic zebrafish line in the live imaging of macrophage activation in response to multiple infection-associated model systems, as demonstrated through LPS injection into the hindbrain or otic vesicle and through injection of a human breast cancer xenograft. It is expected that this unique transgenic line will be of use to numerous other models of diseases that involve macrophage responses and pathologies where modulation of macrophage phenotype is a desirable strategy for therapeutic treatment.
5 Investigating downstream consequences of attenuated Irg1 activity within macrophage-lineage cells through RNA-Seq

The expression of irg1 has been linked to the metabolic reprogramming of macrophage-lineage cells through the promotion of fatty acid β-oxidation and an increased production of pro-inflammatory mediators, including mROS and itaconic acid (Hall et al., 2013; Michelucci et al., 2013). In the context of these studies, this elevated production of mROS was found to contribute to increased bactericidal macrophage capacity. Given the range of signalling and effector functions mediated by mROS, it is anticipated that Irg1-dependent mROS production also contributes to other macrophage functions. For example, mROS has, to date, demonstrated direct bactericidal capacity, functions in regulating the transcription of immune response genes including il-6, mmp9, and tnfα, and contributions to the maturation of inflammatory cytokines such as IL-1β and IL-18 through its role in activating the NLRP3 inflammasome (Sena & Chandel, 2012; West et al., 2011; Woo et al., 2004; Zhou et al., 2011). It is also likely that a number of Irg1-driven, mROS-regulated activities remain undiscovered or unattributed. Insight into the signalling pathways activated by the presence of Irg1 may be gained by assessing transcriptome changes within macrophage-lineage cells that result from knocking down Irg1 in an otherwise intact in vivo environment. These data will increase our understanding of the consequences of Irg1-driven metabolic reprogramming of macrophage-lineage cells and may help elucidate the relevance of Irg1 to inflammatory diseases where macrophage-lineage cells may contribute. These insights may be used to inform new approaches to the treatment of inflammation and disease through the manipulation of Irg1 signalling.
5.1 Rationale

Cell phenotype is indicated by the sum of DNA sequences expressed at any given point in time and their relative expression levels (Jacquier, 2009). These comprise a cell’s transcriptome. Comparing the transcriptome of a population of cells under different circumstances has the power to highlight changes in transcript expression levels and shifts in preferences for cell processes and responses that suggest changes in cell functions (Costa, Angelini, De Feis, & Ciccodicola, 2010). By examining all genes within an annotated genome, these data may be applied to gene set databases to investigate skewed representation of different cellular responses or processes by examining all relevant genes cumulatively, producing a more holistic and meaningful view of the cell phenotype than pursuing changes to the expression of any one individual gene.

RNA-Sequencing (RNA-Seq) is a technique for transcriptome analyses that has already proven effective in identifying infection-responsive genes in zebrafish embryos (Ordas et al., 2011). In this technique, RNA transcripts are sequenced directly (Costa et al., 2010). This avoids the drawbacks of hybridisation approaches in that it allows for differential analysis of alternate splicing events, the detection of SNPs, and can report new, unknown transcripts, as it does not rely on prior knowledge of the genome sequence (Costa et al., 2010). Consequently, RNA-Seq also has much wider coverage than traditional techniques like microarray analysis. RNA-Seq has previously been successfully applied to assessing the immune response of zebrafish embryos to infection by Salmonella enterica serovar Typhimurium, Staphylococcus epidermidis, or Mycobacterium marinum revealing genes of both known and anticipated importance to innate immune response (Ordas et al., 2011; Veneman et al., 2014; Veneman et al., 2013).
Given that infection-responsive irg1 expression has been linked to the promotion of fatty acid β-oxidation in macrophage mitochondria (Hall et al., 2013), which in turn elevates mROS production, and considering the broad range of mROS-driven immune functions, it is anticipated that irg1-driven mROS production contributes to a range of immune functions (FIG 5.1). This has been supported by data presented in Chapter 4, where, following injection of either LPS or a tumour xenograft, expression of the -4.7irg1:EGFP transgene was observed in Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)β23 larvae, indicating induction of irg1 expression in response to varied inflammatory stimuli. By using RNA-Seq to assess the transcriptome changes that result from inhibiting irg1 expression during the immune response of activated macrophage-lineage cells, pathways dependent on Irg1 and Irg1-driven mROS production may be revealed.

**FIGURE 5.1: Model illustrating how Irg1-dependent mROS production may drive transcriptome changes in activated macrophage-lineage cells**

During activation of macrophage-lineage cells in response to infection, irg1 expression promotes fatty acid β-oxidation (orange), which feeds into the citric acid cycle and electron transport chain (pink), producing mROS. Contributions of mROS to the inflammatory response of macrophage-lineage cells include antimicrobial activities, inflammasome activation, and transcription regulation, leading to changes in the transcriptome that dictate other immune functions. Blocking irg1 expression (blue) will reduce mROS-mediated effects, revealing infection-responsive, Irg1-dependent functions in activated macrophage-lineage cells. Abbrev. CAC, citric acid cycle; ETC, electron transport chain; FFA, free fatty acids; mROS, mitochondrial reactive oxygen species.
The \textit{Tg(irg1:EGFP)nz4} transgenic developed in this thesis is a unique zebrafish reporter line that allows isolation of a discrete population of activated macrophage-lineage cells, as indicated by their expression of the -4.7\textit{irg1:EGFP} transgene following stimulation by hindbrain injection with LPS. Selecting this population using FACS from a suspension of single cells made from dissociated whole \textit{Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23} larvae following injection with LPS allows analysis of the transcriptome of activated macrophage-lineage cells in isolation. Outcrossing the \textit{Tg(irg1:EGFP)nz4} transgenic line to the \textit{Tg(mpeg1:mCherry)gl23} reporter, labelling all macrophage-lineage cells with fluorescent mCherry, adds additional confidence to the purity of this macrophage population, as \textit{irg1} expression has been detected in non-macrophage tissues in other animal systems (Cheon et al., 2003; Hall et al., 2014) though the only evidence of non-macrophage-lineage expression observed thus far in \textit{Tg(irg1:EGFP)nz4} has been weak transgene expression in the forebrain. Assessing the changes that occur during the activation of macrophage-lineage cells in response to changing circumstances, such as the presence or absence of functional Irg1 during an infection response, will elucidate the process of phenotype switching within these cells and the role that Irg1 plays in driving their effector functions.

Depletion of Irg1 can be accomplished through the use of splice-blocking morpholinos. Splice-blocking morpholinos are stable, antisense oligomers that act by binding splice donor or acceptor sites, preventing their recognition (FIG 5.2) (Moulton & Yan, 2008). Typically, use of the next available splice junction leads to exclusion of the exon adjacent to the targeted site or inclusion of the adjacent intron (FIG 5.2 B,C). Rarely, activation of cryptic splice sites leads to alternate splicing events (FIG 5.2 D). Transcripts targeted by
A splice-blocking morpholino will often lead to the production of inactive, truncated protein (Moulton & Yan, 2008).

**FIGURE 5.2: Potential effects of splice-blocking morpholinos on pre-mRNA splicing**

**A.** Correct splicing of an example pre-mRNA retains all exons (blue rectangles) while excluding all introns (adjoining lines and orange rectangles). **B.** Splice-blocking morpholinos bind to targeted splice donor or acceptor sites, preventing their recognition. Use of the next available site may result in exclusion of the targeted exon. **C.** Alternatively, an adjacent intron may be included. **D.** In some instances, preventing binding to a splice acceptor or donor site activates use of a cryptic splice site, leading to inclusion of a partial exon. Black lines indicate splicing. Red lines indicate splice-blocking morpholinos binding splice junctions. Abbrev. E, exon; I, intron.

As expression of the -4.7irg1:EGFP transgene produces EGFP transcript following stimulation of the *irg1* promoter region, fluorescent protein will still mark macrophage-lineage cells within *irg1*-targeting, splice-blocking morpholino-injected larvae that receive activation signals but have compromised Irg1 function. This means that both Irg1-replete and Irg1-depleted macrophage-lineage cells can be specifically isolated by observing expression of the -4.7irg1:EGFP transgene. Additionally, by using two *irg1*-targeting, splice-blocking morpholinos in separate treatment groups, potential off-target effects from using morpholinos, though not anticipated, may be accounted for by
determining similarly-regulated sequences when compared with the group injected with a control, non-targeting morpholino.

Macrophage-lineage cells with reduced Irg1 activity have previously been shown to have impeded bactericidal activity, leading to an increased bacterial load (Hall et al., 2013). The greater presence of an active immune insult within these cells is anticipated to lead to increased stress that may activate cell programs not directly related to the presence or absence of Irg1 activity. For example, when assessing macrophage response to the intracellular zebrafish pathogen *Mycobacterium marinum*, Roca and Ramakrishnan reported that while the initial induction of ROS conferred microbicidal functions to macrophages, extended production of this reactive compound lead to the induction of necroptosis and further dispersion of bacteria (Roca & Ramakrishnan, 2013). In order to more specifically examine the functions conferred to macrophages through Irg1 activity, a method of induction that does not generate a propagating stress response is required. To this aim, hindbrain injection of LPS has been shown to effectively induce *irg1* expression in zebrafish macrophage-lineage cells (Hall et al., 2013). Figure 5.3 describes the approach taken for transcriptome analysis in the present study.
FIGURE 5.3: Rationale for assessing the transcriptome contributions of Irg1, using the Tg(irg1:EGFP)nz4 transgenic reporter line

1,2. Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 embryos are injected at the 1-2 cell stage with splice-blocking morpholinos targeting irg1 transcripts or a non-targeting morpholino control. All samples are generated in biological triplicate. 3. Larvae are stimulated at 2 dpf through hindbrain injection with LPS. 4. The promoter region that drives irg1 expression within activated macrophage-lineage cells is induced by 2 hpi, leading to transcription of irg1 and the -4.7irg1:EGFP transgene. EGFP transcript is translated into functional fluorescent protein in all groups, while injected splice-blocking morpholinos targeting irg1 inhibit the correct splicing of irg1 pre-mRNA, leading to depletion of functional Irg1 protein. 5. Whole larvae are dissociated into single-cell suspensions. 6. A discrete population of EGFP+mCherry+ activated macrophage-lineage cells are collected from each sample via FACS. 7. RNA is isolated from each sample. 8. RNA-Seq is used to compare the transcriptomes of activated macrophage-lineage cells with or without the functional contribution of Irg1. 9. Differential sequence analysis identifies macrophage-specific, Irg1-
dependent transcriptome changes by determining common, differentially-expressed sequences to both splice-blocking morpholino-injected groups. Abbrev. CTRLMO, control morpholino; dpf, days post-fertilisation; FACS, flow assisted cell sorting; hpi, hours post-injection; IRG1SBMO1, irg1 splice-blocking morpholino 1; IRG1SBMO3, irg1 splice-blocking morpholino 3.

5.2 Experimental Design

5.2.1 Morpholino design and characterisation

Embryos were injected with morpholinos at the 1-2 cell stage. Splice-blocking morpholinos were chosen to prevent proper splicing of irg1 pre-mRNA, as validated by RT-PCR revealing altered open reading frame sizes reflective of intron inclusions or exon exclusions. As there is currently no available antibody against zebrafish Irg1, ATG-targeting morpholinos, which prevent mRNA translation (Moulton & Yan, 2008), cannot be validated and so were not used. These embryos were compared with a control group injected with a non-targeting morpholino (Hall et al., 2013). As an additional control to account for potential off-target effects, two splice-blocking morpholinos, targeting different splice junctions of the irg1 transcript, were used in separate treatment groups. Differentially-expressed sequences common to both splice-blocking morpholino-injected groups, when compared with the control morpholino-injected group, were anticipated to indicate expression changes specifically resulting from reduced Irg1 activity within macrophage-lineage cells.

Splice-blocking morpholinos were designed and selected based on their ability to affect normal splicing of irg1 pre-mRNA while retaining low toxicity. Figure 5.4 shows the exon-intron structure of the irg1 pre-mRNA, with details about splice-blocking morpholino target sites and the products resulting from the induced alternate splicing.
FIGURE 5.4: Analysis of the irg1 gene sequence for splice-blocking morpholino design

A. The irg1 gene consists of five exons (blue rectangles) and four introns (adjoining lines). Morpholinos targeting the intron1/exon2, intron2/exon3, and exon4/intron4 splice junctions (indicated and named in orange) were generated. IRG1SBMO2 was found to be highly toxic at low doses. B. The wild type irg1 transcript contains a 1434bp open reading frame and encodes a predicted 477aa protein. C. The presence of IRG1SBMO1 results in inclusion of intron2 (orange rectangle), producing a 1523bp alternate splice product containing a premature stop codon that truncates the protein at 78aa. D. The presence of IRG1SBMO3 results in exclusion of exon4, producing a 1234bp alternate splice product and a premature stop codon truncating the protein at 105aa. The locations of primers used in RT-PCR are indicated and named in blue. Black lines indicate correct splicing, as seen when splice-blocking morpholinos are not present. Purple lines indicate translated sequence length with asterisks demarkating stop codons. Abbrev. aa, amino acid; bp, base pair; E, exon; I, intron; ORF, open reading frame; WT, wild type.
Previous studies from our laboratory characterised the splice-blocking morpholino IRG1SBMO1, which targets exon three leading to inclusion of the preceding intron (Hall et al., 2013). This creates a premature stop codon predicting a truncated protein only 78aa long, compared to the 477aa wild type protein (FIG 5.4 C). Additional morpholinos targeting exons two and four, IRG1SBMO2 and IRG1SBMO3 respectively, were synthesised and assessed for this study. Injection with IRG1SBMO2 resulted in high embryo toxicity at doses as low as 0.4nmol, as evidenced by hindbrain and cardiac oedema and crooked notochords in over 90% of embryos at 2 dpf. Conversely, IRG1SBMO3 was well tolerated, with doses up to 2nmol eliciting only mild hindbrain oedema in approximately 10% of the sample at 2 dpf. Titrations of both IRG1SBMO1 and IRG1SBMO3 lead to an optimised dose of 1nmol for each, supplied in a 1.5 nL injection volume. IRG1SBMO2 was not used in this study due to its high rate of toxicity.

RT-PCR analysis using primers flanking the targeted irg1 splice junction sites suggested that injection with IRG1SBMO3 lead to exclusion of exon four (FIG 5.5). This was confirmed through sequencing of the alternatively spliced product. This shortened alternate splice product contained a premature stop codon that coded for a truncated, 105aa protein, compared to the wild type 477aa protein (FIG 5.4 D).

![DNA ladder](image)

**FIGURE 5.5: Analysis of irg1 mRNA splice products from 2 dpf larvae injected with either control morpholino, IRG1SBMO1, or IRG1SBMO3, two hours following hindbrain injection with LPS**

Irg1 Do2 and Irg1 Up6 primers produced a band running at 1265bp in all samples, indicating wild type splice product. IRG1SBMO1 morphants produced a larger, 1354bp band consistent with inclusion of the 89bp intron2. IRG1SBMO3 morphants produced an additional band running at 1065bp, consistent with exclusion of the 200bp exon4. White arrow indicates position of wild type splice products. Black arrows indicate alternate splice products. Abbrev. bp, base pairs; CTRLMO, control morpholino; MO1, IRG1SBMO1; MO3, IRG1SBMO3
RT-PCR analysis on IRG1SBMO1 morphants under similar conditions supported previous reports of IRG1SBMO1 injection leading to inclusion of intron two, leading to reduced Irg1 activity (FIG 5.5) (Hall et al., 2013). This produced a longer alternate splice product with a premature stop codon after 78aa.

5.2.2 Immune stimulation and dissociation

Following injection of 1-2 cell stage $Tg(\text{irg1:EGFP})^{naz4}/Tg(\text{mpeg1:mCherry})^{gl23}$ embryos with morpholino oligonucleotides, embryos were manually dechorionated at 30 hpf. While more labour-intensive, manual dechorionation minimises the potential stresses and immune stimulation that may result from enzymatic digestion protocols (Henn & Braunbeck, 2011). At 45.5 hpf, all larvae were arrayed in 3% methyl cellulose prior to hindbrain injection with LPS. The number of larvae injected per treatment on each experiment day was limited to approximately 200. Figure 5.6 outlines the experiment timeline from embryo collection to RNA extraction. Each morpholino-injected group was assessed in biological triplicate, with each experiment day generating one replicate of each group, ensuring comparable stimulation levels across samples from each experiment day.
1. **Tg(irg1:EGFP)etz/Tg(mpeg1:mCherry)j123** embryos were injected with either IRG1SBMO1 or IRG1SBMO3 splice-blocking morpholinos or a non-targeting control morpholino at the 1-2 cell stage. A minimum of 300 embryos were injected per morpholino to account for losses from poor fertilisation or damage at later steps. 2. Embryos were manually dechorionated at approximately 30 hpf. 3. At 45.5 hpf, 200 embryos from each morpholino group were mounted in 3% methyl cellulose, a process taking approximately 2h. 4. Mounted embryos were injected with LPS into the hindbrain over the course of approximately 30 min. 5. Stimulated embryos were incubated for 2 h at 28°C. 6. Dissociation of whole larvae began at 2 hpi and took approximately 3h, producing a single cell suspension. 7. FACS was used to isolate EGFP⁺mCherry⁺ macrophage-lineage cells. Sorting all samples typically took 2h. 8. Cells were sorted into Trizol LS for later RNA extraction. Each sample was produced in biological triplicate. Abbrev. dpf, days post-fertilisation; FACS, fluorescence-activated cell sorting; hpf, hours post-fertilisation.

**FIGURE 5.6: Workflow for processing larvae prior to RNA extraction for RNA-Seq**

1. Collect Tg(irg1:EGFP)etz/Tg(mpeg1:mCherry)j123 embryos and inject with IRG1SBMO1, IRG1SBMO3, or CTRLMO at 1-2 cell stage (1h).
2. Manually dechorionate (2h).
3. Mount in methyl cellulose (2h).
4. Stimulate 2dpf larvae with hindbrain injection of LPS (30min).
5. Incubate at 28°C (2h).
6. Begin dissociating larvae into single cell suspension (3h).
7. Begin FACS collection of EGFP⁺mCherry⁺ activated macrophages (2h).
8. Store or process purified cells for RNA isolation.
Dissociation of morpholino-injected $Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{gl23}$ larvae began two hours after hindbrain injection with LPS, in order to produce a single cell suspension. WMISH analyses have shown that 2 hpi is the point of maximum $\text{irg1}$ expression following hindbrain injection with LPS (see section 3.1). Taking into consideration the time to dissociate larvae (approximately three hours), isolation of $-4.7\text{irg1:EGFP;mpeg1:mCherry}$-expressing cells by FACS began approximately five hours after LPS injection and typically took an additional two hours to complete. Flow cytometry analysis of the $Tg(\text{irg1:EGFP})^{nz4}$ reporter line, as discussed in Chapter 3, has shown that this timeframe is sufficient for EGFP production in $Tg(\text{irg1:EGFP})^{nz4}$ macrophage-lineage cells, following injection with LPS, to enable their accurate isolation by FACS.

When analysed on the FACSria II, macrophage-lineage cells comprised approximately 0.1-0.2% of the total cell suspension (FIG 5.7 A). Originally, it was anticipated that cell suspensions would be double-sorted, running collected cells through the FACS machine a second time for increased purity. However, despite sorting with low pressure and using a chilled sample compartment, 40-60% death was typically seen in re-sorted populations (FIG 5.7). The necessity of mounting large numbers of larvae for hindbrain injection of LPS to stimulate activation of the macrophage-lineage cells and expression of the $-4.7\text{irg1:EGFP}$ transgene makes this protocol difficult to scale up for increased source material. Additionally, when larger initial larva numbers were trialled, sorting the larger cell suspension using FACS extended the collection time and tended to display higher rates of cell death, often resulting in minimally increased second-sort yields. Thus, the number of macrophage-lineage cells collected with double sorting appeared capped at approximately 6000 cells.
FIGURE 5.7: Assessing the purity and viability of macrophage-lineage cells isolated from dissociated, LPS-injected $Tg(\text{irg1:EGFP})^{a4}/Tg(\text{mpeg1:mCherry})^{a23}$ larvae using FACS

A. EGFP+mCherry+ macrophage-lineage cells comprised 0.1-0.2% of the total cell suspension. Blue gate indicates sorting gate used for collection.  
B. Forward and side scatter analysis of collected events from A during first sort collection displays heterogeneous size and granularity and very little debris.  
C. Reassessing the forward and side scatter profile of collected cells when sorted a second time displays a large amount of debris (outlined in red), indicating cell death.  
D. 85-95% of the live cells isolated through a second sort demonstrated EGFP+mCherry+ double fluorescence. Abbrev. FFC, forward scatter; SSC, side scatter.

Due to the length of time required to collect such a relatively rare population, and the resulting high rate of death in subsequent sorts through the machine (FIG 5.7 C), it was decided that the potential benefits of double sorting were outweighed by the reduced yield and probable transcriptome alterations incurred by the extended stress. Single sorting resulted in average yields of 10k cells per sample. Cells were sorted directly into Trizol LS and processed with a combined phenol-chloroform extraction and silica
column purification. Each sample type was produced in biological triplicate, with one replicate of each morpholino-injected group collected on each of three experiment days.

5.2.3 RNA quantification and preparation of cDNA libraries

Original experiment design anticipated using the TruSeq Stranded mRNA Sample kit from Illumina to prepare libraries for transcriptome sequencing. This kit has been optimised for sample sizes ranging from 0.1-4 μg of RNA. Estimating a yield of 1-10pg RNA per cell, this would require the collection of a minimum of 10k-100k macrophage-lineage cells. Given the average collection of macrophage-lineage cells from 200 dissociated Tg(ireg1:EGFP)dx4/Tg(mpeg1:mCherry)gl23 larvae following LPS-injection was only 10k cells using only a single sort, this suggested a final yield of just 10-100 ng per sample, assuming 100% retention. As such, quantification of the RNA isolated from these samples, via standard methods using Qubit or Nanodrop, was unfeasible, as the respective detection limits of 5 ng and 2 ng/μL for these machines required too great a proportion of the total sample (Life Technologies; Thermo Scientific). Instead, quantity and quality were assessed using the RNA 6000 Pico kit on an Agilent Bioanalyzer 2100. The RNA integrity number (RIN) values varied between 8.3 and 9.5 on a possible scale of 1-10, indicating high-quality samples (Mueller, Lightfoot, & Schroeder, 2004). There was no indication of either protein or genomic DNA contamination in the sample spectra. Total RNA yields varied between 4 and 25 ng per sample.

As a consequence of the limited amount of source material, RNA samples were unable to be processed into libraries using the TruSeq Stranded mRNA Sample kit and instead required amplification and processing into cDNA libraries using the NEBNext Ultra RNA Library Prep Kit for Illumina. This kit has been shown to have even transcript coverage
for a wide range of starting sample amounts and has been tested with inputs as low as 10 ng of total RNA (New England Biolabs). Library quality was confirmed through use of a bioanalyzer, additionally revealing an average concentration of 16000pM.

5.2.4 RNA-Seq analysis

Library sequencing and bioinformatics were carried out by New Zealand Genomics Limited (NZGL), including inter- and intra-sample normalisation, reference-guided assembly to the zebrafish genome, and differential expression analysis. Samples were sequenced in one lane of the Illumina HiSeq 2500. Sequence reads were mapped using TopHat v2.0.12 against the zebrafish reference genome Zv9, obtained from Ensembl (D. Kim et al., 2013). Gene expression was measured using fragments per kilobase of transcript per million mapped reads (FPKM), a method of normalising read counts that takes into consideration paired-end sequencing and the variability in transcript lengths (Garber, Grabherr, Guttman, & Trapnell, 2011). Differential expression analysis was performed using Cuffdiff, part of the open source transcriptome assembly software program, Cufflinks (C. Trapnell et al., 2010). The false discovery rate (q-value) was also taken into consideration for genes with significantly different normalised expression levels when comparing control and experimental groups. Genes were considered significant hits when both their $P$- and $q$- probabilities were calculated as less than 0.05.
5.3 Results

5.3.1 Abrogating expression of Irg1 using the splice-blocking morpholino IRG1SBMO3 significantly alters transcription of 99 genes in activated Tg(ig1:EGFP)nz4/Tg(mpeg1:mCherry)g123 macrophage-lineage cells following LPS injection.

Sequencing produced a total of 163,653,422 paired-end, 100bp reads across all nine samples, 139,531,521 (85.26%) of which passed quality filtering and were used in downstream analyses. Reads across the nine samples mapped to an average of 84.26% of the 56,754 known Ensembl transcripts (representing 33,473 genes) present in the zebrafish reference genome Zv9.

Differential expression analysis revealed 99 genes that differed between the control morpholino and IRG1SBMO3-injected groups. Within these, 74 were significantly downregulated while 25 demonstrated increased expression (Appendix 5). No significantly dysregulated genes were apparent when comparing control morpholino-injected with IRG1SBMO1-injected samples.

Sample variance across the IRG1SBMO1-injected samples tended to be higher than that seen in control morpholino-injected samples (FIG 5.8 A), while IRG1SBMO3-injected larvae had generally less variance between samples (FIG 5.8 B).
FIGURE 5.8: Normalised coefficient of variation measurements across samples in each morpholino group, relative to the number of reads per sequence as measured by FPKM
A. IRG1SBMO1-injected samples displayed higher inter-sample variation when compared with control-morpholino-injected samples.  B. IRG1SBMO3-injected samples displayed lower inter-sample variation when compared with control-morpholino-injected samples. Abbrev. CTRL MO, control morpholino; CV, coefficient of variation; FPKM, fragments per kilobase of transcript per million mapped reads; IRG1SBMO1, irg1 splice-blocking morpholino 1; IRG1SBMO3, irg1 splice-blocking morpholino 3.

As expected, all samples demonstrated a higher coefficient of variation across genes with fewer mapped reads (FPKM), and a gradual reduction in variance as mapped reads became more numerous.

5.3.1 Inflammatory cytokines and targets of NFκB signalling are significantly affected by Irg1 depletion in macrophage-lineage cells following use of the splice-blocking morpholino IRG1SBMO3

Among the 99 differentially-expressed genes identified by comparing the transcriptome of macrophage-lineage cells from IRG1SBMO3- and LPS-injected larvae with that of macrophage-lineage cells from CTRLMO- and LPS-injected larvae were seven genes identified within the TRANSFAC gene set V$NFKAPPAB65_01. TRANSFAC is a curated database containing information regarding eukaryotic transcription factors, their DNA recognition sites, binding partners, and downstream regulated genes (Wingender, 2008). The V$NFKAPPAB65_01 gene set contains downstream targets of inflammatory NFκB
signalling. From the 99 differentially-expressed genes identified during this transcriptome comparison, amyloid precursor-like protein 2 (aplp2), ATPase class 1 type 8b member 6 (atp8b4), carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 1 (ctdsp1), GTP-binding protein 4 (gtpb4), interleukin 1 beta (il-1β), interferon regulatory factor 8 (irf8), and TNF-receptor-associated factor 3 (traf3) are all contained within this gene set (FIG 5.9). A regulator of NFκB function, B-cell lymphoma 3-encoded protein (bcl3), known to function as both an inhibitor and promoter of NFκB signalling through direct binding interactions with NFκB subunits, depending on environmental cues and post-translational modification, was also highlighted as a differentially-expressed gene following depletion of Irg1 (FIG 5.9) (Bundy & McKeithan, 1997; Oeckinghaus & Ghosh, 2009; Richard, Louahed, Demoulin, & Renauld, 1999). Similarly, traf3 and B-cell CLL/Lymphoma 10 (bcl10), another differentially regulated gene (FIG 5.9), have been associated with NFκB activation through their interactions with tumour necrosis factor receptor type 1-associated death domain (TRADD) (Guiet & Vito, 2000; Michallet et al., 2008).
FIGURE 5.9: Differentially-expressed genes between CTRLMO-injected and IRG1SBMO3-injected samples with established links to NFκB signalling and inflammatory response

Differential expression between morpholino groups was considered statistically significant when both the $P$ and $q$ values were calculated as less than 0.05 when using Cuffdiff. For all genes shown here, $P < 0.0005$. Error bars indicate standard deviation. Abbrev. *, $q<0.05$; **, $q<0.01$; CTRLMO, control morpholino; FPKM, fragments per kilobase of transcript per million mapped reads; IRG1SBMO3, irg1-targeting splice-blocking morpholino 3.

NFκB signalling has long been linked to the propagation of inflammation and immune cell effector functions (Hayden, West, & Ghosh, 2006). Inflammatory cascades mediated through NFκB have also been more specifically implicated in the inflammatory processes that underlie metabolic diseases and the pro-tumoral microenvironment (Baker, Hayden, & Ghosh, 2011; Carlsen et al., 2009), with recent data suggesting that this may, in part, be through the regulation of genes involved in glucose metabolism (Moretti, Bennett, Tornatore, Thotakura, & Franzoso, 2012). As such, a role for Irg1 in the regulation NFκB and NFκB-mediated pathways is not unexpected and further describes potential routes of enacting Irg1-associated macrophage functions.
Also included in the group of differentially-expressed sequences identified by comparing the transcriptomes of macrophage-lineage cells from IRG1SBMO3- and CTRLMO-injected larvae, following stimulation with LPS-injection, were the potent inflammatory cytokines *tumour necrosis factor alpha* (*tnfα*), *colony-stimulating factor 3* (*csf3*), and *interleukin-1 beta* (*il-1β*), as well as the negative regulator of cytokine signalling, *suppressor of cytokine signalling 3a* (*socs3a*). Each of these have well-established roles in the propagation or regulation of inflammation and inflammatory disease, and their altered expression following inflammatory stimulation when Irg1 activity has been abrogated indicates that Irg1 may confer inflammatory macrophage effector functions and phenotype regulation through these molecules following infection (Bradley, 2008; Dinarello, 2011; Hamilton, 2008).

### 5.3.2 Assessing global transcription within macrophage-lineage cells from IRG1SBMO3-injected Tg(irg:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae supports a trend towards a dysregulated inflammatory response

Gene set enrichment analysis seeks to determine whether there is a significant shift in biological phenotype between states by examining the expressions of groups of related genes together (Subramanian et al., 2005). GeneSetDB is a meta-database that facilitates this investigation by enabling the analysis of user-defined gene lists, such as transcriptome expression data, simultaneously against multiple gene lists defined in public databases as related to a particular process or response (Araki et al., 2012). Examining the expression changes of all genes mapped within IRG1SBMO3-injected larvae using GeneSetDB demonstrated a trend towards the global dysregulation of inflammatory response genes within activated, macrophage-lineage cells when Irg1 activity had been depleted (FIG 5.10).
FIGURE 5.10: Gene set enrichment analysis of the transcriptome of activated, macrophage-lineage cells with reduced Irg1 function, using GeneSetDB

Changes in gene expression within the transcriptome of activated macrophage-lineage cells from IRG1SBMO3-injected, LPS-injected larvae when compared with CTRLMO-injected, LPS-injected larvae suggest global dysregulation of inflammatory response genes. This is indicated by a clustering of sequences to both the left (upregulation) and right (downregulation) in inflammatory response pathways defined by the SIDER (A), MPO (B), and WikiPathways (C) databases. Each vertical line indicates a unique sequence from the transcriptome data set that is included within the given gene list. Differential expression is depicted as a horizontal continuum of P-value significance, from the most significantly upregulated gene on the left to the most significantly downregulated on the right. Curved lines indicate enrichment trends across the gene set, varying from a normal distribution as indicated by the dotted line.
While the $P$-value calculating gene enrichment for inflammatory response genes in the WikiPathways gene set WP453 is below the 0.05 confidence level indicating significance, the low representation of mapped sequences from the assessed transcriptome of Irg1-ablated, activated macrophage-lineage cells within this pathway prevents conclusive observations (FIG 5.10). This may also suggest either poor mapping of sequence reads or poor annotation of the zebrafish genome, preventing accurate identification of genes present in each gene set from within the mapped sequences of the assessed transcriptome. As the mapping rate was reported at an average of 84.26%, it is unlikely that this is responsible for the poor representation. Taken together however, these three gene sets indicated similar clustering of both strongly up and downregulated inflammation-associated genes within activated macrophage-lineage cells following Irg1 depletion.

5.3.3 Comparing differential expression data between macrophage-lineage cells from IRG1SBMO1-injected and IRG1SBMO3-injected $Tg(\text{irg}:\text{EGFP})^{a24}$/$Tg(\text{mpeg1}:\text{mCherry})^{\text{gl23}}$ larvae following LPS-injection suggests a potential role for glycine N-methyltransferase in promoting an anti-inflammatory macrophage phenotype.

Comparing the transcriptome of macrophage-lineage cells from IRG1SBMO1- and LPS-injected larvae with that from CTRLMO- and LPS-injected larvae did not reveal any sequences with significantly different expression levels. However, the gene with the most substantially altered expression, glycine $N$-methyltransferase ($gnmt$), with a $q$-value of 0.0508, was also represented in the list of 99 genes that were significantly altered when comparing macrophage-lineage cells from IRG1SBMO3- and LPS-injected larvae with those of CTRLMO- and LPS-injected larvae, with a $q$-value of 0.0098 (FIG 5.11). This gene encodes an enzyme involved in cholesterol metabolism (C. Y. Chen et al., 2012).
Assessing the expression levels of genes found to be differentially represented in IRG1SBMO3-injected samples as compared with CTRLMO-injected samples, including *gnmt*, *tnfa*, *il-1β*, *csf3*, *bcl3*, and *aplp2*, it appeared that experiment day number one, where one sample from each morpholino group was collected, may not have received as great an inflammatory stimulation from the LPS-injection as experiment days number two and three. It was also apparent that, in the majority of instances, IRG1SBMO1-injected samples closely mirrored the expression levels seen in CTRLMO-injected samples. For the genes shown, differences in expression between CTRLMO and IRG1SBMO1 samples did not reach the level of significance ($P < 0.05$), while comparisons between CTRLMO and IRG1SBMO3 all had $P$-values below 0.0005. Statistical analyses were performed using Cuffdiff. Abbrev. **, $q < 0.01$; CTRLMO, control morpholino; FPKM, fragments per kilobase of transcript per million mapped reads; IRG1SBMO1, *irg1*-targeting splice-blocking morpholino 1; IRG1SBMO3, *irg1*-targeting splice-blocking morpholino 3.

Assessing trends across biological replicates and between morpholino groups for the expression of *gnmt* and a selection of the top inflammatory genes described in 5.3.1, including *tnfa*, *il-1β*, *csf3*, *bcl3*, and *aplp2*, it appears that in all instances but for *gnmt*, IRG1SBMO1-injected samples closely mirror the expression levels seen in the CTRLMO-injected samples (FIG 5.11). Also, as one biological replicate of each morpholino-injected group was collected on each of three experiment days, with comparable treatment within each experiment, it is also possible to assess trends across each experiment day. This revealed that samples within experiment day 1 may have received a lower inflammatory stimulation than those in experiments 2 and 3, as the expression
level of inflammatory genes in the CTRLMO-injected sample for this replicate is consistently low and there is very little difference between the three morpholino groups for this replicate as compared with replicates 2 and 3.

5.4 Conclusions

The Tg(irg1:EGFP)nz4 transgenic reporter line, marking activated macrophage-lineage cells within zebrafish larvae following appropriate immune stimulation, has enabled previously unfeasible examination of the transcriptome of this distinct population as it responds to LPS injection either with or without the downstream consequences of Irg1 activity. By identifying differences in gene expression, achieved through abrogating Irg1 activity through the use of irg1 pre-mRNA-targeting splice-blocking morpholinos, a role for Irg1 in the regulation of the inflammatory response within macrophage-lineage cells has been supported. This role includes regulatory effects on the transcription of inflammatory cytokines, as indicated by effects on the expression levels of tnfα, il-1β, and socs3a, and targets of NFκB signalling pathways, such as traf3 and irf8. While these regulatory effects are anticipated to be mediated through Irg1-driven mROS production, this connection requires further validation. Inflammatory mediators described here are among 99 differentially regulated sequences following LPS injection in Irg1 replete and depleted larvae, potentially implicating other members of this group in the regulation, direction, or presentation of macrophage activation. Together, analyses described here have suggested some of the signalling networks employed by Irg1 to exert effector functions within macrophage-lineage cells responding to infection. Similarly, these data have provided insight into the potential consequences of manipulating Irg1 activity for the treatment of macrophage-mediated inflammatory disease.
6 Identification of small molecule modulators of $irg1$ expression through a drug-repurposing screen

Given the strong connection between $irg1$ expression and inflammatory macrophage phenotypes implicated in the pathogenesis of a variety of disorders, as observed in models of neurotoxicity (H. Li et al., 2006) and implicated in obesity (Amano et al., 2014), small molecule modulation of Irg1 presents as a potential therapeutic strategy for limiting inflammatory macrophage activation and thus reducing the deleterious inflammatory state of these disorders.

6.1 Rationale

Characteristics underpinning an ideal drug target include unique expression in the disease state(s), limited necessity in homeostasis, “assayability” facilitating high throughput screening, and the existence of a specific biomarker that can be used to monitor treatment efficacy (Gashaw, Ellinghaus, Sommer, & Asadullah, 2011). Satisfying these criteria simplifies the identification of candidate drugs and reduces the likelihood of serious off-target complications. The robust, infection-responsive expression pattern of $irg1$ is readily observed in zebrafish by way of WMISH (Hall et al., 2013) or $irg1$-promoter-driven EGFP in the Tg($irg1$:EGFP)$_{nz4}$ reporter line. Paired with the advantages in throughput, visualisation, and in vivo analyses offered by the zebrafish drug-screening platform (Zon & Peterson, 2005), zebrafish $irg1$ is uniquely positioned as a specific phenotypic readout for small-molecule modulation of macrophage activation. In turn, information pertaining to the regulation of macrophage activation resulting from such a screen may be pertinent to the development of treatments for relevant metabolic diseases.
Compound discovery through the use of a drug-repurposing approach builds off the hypothesis that seemingly disparate diseases often share common molecular pathways (Hall et al., 2014; J. Li & Lu, 2013). This means that libraries of compounds deemed already effective for treating disease are more likely to produce hits with biological significance than libraries of \textit{de novo} generated molecules. In this approach, the small molecule library screened is comprised of drugs of known effects and toxicities. Many of these compounds may still be in clinical circulation or off the market simply because more effective compounds have been developed for their initial application (Arrowsmith & Harrison, 2012). These libraries are particularly attractive because identified hits are less likely to fail safety trials (Arrowsmith & Harrison, 2012; Cavalla, 2013). Given their previous characterisation, candidate compounds often carry a wealth of information that may be used to elucidate mechanisms of action (Keiser et al., 2009). In the case of \textit{irg1}, a potentially disease-relevant gene with limited prior characterisation, this data may lead to the discovery and further understanding of which signalling pathways regulate its activity.

Compounds that reduce or ablate \textit{irg1} expression following stimulation are the most likely to have significant clinical applications, as metabolic disease appears to be linked to the presence of macrophage-lineage cells exhibiting pro-inflammatory functional states indicative of Irg1 activity (Amano et al., 2014; H. Li et al., 2006). It is expected that blocking \textit{irg1} expression will prevent the metabolic reprogramming of macrophage-lineage cells to the pro-inflammatory activation state that contributes to disease progression in these environments (FIG 6.1).
FIGURE 6.1: Suppressing metabolic reprogramming of macrophages through chemical inhibition of irg1 expression as a strategy for treating obesity-associated disease

1. Irg1 is a specific marker for inflammatory macrophage phenotypes. It is hypothesised that expression of irg1 in obesity has the potential to further amplify inflammation and disease by directing metabolic reprogramming through mROS-mediated transcriptional regulation of inflammation-associated genes. This leads to the production of inflammatory cytokines such as TNFα and IL-1β. These, in turn, contribute to chronic inflammation and an increased risk of developing obesity-associated disease. 2. Chemical inhibition of irg1 expression may prevent undesired macrophage reprogramming and activation, reducing inflammation and ameliorating disease symptoms.

In the instance of obesity for example, adipocyte hypertrophy and subsequent release of FFAs and inflammatory cytokines leads to the inflammatory activation of macrophages (Chawla et al., 2011). These ATMs further contribute to the pro-inflammatory environment that increases the risk of patients developing secondary conditions such as diabetes and hypertension (FIG 6.1-1) (Chawla et al., 2011). Blocking Irg1 activity is expected to reduce the inflammatory response to FFAs by restricting their uptake and oxidation in the mitochondria. This in turn would limit the production of mROS and inhibit the positive feedback loop that is the pro-inflammatory, mROS-dependent...
detection of FFAs by the NLRP3 inflammasome (Legrand-Poels et al., 2014). It is also
expected that reduced Irg1-dependent mROS activity will abrogate mROS-mediated
transcriptional regulation of pro-inflammatory genes. Thus, blocking Irg1 may prevent
the conversion of macrophages to a pro-inflammatory phenotype, reduce the level of
inflammatory cytokines produced, and thus treat or limit the likelihood of developing
obesity-associated disorders (FIG 6.1-2) (Donath, 2014).

This hypothesis is especially significant considering recent observations of
improvements in metabolic diseases such as diabetes when treated with anti-
inflammatory compounds including anakinra, an IL-1 receptor antagonist, and salsalate,
an NFκB inhibitor (Donath, 2014; Fleischman, Shoelson, Bernier, & Goldfine, 2008;
Larsen et al., 2009). A better understanding of the propagation of inflammation during
these diseases will allow for the development of stronger, more specific therapies.
Tackling the specific biochemical bases of macrophage activation and metabolic
reprogramming is an under-developed approach and it is anticipated that chemical
control of irg1 expression offers a strategy for elucidating this process.

Drugs found to be effective in modulating expression of irg1 may also present as useful
research tools for further investigations of the regulatory mechanisms surrounding Irg1
activity. In the study of irg1 in zebrafish, molecules that reduce its expression may
provide an alternative or complementary approach to morpholino knockdowns - which
have limited longevity within the larva and in some models have been shown to be less
robust or specific as knockout models (Schulte-Merker & Stainier, 2014). A chemical
method of irg1 inhibition would also enable more temporally precise control of Irg1
activity with the potential for studies at later timepoints in development, while providing
additional validation of morphant phenotypes.
6.2 Experimental Design

For many applications of chemical screens in zebrafish, simple immersion of embryos in solutions of drug is sufficient to measure readout and assess their effects (Zon & Peterson, 2005). Observations of immune responses however, often require an immunogenic stimulus. Fin clipping is a relatively common technique for inducing sterile inflammation (H. Meijer & P. Spaink, 2011), though the infection-responsive nature of \textit{irg1} expression suggests that stimulation with LPS will be required (Hall et al., 2013). Static immersion in solutions of LPS has proven insufficient to drive \textit{irg1} expression in macrophage-lineage cells (FIG 6.2 A), likely due to the aquatic adaptations of the zebrafish. For example, the epidermis of the fish is comprised of living cells actively producing a surface layer of mucus (Ebran et al., 1999). This mucus protects by acting as both a physical barrier to pathogens and as a reservoir for antimicrobial compounds. However, while fin clipping followed by immersion may present an effective method to stimulate \textit{irg1} expression, combining these techniques would be both laborious and reagent intensive. Thus, direct injection of an immunogen was chosen to induce expression of \textit{irg1}. The hindbrain is a well-established infection site in the zebrafish that enables analysis of systemic signalling events and immune cell migration (Benard et al., 2012; Hall et al., 2013; Takaki, Davis, Winglee, & Ramakrishnan, 2013). Current hindbrain injection protocols involve positioning anaesthetised larvae in a viscous solution of methylcellulose or mounting them in agarose (Benard et al., 2012; Hall, Flores, Crosier, & Crosier, 2009; Westerfield, 2000). These methods are time-consuming and may introduce additional mechanical stress on the larva through physical manipulation during orientation.
A second consideration is the speed with which *irg1* expression is induced following stimulation. As described in **Chapter 3**, WMISH analysis of *irg1* expression following hindbrain injection with LPS revealed distinct staining as early as 1 hpi that returned to homeostatic levels by 4 hpi, with peak expression observed at 2 hpi (**FIG. 3.1**). Based on these data, a two-hour stimulation period was chosen before assessing readout to maximise the potential range of drug effects detectable. This rapid time course also highlighted the importance of limiting the interval between inflammatory stimulation and drug application, as false negatives may result from the inadequate opportunity of applied compounds to prevent, degrade, or restrict *irg1* expression. This would lead to limited visible differences in treatment groups compared to negative controls.

Treating larvae with drugs continuously from an initial pre-treatment period through stimulation and until collection would avoid complications with respect to timing, though this is challenging to apply to current protocols as described above. The considerable time required to array larvae in methylcellulose and subsequently inject and free them contributes substantially to the interval between stimulation and drug treatment. Recent data from Wang *et al.* reiterates the potential complications of limited drug exposure through the observation that drug pre-treatment was necessary to observe significant effects in an assay testing inhibitors of neutrophil recruitment (X. Wang *et al.* 2014), an observation similarly noted in screens from the Crosier group (Hall *et al.*, 2014). This pre-treatment had to be carefully timed, as Wang and colleagues observed that known inhibitors of neutrophil recruitment were unable to exert a noticeable affect when pre-treatment lasted only one hour. While three and five-hour pre-incubations were sufficient to observe the expected effect, the five-hour treatment was more likely to display toxic side effects (X. Wang *et al.*, 2014). Based on these considerations, a three-hour pre-injection treatment was chosen to study drug effects on LPS-induced *irg1* expression.
To successfully carry out a small molecule screen for modulators of *irg1* expression, a novel injection protocol needed to be developed that allowed medium throughput screening with minimal manual labour and continuous drug exposure at exact concentrations during pre-treatment and immune stimulation.

6.2.1 Development of a novel hindbrain microinjection platform

An injection mould was developed to produce wells that maintained embryos in drug solution continuously, while supporting them for hindbrain LPS injection. Injecting into the hindbrain at 2 dpf requires mounting and orientation of the larva in such a way as to facilitate access of the needle into the hindbrain. At 2 dpf the larval yolk sac is pronounced, resulting in anaesthetised larvae tending to rest dorsal-side-down with their yolk presented and hindbrain inaccessible (FIG 6.2 B).

![Diagram of hindbrain injection platform](image)

**FIGURE 6.2: Orientation of 2 dpf larvae for hindbrain injection**

**A.** Schematic illustration of a plastic embryo injection tray. **B.** Anaesthetised larva immersed in E3 medium resting on the bottom of a culture dish (flat surface) with obstructed access to the hindbrain from above. **C.** Anaesthetised larva resting in the trough of a plastic embryo injection tray (angled surface). The shape of the trough positions the yolk sac downwards, presenting the hindbrain for injection from above. Arrows indicate hindbrain injection site. Scale bar 100 μm in **B.** Abbrev. dpf, days post-fertilisation. Larvae oriented anterior to left.

Proper orientation for hindbrain access required a resistant or solid support. Existing injection setups, such as the plastic injection trays used for injection of young zebrafish
embryos (FIG 6.2 A), proved either too small to house larger larvae or were unable to simultaneously house aqueous drug solution. Additionally, due to capillary action within the trenches of the plastic injection tray, it is difficult to array large numbers of larvae prior to injection.

A solution was engineered by developing moulds that, when floated in liquid agarose in six-well culture dishes, generated troughs with similar proportions to those found in the plastic embryo injection tray (FIG 6.2).

![Figure 6.3: Mould design for aqueous larval hindbrain injections]

**FIGURE 6.3: Mould design for aqueous larval hindbrain injections**

A. Schematic representation of the mould developed to produce agarose wells capable of containing 500 μL of liquid while supporting and positioning anaesthetised larvae for hindbrain injection (B). C. Larvae arrayed in the troughs of an agarose well. D. Microinjection needle inserted into the hindbrain of an anaesthetised larva. Scale bar 100 μm in D. Larva oriented anterior to left in D.
Agarose wells resulting from the use of these new moulds were able to contain up to 500 μL of liquid while facilitating correct larval orientation. These wells allowed many larvae to be arrayed prior to injection (FIG 6.3 C). Of note, the individual wells created through the use of six-well plates allowed multiple parallel drug treatments with constant exposure throughout stimulation and treatment periods.

6.2.2 Small molecule drug library

A drug-repurposing approach was taken for this screen, using The Prestwick Chemical Library® of FDA-approved drugs. This library has been used previously by our research group in drug-repurposing screens using zebrafish to successfully identify potential new anti-inflammatory agents for the treatment of neutrophilic inflammation and inhibitors of lymphangiogenesis with potential applications to cancer therapy (Astin et al., 2014; Hall et al., 2014). The Prestwick Chemical Library® consists of 1120 compounds proven safe for use in humans and chosen for their broad range of chemical and pharmacological properties. Given the number of compounds being tested, it was not practical to optimise dosage of each compound individually in the first pass so a standard concentration was chosen that had previously proven effective for the identification of numerous hits for other indications. Drugs were provided at a concentration of 2 mg/mL in DMSO and diluted to a final concentration of 5 μg/mL in screening media, as described in the recent screen published by our research group (Astin et al., 2014). It was understood that some potential leads may be screened out due to toxicity or be missed due to insufficient dosing.
6.2.3 Positive and negative controls

Given the limited information available about regulation of \textit{irg1} expression, an effective inhibitor for use as a chemical positive control was not immediately apparent. Preliminary experiments trialled cycloheximide (CHX), a protein synthesis inhibitor, as a potential positive control compound. It was expected that CHX would inhibit \textit{irg1} expression by preventing the translation of required inducers and promotors, as previously described by the Crosier group (Hall et al., 2013). However, these studies deemed it inappropriate and gave unanticipated results that led to new insights into the regulation of \textit{irg1} expression, which are further discussed in 6.2.4. As glucocorticoid and JAK-STAT signalling are also required for \textit{irg1} expression (Hall et al., 2013), known chemical inhibitors of these pathways were also tested as potential positive controls (FIG 6.4).

**FIGURE 6.4**: Treatment with known inhibitors of JAK-STAT signalling does not abrogate \textit{irg1} expression at 2 hpi when induced by hindbrain injection with LPS

A. WMISH staining of a DMSO-treated vehicle control showed \textit{irg1} expression throughout the body following LPS-injection. B, C. Treatment with 25 μM STAT3 inhibitor peptide or 500 μM AG490 did not significantly reduce LPS-induced expression of \textit{irg1}. Arrows indicate hindbrain injection site. Scale bar 100μm in A. Numbers indicate the proportion of larvae that displayed the presented phenotype. Abbrev. DMSO, dimethyl sulfoxide; dpf, days post-fertilisation; hpi, hours post-injection. Larvae oriented anterior to left.

Neither AG490 nor STAT3 inhibitor peptide, inhibitors of JAK and STAT3 signalling respectively (Funakoshi-Tago, Tago, Sato, Tominaga, & Kasahara, 2011; Y. Wang, Mohsen, Mihalik, Goetzman, & Vockley, 2010), were able to consistently ablate \textit{irg1}
expression as detected by WMISH two hours following stimulation with LPS (FIG 6.4). The screen proceeded using PBS-injected, DMSO-only (vehicle) treated larvae as a control for steady state *irg1* expression and LPS-injected, DMSO-only (vehicle) control larvae as a measure of LPS-induced *irg1* expression.

6.2.4 Expression of *irg1* is partially regulated by a transcriptional repressor.

Preliminary studies investigated the possibility of using CHX as a positive drug control to abrogate *irg1* expression in this screen when compared to LPS-injected larvae treated with only a DMSO vehicle control. Consistent with previous studies from our research group (Hall et al., 2013), CHX pre-treatment appeared to reduce LPS-driven *irg1* expression when assessed at 1 hpi by WMISH (FIG 6.5 A, A’). However, when analysing the effect of CHX on LPS-induced expression of *irg1* over a four-hour time course, this initial reduction was followed by a dramatic increase in *irg1* transcript production that extended beyond the timepoint at which detection levels typically returned to steady-state (FIG 6.5). As *irg1* expression in macrophage-lineage cells had previously only been observed in instances of infection-driven inflammation, it was hypothesised that a translational inhibitor would block the production of proteins responding to the stimulus and contributing to induction of *irg1* expression. Since *irg1* expression continued in the presence of CHX, it indicated that while the translation of some proteins involved in inducing *irg1* expression may have been blocked by CHX, contributing to an initial reduction in LPS-responsive *irg1* expression, suppressing translation might also have prevented the translation of transcriptional repressors that directly control *irg1* expression.
FIGURE 6.5: Temporal analysis of \textit{irg1} expression in 2 dpf, LPS-injected larvae treated with CHX or DMSO

Larvae were assessed via WMISH 1-4 h post-hindbrain injection with LPS while exposed to 100 $\mu$g/mL CHX ($A'$, $B'$, $C'$, $D'$) or 1% DMSO vehicle control ($A$, $B$, $C$, $D$). Arrows indicate hindbrain injection site. Scale bar 100 $\mu$m in $A$. Numbers indicate the proportion of larvae that displayed the presented phenotype. Abbrev. CHX, cycloheximide; DMSO, dimethyl sulfoxide; dpf, days post-fertilisation; hpi, hours post-injection. Larvae oriented anterior to left.

Transcriptional repressor proteins are a common mechanism for the regulation of early response genes (Hargreaves, Horng, & Medzhitov, 2009). They enable rapid induction while providing an additional level of control to prevent erroneous, signal-independent expression. Hall \textit{et al.} have already suggested the existence of repressors in a signalling pathway associated with \textit{irg1} expression, reporting increased \textit{cebpβ} expression in the macrophage-lineage cells of both infected and PBS-injected zebrafish larvae following treatment with CHX (Hall \textit{et al.}, 2013). This indicated that a constitutively active transcriptional repressor was acting at the level of \textit{cebpβ} expression. Results described here, indicating a similar mechanism is at play with respect to \textit{irg1} expression, add to the current model of infection-driven Irg1 expression (FIG 6.6).
Previous data has indicated the existence of a constitutively-expressed transcriptional repressor acting on \textit{cebp}β, which in turn inhibits downstream \textit{irg1} transcription. New data presented here points to an additional inhibitor acting at the level of \textit{irg1} transcription. Abbrev. CAC, citric acid cycle; C/EBPβ, CCAAT/enhancer binding protein β; ETC, electron transport chain; FFA, free fatty acids; GR, glucocorticoid receptor; mROS, mitochondrial reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TLR, toll-like receptor.

To assess whether the transcriptional repressor acted in the absence of infection, \textit{irg1} expression was determined in the presence of CHX in non LPS-injected larvae. Expression of \textit{irg1} was detected after five hours of immersion in CHX (FIG 6.7), demonstrating that gene expression is not strictly infection-inducible and indicating that the putative transcriptional repressor acting on \textit{irg1} is constitutively active.
FIGURE 6.7: Analysis of *irg1* expression via WMISH in non-stimulated 2 dpf larvae following treatment with CHX

Embryos were treated with 100 μg/mL CHX (in 1% DMSO) for 3, 4, and five hours. **A,B.** No *irg1* expression was observed after three or four hours of treatment. **C.** By five hours post-exposure to CHX, *irg1* expression was seen throughout the larvae, despite lacking immune stimulation with either bacteria or LPS. Scale bar 100 μm in **A.** Numbers indicate the proportion of larvae that displayed the presented phenotype. Abbrev. CHX, cycloheximide; DMSO, dimethyl sulfoxide; dpf, days post-fertilisation; WMISH, whole-mount in situ hybridisation. Larvae oriented anterior to left.

By assessing the potential of using CHX as a positive control in a screen for inhibitors of *irg1* expression, an additional mechanism for its regulation was revealed instead. This further highlights the multi-faceted regulatory networks surrounding *irg1* expression and the resulting potential sensitivity of *irg1* to various microenvironmental signals.
6.2.5 Chemical screen workflow

Taking into consideration the design elements discussed above, it was decided to carry out a chemical screen using the workflow described in Figure 6.8. This incorporated a 3 h drug pre-treatment prior to hindbrain injection with LPS using the novel agarose microinjection platform. Embryos were assessed at 2 hpi using WMISH comparing irg1 expression to PBS- and LPS- injected no-drug controls.

**FIGURE 6.8: Drug screen workflow**

1. Prestwick Chemical Library® is diluted to 0.5 mg/mL and dispensed using the Eppendorf epMotion robot.  
2. 2 dpf larvae are pre-treated in drug for three hours at a final concentration of 5 μg/mL.  
3. Larvae and drug solution were transferred together into agarose wells.  
4. LPS was injected into the larval hindbrain to stimulate macrophage-specific irg1 expression.  
5. Larvae were fixed at 2 hpi and processed using the Intavis InsituPro VSi robot to stain for irg1 expression.  
6. Stained larvae were scored by eye, comparing drug-treated samples to PBS- and LPS- injected no-drug controls.  

Abbrev. dpf, days post-fertilisation; hpi, hours post-injection; WMISH, whole-mount in situ hybridisation.
Workflow was automated where possible to increase throughput. WMISH expression analysis was carried out using the Intavis InsituPro VS i robot with an optimised protocol taking up to 60 samples from initial rehydration through to staining buffer in only 34 h (Appendix 1). Samples were manually placed into staining solution so staining progress could be closely monitored, with final washes and clearing steps performed by hand. An overview of the drug screen approach and timeline is given in Appendix 6.

6.3 Results

6.3.1 Treating LPS-injected larvae with compounds from the Prestwick Chemical Library reveals multiple effects on irg1 expression.

Larvae were observed for changes in irg1 expression level within macrophage-lineage cells, broadly classifying observed effects into one of three categories: reduction, ablation, or increase. Examples from each of these groups are shown in Figure 6.9. The first-pass screen identified 146 drug hits, of which 108/146 displayed a reduction in irg1 expression and 3/146 demonstrated near-complete ablation. While chemicals that strongly reduce or ablate irg1 expression are of greatest interest, 35/146 of the compounds identified in the first pass exhibited an enhancing effect on irg1 expression. Due to variations observed between inter-experiment vehicle controls, each example is shown adjacent to a batch-specific LPS-injected, no-drug, DMSO control larva (FIG 6.10).
Chemicals were also scored for toxicity at the time of injection three hours post-exposure. Toxicity was observed as death (physical disintegration or systemic opacity and loss of circulation), gross morphological deformity, cranial necrosis, laboured or absent circulation, and cranial or cardiac oedema. Within the Prestwick library, 21/1120 compounds were deemed toxic at 5 μg/mL. Compounds causing slight oedema were considered for downstream analysis while chemicals with other toxic effects were not progressed.
6.3.2 Established antibacterial, anti-inflammatory, and antifungal compounds affect \textit{irg1} expression with increased frequency

Following initial screening using six larvae per treatment and one set of controls per 25 drug samples, 146 promising “first pass” hits were identified (Appendix 7). These first pass hits were further triaged by performing a secondary screen using a larger number of larvae and more stringent controls (12 larvae per treatment, one LPS-injected no-drug DMSO control per treatment, one PBS-injected no-drug DMSO control per 6 treatments). This revealed 20 compounds with robust effects on \textit{irg1} expression (the “second pass hits”) Figure 6.10 compares the therapeutic drug classifications of the Prestwick Library as a whole to those of the first and second pass hits.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure610.png}
\caption{Analysis of the Prestwick library, first pass hits, and second pass hits by therapeutic group, as annotated by Prestwick Chemical}
\end{figure}

The increasing proportion of known anti-bacterial, anti-inflammatory, and anti-fungal drugs at each stage suggests that drugs with such properties are preferentially selected in this screen.
Comparing the primary therapeutic categories of compounds identified in the second pass hits to those of the first pass hits and complete Prestwick library, enrichment for known anti-bacterial, anti-inflammatory, and anti-fungal drugs is apparent. While the representation of anti-bacterial compounds increases from 15-25%, the proportion of anti-inflammatory compounds nearly doubles, rising from 8.1-15%, while anti-fungal compounds increase from 2.9-15%. The primary therapeutic groups and identities of the 20 compounds that comprise the second pass hits are shown in Table 6.1.

**TABLE 6.1: Summary of second pass hits identified for their strong modulating effects on LPS-induced irg1 expression**

<table>
<thead>
<tr>
<th>Effect on irg1 expression</th>
<th>Drug Identity</th>
<th>Formula</th>
<th>Structure</th>
<th>Therapeutic Group</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ablate</td>
<td>Piperlongumine</td>
<td>C_{17}H_{19}NO_{5}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-fungal, Anti-cancer</td>
<td>(Adams et al., 2012)</td>
</tr>
<tr>
<td>Ablate</td>
<td>Chrysin (5,7-Dihydroxy flavone)</td>
<td>C_{13}H_{10}O_{4}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-fungal</td>
<td>5-lipoxygenase inhibitor</td>
</tr>
<tr>
<td>Ablate</td>
<td>Camptothecine (S+)</td>
<td>C_{20}H_{16}N_{2}O_{4}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-tumour agent</td>
<td>Topoisomerase I inhibitor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Urapidil hydrochloride</td>
<td>C_{20}H_{30}ClN_{5}O_{3}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-hypertensive</td>
<td>α1-adrenoceptor antagonist, 5-HT1A receptor agonist</td>
</tr>
<tr>
<td>Reduce</td>
<td>Sparteine (-)</td>
<td>C_{16}H_{15}N_{2}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-arrhythmic</td>
<td>Postganglionic transmission inhibitor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Isoniazid</td>
<td>C_{4}H_{7}N_{3}O</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-bacterial</td>
<td>Mycolic acid synthesis inhibitor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Aztreonam</td>
<td>C_{13}H_{11}N_{3}O_{5}S_{2}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-bacterial</td>
<td>Bacterial transpeptidase inhibitor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Azlocillin sodium salt</td>
<td>C_{6}H_{12}N_{3}NaO_{3}</td>
<td><img src="image" alt="Structure" /></td>
<td>Anti-bacterial</td>
<td>Bacterial transpeptidase inhibitor</td>
</tr>
<tr>
<td>Reduction</td>
<td>Compound Name</td>
<td>Chemical Formula</td>
<td>Molecular Weight</td>
<td>Biological Effect</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Reduce</td>
<td>Nafcillin sodium salt monohydrate</td>
<td>C₂₁H₂₅N₂NaO₆S</td>
<td></td>
<td>Anti-bacterial</td>
<td>Bacterial transpeptidase inhibitor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Sulconazole nitrate</td>
<td>C₁₈H₁₆Cl₃N₃O₃S</td>
<td></td>
<td>Anti-fungal</td>
<td>Inhibitor of ergosterol synthesis</td>
</tr>
<tr>
<td>Reduce</td>
<td>Kaempferol</td>
<td>C₁₅H₁₀O₆</td>
<td></td>
<td>Anti-inflammatory</td>
<td>Topoisomerase 1 inhibitor, tyrosine kinase, xanthin oxidase</td>
</tr>
<tr>
<td>Reduce</td>
<td>Dexamethasone acetate</td>
<td>C₂₉H₃₇FO₅</td>
<td></td>
<td>Anti-inflammatory</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>Reduce</td>
<td>Alclometasone dipropionate</td>
<td>C₂₉H₃₇ClO₇</td>
<td></td>
<td>Anti-inflammatory</td>
<td>Corticosteroid</td>
</tr>
<tr>
<td>Reduce</td>
<td>Haloperidol</td>
<td>C₂₁H₂₃ClFNO₂</td>
<td></td>
<td>Anti-psychotic</td>
<td>Alpha-blocker, dopamine antagonist</td>
</tr>
<tr>
<td>Reduce</td>
<td>Methantheline bromide</td>
<td>C₂₁H₂₆BrNO₃</td>
<td></td>
<td>Anti-spasmodic</td>
<td>Anti-muscarinic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Aminophylline</td>
<td>C₁₆H₂₄N₁₀O₄</td>
<td></td>
<td>Bronchodilator</td>
<td>Benzodiazepine antagonist, mast cell degranulation inhibitor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Bucladesine sodium salt</td>
<td>C₁₈H₂₅N₃NaO₄P</td>
<td></td>
<td>Cardiotonic</td>
<td>Adenylate cyclase modulator</td>
</tr>
<tr>
<td>Reduce</td>
<td>Iodixanol</td>
<td>C₃₅H₄₄I₆N₆O₁₅</td>
<td></td>
<td>Diagnostic aid</td>
<td>Unknown</td>
</tr>
<tr>
<td>Increase</td>
<td>Cycloheximide</td>
<td>C₁₂H₁₂NO₄</td>
<td></td>
<td>Anti-bacterial</td>
<td>Protein synthesis inhibitor</td>
</tr>
<tr>
<td>Increase</td>
<td>Fluvastatin sodium salt</td>
<td>C₂₄H₂₅FNNaO₄</td>
<td></td>
<td>Anti-hyperlipoproteinemic</td>
<td>HMG CoA reductase inhibitor</td>
</tr>
</tbody>
</table>

Compound information supplied by Prestwick Chemical unless otherwise cited.
6.3.3 Assessing effects of second pass hits on whole larvae macrophage numbers.

To assess the possibility that reductions in irg1 expression were secondary to an ablative effect of drug treatment on macrophage-lineage cells, whole larvae numbers of macrophage-lineage cells were quantified by flow cytometry using $Tg(mpeg1:EGFP)^{gl22}$ larvae following drug treatment and compared to a DMSO-treated control group (FIG 6.11). Three biological replicates of 30 $Tg(mpeg1:EGFP)^{gl22}$ larvae per drug were incubated with drug for five hours prior to dissociation and quantification of fluorescent macrophage-lineage cells by flow cytometry. This five-hour timepoint mirrored the three-hour pre-treatment and two hour incubation experienced by larvae in the chemical screen.

FIGURE 6.11: Effect of drug treatment on the number of macrophage-lineage cells in whole larvae

Macrophage-lineage cells were quantified using the $Tg(mpeg1:EGFP)^{gl22}$ reporter line and BD LSR II flow cytometer, gating on EGFP$^+$ cells. Significance was determined by one-way ANOVA with multiple comparisons relative to the DMSO control. Bars indicate average and standard deviation. n=3 biological replicates. Abbrev. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.
When compared with DMSO-treated control samples, 11 of the 20 top hits showed no statistically significant change in whole-larvae macrophage numbers (**FIG 6.11**). Of note, piperlongumine treatment resulted in a significant increase in macrophage number. In contrast, treatment with isoniazid, aztreonam, sparteine, bucladesine sodium salt, sulconazole nitrate, aminophylline, cycloheximide, or camptothecine resulted in a significant reduction in whole-larvae macrophage numbers (**FIG 6.11**). The two most significant of these compounds, cycloheximide and camptothecine, are known to block protein synthesis and DNA replication respectively (Prestwick Chemical). This indicated that these drugs might act, at least in part, by preventing the natural growth and replication of macrophage-lineage cells.

6.3.4 Assessing mechanism of *irg1* inhibition used by second pass hits using the *Tg(irg1:EGFP)* <sup>nz4</sup> transgenic reporter line

It has previously been reported that altered *irg1* transcript stability may be an active means of regulating *irg1* expression (Basler et al., 2006; Lee et al., 1995). Given that stimulation of the *irg1* promoter within *Tg(irg1:EGFP)* <sup>nz4</sup> transgenic larvae drives production of EGFP regardless of *irg1* transcript fate (**FIG 6.12**), a characteristic also exploited in **Chapter 5**, *Tg(irg1:EGFP)* <sup>nz4</sup> larvae were treated with second pass hits and stimulated with hindbrain injection of LPS to assess whether these compounds may be acting by inhibiting transcription of *irg1*, leading to the reduction of both *irg1* transcript and fluorescent protein, or by contributing to the degradation or instability of *irg1* transcript, allowing production of the EGFP fluorescent reporter.
FIGURE 6.12: Differing methods of irg1 inhibition affect production of the EGFP reporter

A. Following hindbrain injection with LPS, applied chemical regulators of irg1 expression may act by preventing induction of the irg1 promoter region, resulting in no production of either irg1 or -4.7irg1:EGFP mRNA or protein. B. Alternatively, drugs may act by altering the stability of the irg1 transcript, resulting in enhanced degradation and reduced Irg1 protein yield, while production of the EGFP transcript and protein remain unhindered.

To ensure full fluorescence development for accurate comparisons, transgenic embryos were exposed to drugs at 48 hpf for three hours prior to LPS injection and then maintained in drug for an additional 16 hours. At this timepoint, LPS-injected Tg(irg1:EGFP)nz4 larvae with no drug treatment exhibited strong, distinct transgene expression (FIG 6.13 A’). While the majority of drugs recapitulated the expression profiles of irg1 as detected by WMISH at 2 hpi, a small number of discrepancies were observed (FIG 6.13 C’,H’,M’,S’).
<table>
<thead>
<tr>
<th>DMSO LPS</th>
<th>WMISH 2hpi</th>
<th>Tg(ig1:EGFP)nz4 16hpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A’</td>
<td>9/11</td>
</tr>
<tr>
<td>No Drug</td>
<td>B</td>
<td>10/10</td>
</tr>
<tr>
<td>DMSO PBS</td>
<td>15/15</td>
<td>8/8</td>
</tr>
<tr>
<td>ablate</td>
<td>C</td>
<td>14/14</td>
</tr>
<tr>
<td>Chrysin</td>
<td>C’</td>
<td>9/10</td>
</tr>
<tr>
<td>reduce</td>
<td>D</td>
<td>13/16</td>
</tr>
<tr>
<td>Urapidil Hydrochloride</td>
<td>D’</td>
<td>5/10</td>
</tr>
<tr>
<td>reduce</td>
<td>E</td>
<td>10/16</td>
</tr>
<tr>
<td>Spartaine</td>
<td>E’</td>
<td>8/9</td>
</tr>
<tr>
<td>reduce</td>
<td>F</td>
<td>18/18</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>F’</td>
<td>9/9</td>
</tr>
<tr>
<td>reduce</td>
<td>G</td>
<td>13/15</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>G’</td>
<td>12/12</td>
</tr>
<tr>
<td>reduce</td>
<td>H</td>
<td>16/17</td>
</tr>
<tr>
<td>Azlocillin Sodium Salt</td>
<td>H’</td>
<td>7/10</td>
</tr>
<tr>
<td>WMISH 2hpi</td>
<td>Tg(lrg1:EGFP)(n24) 16hpi</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------</td>
<td></td>
</tr>
<tr>
<td>I reduce Nafcillin Sodium Salt</td>
<td>I' 12/17</td>
<td></td>
</tr>
<tr>
<td>J reduce Sulconazole Nitrate</td>
<td>J' 8/10</td>
<td></td>
</tr>
<tr>
<td>K reduce Kaempferol</td>
<td>K' 4/10</td>
<td></td>
</tr>
<tr>
<td>L reduce Dexamethasone Acetate</td>
<td>L' 7/12</td>
<td></td>
</tr>
<tr>
<td>M reduce Alclometasone Diprionate</td>
<td>18/18</td>
<td></td>
</tr>
<tr>
<td>N reduce Haloperidol</td>
<td>N' 8/10</td>
<td></td>
</tr>
<tr>
<td>O reduce Methantheline Bromide</td>
<td>O' 7/10</td>
<td></td>
</tr>
<tr>
<td>P reduce Aminophylline</td>
<td>P' 8/10</td>
<td></td>
</tr>
<tr>
<td>WMISH 2hpi</td>
<td>Tg(irg1:EGFP)$^{nz4}$ 16hpi</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Q</strong></td>
<td>Q'</td>
<td></td>
</tr>
<tr>
<td>reduce</td>
<td>12/14</td>
<td>6/10</td>
</tr>
<tr>
<td>Bucladesine Sodium Salt</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>R</strong></td>
<td>R'</td>
<td></td>
</tr>
<tr>
<td>reduce</td>
<td>16/16</td>
<td>8/10</td>
</tr>
<tr>
<td>Iodixanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S</strong></td>
<td>S'</td>
<td></td>
</tr>
<tr>
<td>increase</td>
<td>12/12</td>
<td>6/10</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T</strong></td>
<td>T'</td>
<td></td>
</tr>
<tr>
<td>increase</td>
<td>11/15</td>
<td>5/5</td>
</tr>
<tr>
<td>Fluvastatin Sodium Salt</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U</strong></td>
<td>U'</td>
<td></td>
</tr>
<tr>
<td>ablate</td>
<td>12/18</td>
<td></td>
</tr>
<tr>
<td>Piperlongumine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>V</strong></td>
<td>V'</td>
<td></td>
</tr>
<tr>
<td>ablate</td>
<td>16/16</td>
<td></td>
</tr>
<tr>
<td>Camptothecine (S,+), Toxic at 16hpi</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 6.13:** Comparison of effects of second pass hits on irg1 expression as detected by WMISH at 2 hpi and by fluorescence microscopy of Tg(irg1:EGFP)$^{nz4}$ larvae at 16 hpi following LPS injection. A-V. WMISH detection of irg1 expression in drug-treated larvae two hours post-injection with LPS. A'-T'. Tg(irg1:EGFP)$^{nz4}$ transgenic larvae treated with second pass hits and live imaged 16 hours post-injection with LPS. U',V'. Larvae treatment with 5 µg/mL piperlongumine or camptothecine did not survive the extended treatment. Labels on the left indicate the effect had on irg1 expression as assessed by WMISH. Red arrows in A, A' indicate hindbrain injection site (common for all frames). Green arrow in A' indicates constitutive transgene expression. Scale bars 100µm in A, I, Q. Numbers indicate the proportion of larvae that displayed the presented phenotype. Larvae oriented anterior to left.
An expected difference between the *irg1* expression profile seen by WMISH and the -4.7*irg1*:EGFP transgene expression seen in *Tg(irg1:EGFP)*nz4 transgenic embryos was that seen in response to treatment with cycloheximide (FIG 6.13 S,S'). Cycloheximide acts by blocking global protein production. This has previously indicated the presence of a repressor protein regulating *irg1* expression, as discussed in 6.2.4. While blocking the production of repressor proteins led to an increase in *irg1* transcription, it also prevented the translation of transgene mRNA, leading to an absence of EGFP protein despite stimulation with LPS.

The opposite association was seen in larvae treated with chrysin, azlocillin sodium salt, or alclometasone dipropionate, three compounds that ablate or reduce *irg1* expression as detected by WMISH. When assessed using *Tg(irg1:EGFP)*nz4 transgenic larvae, each of these three appeared to have substantially more expression of the -4.7*irg1*:EGFP transgene than had been implied by the level of *irg1* expression seen in their respective WMISH analysis (FIG 6.13 C,C', H,H', M,M'). This indicates that these drugs may act on *irg1* transcript post-transcriptionally.

6.3.5 Chrysin treatment reduces *irg1* expression in stimulated larvae in a dose-dependent manner with minimal effect on known regulatory genes.

Second pass hits were further analysed in a preliminary qPCR assessing *irg1* transcript levels following drug treatment at a single concentration (5 μg/mL) (FIG 6.14)
Relative to the LPS-injected DMSO control, piperlongumine, iodixanol, kaempferol, chrysin, and camptothecine all had significantly reduced irg1 expression in larvae two hours post-injection with LPS, following a three-hour drug pre-treatment, while methaneline bromide demonstrated significantly increased expression of irg1. Assessment by t-test between DMSO LPS control and each drug treatment group. Error bars indicate average and standard deviation.

The four compounds resulting in the most significantly reduced expression of irg1 following injection with LPS were selected for further analysis. These included piperlongumine, an anti-fungal plant extract with possible anti-cancer properties; kaempferol, an anti-inflammatory flavonoid; camptothecine, an alkaloid with anti-tumour properties; and chrysin, another anti-fungal plant extract. These compounds were next tested to assess whether their ability to reduce irg1 followed a dose-dependent response (FIG 6.15).
LPS-injected larvae demonstrated significant, dose-dependent reductions in \textit{irg1} expression when treated with either piperlongumine or chrysin at 5, 10, and 20 µg/mL. By contrast, kaempferol did not exhibit dose-dependent reductions in \textit{irg1} and camptothecine treatment resulted in \textit{irg1} expression at the level of the PBS control sample at all concentrations tested. Larvae were pre-treated with drug for 3 h prior to LPS injection and remained in drug until sample collection two hours later. ANOVA with multiple comparisons relative to the DMSO LPS control. Error bars indicate average and standard deviation. \( n \)=3-5 biological replicates. Abbrev. *, \( P <0.05 \); **, \( P <0.01 \); ***, \( P <0.001 \); ****, \( P <0.0001 \).

Following a three-hour pre-treatment in drug, 2 dpf larvae were injected with LPS to induce expression of \textit{irg1}. Larvae remained in drug for an additional two hours prior to sample collection. Both piperlongumine and chrysin produced significant, dose-dependent reductions in \textit{irg1} expression when assessed across three drug concentrations (5, 10, and 20 µg/mL). By contrast, treatment with increasing concentrations of kaempferol appeared to slightly enhance expression of \textit{irg1}, while \textit{irg1} expression in camptothecine-treated samples was at the level of the PBS control at all concentrations tested, suggesting that it may be effective at a much lower dose.

### 6.3.6 Structural similarity analysis

The chemical structures of piperlongumine, kaempferol, camptothecine, and chrysin were then compared to those of all the compounds present in the Prestwick library to determine the potential for structure-based functional analyses. The ligand preparation module in SYBYL-X2.1.1 was used to strip out non-biological counter ions and then
compounds within the library were clustered using the structure activity relationship mapping (SARMAP) function of the Molecular Data Explorer (SYBYL-X2.1.1, Certara) (Dakshanamurthy et al., 2012). Related compounds were grouped, revealing no significant clusters for piperlongumine or camptothecine, and a cluster of seven compounds that contained both kaempferol and chrysin. This cluster also included apigenin, a known anti-inflammatory that acts as a MAP kinase inhibitor (Prestwick Chemical) and has been reported to have significant anti-oxidant properties (Jung, 2014), which was selected in the first pass hits. The remaining four compounds were acacetin, luteolin, myricetin, and quercetine dihydrate. Acacetin is an antioxidant that acts as a topoisomerase and glutathione reductase inhibitor, luteolin is an expectorant, myricetin is a neutral endopeptidase inhibitor, and quercetine dihydrate is a lipoxygenase (Prestwick Chemical). Statistical analysis using a hypergeometric distribution determined that the probability of selecting two compounds from this group in the top four hits from this screen by random chance is less than 0.02%, while selecting any one specific compound that does not cluster to a group has a probability of 0.36%.

6.4 Conclusions

A novel method for identifying compounds affecting an infection-inducible, rapidly-expressed, immune response gene has been demonstrated using zebrafish larvae. This platform enables aqueous housing of larvae in drug solution during hindbrain injection, ensuring continuous drug exposure. Its application to a small molecule screen designed to identify modulators of irg1 expression in macrophage-lineage cells from a drug-repurposing library of 1120 compounds has revealed a number of strong leads, including
chrysin, kaempferol, piperlongumine, and camptothecine. Complementing WMISH analysis of irg1 expression with observation of transgene expression in drug-treated, LPS-injected Tg(irg1:EGFP)nz4 larvae has suggested that some compounds, including chrysin, may act post-transcriptionally by affecting irg1 transcript stability. Together, these data have identified strong candidates for the attenuation of irg1 expression, which hold potential for the treatment of inflammatory and metabolic diseases through manipulation of macrophage phenotype.
7 Discussion

Macrophages are important effectors in the immune response to injury, infection, and disease (Mosser & Edwards, 2008). Their broad functional plasticity allows them to tailor their behaviours to the specific homeostatic or immune needs of their microenvironment (Mosser & Edwards, 2008). An understanding of the drivers that determine macrophage phenotype is essential to interpret the roles of macrophages in different immune environments and to inform how the process of phenotype switching may be modulated for therapeutic benefit. Growing evidence suggests that shifts in metabolic preference are central to this process (S. K. Biswas & Mantovani, 2012). This thesis has presented irg1 as a metabolic driver of inflammatory phenotype in macrophage-lineage cells responding to infection, through Irg1-driven production of mROS. Generation of the Tg(irg1:EGFP)_nz4 transgenic zebrafish line has enabled unprecedented, in vivo imaging of the process of activation in macrophage-lineage cells within live zebrafish larvae, in response to diverse stimuli, and provides an invaluable tool for the investigation of the functions of these cells during infection and inflammatory disease. Use of the Tg(irg1:EGFP)_nz4 transgenic has led to further discoveries regarding the downstream targets of Irg1 activity and the modes employed by Irg1 to direct pro-inflammatory activation in macrophage-lineage cells. Finally, a number of small molecules have been identified that may have applications in the therapeutic modulation of Irg1 activities in instances where macrophage-driven inflammation is associated with the propagation of disease. Together, this thesis has advanced understanding of the regulation of inflammatory activation in macrophage-lineage cells through Irg1 and has drawn further support for the connection between metabolism and immunity in inflammatory disease.
### 7.1 Promoter elements upstream of *irg1* are highly conserved between zebrafish, mouse, and human

In addition to identification of binding sites for the known *irg1*-regulating transcription factor C/EBPβ (Hall et al., 2013), the observation of additional common transcription factor recognition sequences in the promoter region of *irg1* in human, mouse, and zebrafish suggests that this gene has conserved mechanisms of regulation across species. Further examination of these putative regulatory elements, including recognition sequences for runt-related transcription factor 1 (Runx1), upstream stimulatory factor (USF), cAMP response element-binding protein (CRE-BP), and sterol regulatory element-binding proteins (SREBP), reveals a number of transcription factors that have been linked to specific macrophage phenotypes, as well as a family of regulatory proteins connected to lipid metabolism. The potential involvement of these regulators further supports the positioning of Irg1 as an important driver of metabolic mode as a means of directing macrophage phenotype.

One highly represented binding site is for Runx1, a transcription factor well characterised for its necessity in early haematopoiesis but also linked to microglial phenotype (E. Y. Lam et al., 2009; Zusso et al., 2012). When responding to local nerve damage, murine microglia were observed undergoing morphological changes suggestive of activation. Induction of *runx1* gene expression in a subset of these cells was correlated with their subsequent deactivation (Zusso et al., 2012). Supported by repeated documentation of its functions as both a transcriptional inducer and repressor (Jiang et al., 2005; Komine et al, 2003; Lutterbach et al., 2000; Reed-Inderbitzin et al., 2006), Runx1 recognition sites in the promoter region of *irg1* may indicate a negative regulatory function of the transcription factor on this gene, possibly contributing to its brief expression window.
Upstream stimulatory factor (USF) has also been linked to macrophage and microglial effector function, through the regulation of cytokine expression and MHC Class II molecules (Gobin et al., 2001; L. Zhang et al., 2007). Down-regulation or inhibition of USF in glioma-associated microglia has been observed to stimulate production of the anti-inflammatory cytokine IL-10, leading to a less inflammatory, more M2-type macrophage phenotype (L. Zhang et al., 2007). Conversely, M1-type macrophages demonstrate increased USF levels in inflammatory environments and a corresponding increase in MHC class II molecules (Gobin et al., 2001).

The presence of binding sites for cAMP response element-binding protein (CRE-BP) further supports a connection between Irg1 and the induction of a pro-inflammatory immune response, as this transcription factor has been reported to regulate the expression of pro-inflammatory cytokines *il6* and *tnfa*, while promoting further pro-inflammatory effector functions through the activation of NFkB (Roach et al., 2005; Wen et al., 2010). Within infection-responsive macrophages and monocytes, CRE-BP expression also confers a survival advantage by protecting these cells against pathogen-induced apoptosis (Park et al., 2005; Wen et al., 2010). Interestingly, phosphorylated (activated) CRE-BP has also been found extensively throughout the developing zebrafish brain, where it was deemed necessary for neural development and organisation (Dworkin et al., 2007). While Dworkin et al. did not examine which genes CRE-BP was acting on to direct these processes, it may be possible that Irg1 has a role in neural development or survival. This is supported by studies in the mouse, where CRE-BP has demonstrated crucial roles in neuron survival in both steady state and in response to injury (Dawson & Ginty, 2002; Mantamadiotis et al., 2002), and *Irg1* expression has been shown to confer anti-apoptotic properties to neurons following viral infection (Cho et al.,
A potential connection between \( \text{irg1} \) expression and neural development is discussed in further detail in 7.2.1, in light of the unanticipated, dim expression of \(-\text{4.7irg1:EGFP}\) transgene in the forebrain of non-stimulated \( Tg(\text{irg1:EGFP})^{nz4} \) larvae.

A fourth element found in the upstream sequences of both zebrafish and human \( \text{irg1} \) is recognition sites for sterol regulatory element-binding proteins (SREBPs). Regulation of \( \text{irg1} \) by SREBPs would lend further credence to the association of Irg1 with intracellular metabolic processes, specifically fatty acid \( \beta \)-oxidation, as SREBPs are well known for regulating the expression of many enzymes involved in lipid homeostasis, including the synthesis of cholesterol, fatty acids, triacylglycerols, and phospholipids (Eberlé et al., 2004; Horton et al., 2002). In fact, studies by Shimomura et al. using transgenic mice expressing constitutively-active, truncated SREBPs, determined that expression of SREBPs in the mouse liver was able to activate enzymes involved in the entire process of fatty acid biosynthesis, including, acetyl-coenzyme A carboxylase, fatty acid synthase, and glucose-6-phosphate dehydrogenase (Shimomura et al., 1998).

Taken together, the observation of putative binding sites for transcription factors USF, Runx-1, SREBP, and CRE-BP supports the current model that positions Irg1 as a metabolically sensitive indicator of macrophage phenotype and an integral component of the pro-inflammatory response of these cells, though further analyses are required to substantially validate these connections. While commercial antibodies to many zebrafish proteins are not readily available, an untested zebrafish Runx-1 antibody has been produced (Z-Fish™, Eurogentec). This could be used to assess direct protein-DNA interactions through chromatin immunoprecipitation assays (Lindeman, Vogt-Kielland, Alestrom, & Collas, 2009). For transcription factors where antibodies are not available, information may be inferred through indirect analyses using either morpholino
knockdown or ectopic over-expression by way of mRNA injection. These methods have already proved useful in corroborating the role of C/EBPβ in the induction of zebrafish irg1 expression and may be feasible in studying the effect of SREBP or USF (Hall et al., 2013). Studies using the gonzo zebrafish mutant, lacking the ability to activate SREBP, may also provide a useful background for assessing SREBP contribution to the induction of irg1 expression (Passeri, Cinaroglu, Gao, & Sadler, 2009). Another method of determining the necessity of particular transcription factors to the expression of a gene is by selectively mutating their recognition sites within the promoter region and determining effects on gene expression (Flores, Lam, Crosier, & Crosier, 2008). This can be facilitated through the use of a reporter construct, manipulating the benefits of the light-emitting protein luciferase for example, to provide a more rapidly quantified readout (Flores et al., 2008). A similar method consists of producing multiple reporter constructs using promoter regions of varying lengths, also known as a promoter deletion analysis (D. Jin, Ni, Hou, Rellinger, & Zhong, 2009). Changes in reporter expression between the different constructs may infer the function of the excluded region, providing information about both putative enhancers and repressors (D. Jin et al., 2009). A greater understanding of the promoter elements affecting irg1 expression will improve understanding of its regulation and may also reveal new targets to modulate irg1 expression for therapeutic benefit.

7.1.1 Expression of irg1 is regulated, in part, by a transcriptional repressor

In assessing the possibility of using cycloheximide, a protein synthesis inhibitor, as a positive control for abrogation of irg1 expression during a small molecule screen, it was revealed that instead, expression of irg1 became uncoupled from the infectious stimuli. Not only did transcription of irg1 increase following hindbrain injection of larvae with
LPS, it was observed following extended periods of cycloheximide treatment in the absence of LPS-injection. This suggested that cycloheximide was preventing the synthesis of a necessary transcriptional repressor.

Transcriptional repressors are not unprecedented in the regulation of immune response genes. For example, in the context of the gut, where the natural colonisation by commensal bacteria requires a tolerant immune environment, the Drosophila homolog of the mammalian Oct1 transcription factor, termed Nubbin, acts as a transcriptional repressor of NFκB target genes in the absence of infection (Dantoft et al., 2013). When Nubbin is mutated, uncontrolled production of inflammatory antimicrobial molecules leads to ablation of the gut microbiota. Similarly, the Gfi1 protein in mice has been shown to negatively regulate the expression of inflammatory cytokines TNFα and IL-1β as a means of dampening the immune response to endotoxin (Möröy et al., 2008). Gfi1 deficiency leads to the fatal overproduction of TNFα and IL-1β in mice in response to pulmonary infection. This transcriptional repressor has since been shown to also negatively regulate activation of the NLRP3 inflammasome in macrophages (L. Zhu et al., 2014). These examples illustrate the function of repressor proteins in modulating expression of robust immune responses in inappropriate circumstances. Similarly, given the robust pro-inflammatory responses associated with the expression of irg1, regulation through the use of a transcriptional repressor further ensures highly specific application of these functions.

It is not clear whether the repressor regulating irg1 expression is binding to the DNA itself or to other transcription factors that drive irg1 expression, altering their ability to recognise and bind to the promoter. In either instance, it may be possible to identify this molecule through a yeast 1-hybrid system, examining proteins that bind the promoter
region, or through a yeast 2-hybrid system to assess protein-protein interactions of known transcription factors upstream of irg1 (Ouwerkerk & Meijer, 2001; Toby & Golemis, 2001). Assessing protein-protein interactions may be challenging as it is possible that repressor regulation in this way acts on an as-yet unidentified transcription factor. As described earlier, assessing the function of regions of a promoter through a promoter deletion analysis can be used to identify regions affected by repressors (D. Jin et al., 2009). It is anticipated that deletion of the region of irg1 promoter that interacts with a repressor would lead to constitutive expression of irg1 in the absence of an infectious agent. Sequence analysis of this region may indicate likely protein binding partners, suggesting a specific regulatory protein or a transcription factor whose binding affinity may be controlled elsewhere, producing a repressor phenotype. In the reverse approach, performing a genetic screen for mutant zebrafish that constitutively express irg1 could identify the repressor protein as the product of the mutated gene. Identification of the regulatory elements involved in the induction of irg1 expression can be applied to the modulation of irg1 expression for therapeutic gain, and to further study of the strict regulation of irg1 within specific cells and tissues.

7.2 Infection-induced expression of irg1 in the Tg(irg1:EGFP)nz4 transgenic zebrafish line specifically marks macrophage-lineage cells

Numerous data support infection-induced expression of irg1 in macrophage-lineage cells, though expression of this gene has also been reported in the murine epidermis and uterine epithelium under non-infectious circumstances (Cheon et al., 2003; Hall et al., 2014). While infection-responsive induction of the -4.7irg1:EGFP transgene in the Tg(irg1:EGFP)nz4 reporter line appears to be exclusive to macrophage-lineage cells, it
remains to be seen whether expression may be induced in other cell types under different stimuli. Additionally, the low-level constitutive expression seen in the forebrain requires further characterisation.

7.2.1 Expression of the -4.7irg1:EGFP transgene in the forebrain of Tg(irg1:EGFP)nz4 larvae may indicate a developmental role for early irg1 expression

Weak forebrain expression of the -4.7irg1:EGFP transgene was found to reliably identify transgenic Tg(irg1:EGFP)nz4 larvae from 2 dpf without needing to first inject them with LPS or bacteria. Prior WMISH analyses had not noted any constitutive expression within non-infected or non-LPS-injected zebrafish larvae (Hall et al., 2013). It is unlikely that this expression is the result of the transgene integration site within the genome, as it was seen in all four identified founder lines. However, it is possible that the -4.7kb regulatory region upstream of the irg1 coding sequence used to generate the Tg(irg1:EGFP) transgenic did not capture all of the regulatory elements required to faithfully report endogenous irg1 expression, leading to ectopic transgene expression. Alternatively, necessary regulatory sequences may exist within the introns of the irg1 gene, a regulatory mechanism previously identified to direct tissue-specific gene expression within zebrafish (Camp et al., 2012; H. Li et al., 2012).

The potential requirement for regulatory elements further upstream in the irg1 promoter can be tested by generating transgene constructs using larger regions of the promoter sequence upstream from the irg1 transcription start site and assessing whether forebrain expression is still present in these non-stimulated transgenic larvae at 2 dpf. A second approach, for the determination of distant or intronic regulatory elements, could be bacterial artificial chromosome (BAC)-mediated transgenesis, where
a fluorescent reporter is cloned into the zebrafish \textit{irg1} gene within the BAC, which is then used for transgenesis, resulting in the inclusion of large regions of potential regulatory sequence both up and downstream of the transcription start site (Suster, Abe, Schouw, & Kawakami, 2011).

Despite no previous evidence of \textit{irg1} expression in the forebrain of larvae not injected with either LPS or bacteria, as detected by WMISH, it is still possible that the weak expression of the -4.7\textit{irg1:EGFP} transgene seen in this region in non-stimulated embryos does faithfully report endogenous \textit{irg1} expression. In support of this, the literature provides two studies reporting neural expression of mammalian \textit{Irg1}. Cho and colleagues identified \textit{Irg1} as a differentially expressed transcript when comparing two groups of spatially distinct neurons in mice (Cho et al., 2013). Neurons expressing \textit{Irg1}, either endogenously or ectopically, were found to have increased resistance to neurotropic viruses. More recently, \textit{IRG1} expression in human gliomas was reported to be prognostic for disease severity, with heightened expression correlating with increased cancer cell growth and invasiveness (Pan et al., 2014). In light of the observation that CRE-BP may be involved in driving \textit{irg1} expression, as determined through analysis of the -4.7kb promoter sequence used to produce \textit{Tg(irg1:EGFP)} larvae (see 7.1), and given the prominent role of CRE-BP in brain development, these data together suggest that \textit{irg1} may be involved in neural growth and development. This is intriguing considering that the dominant phenotype of larvae treated with high doses of the splice-blocking, \textit{irg1}-targeting morpholino IRG1SBMO3, as seen during titration of its effective dose, was hindbrain oedema. This swelling may be indicative of a role of \textit{Irg1} in the development of the brain that is compromised by large doses of IRG1SBMO3. However, as hindbrain oedema was only seen in response to one of the two \textit{irg1}-targetting morpholinos, it equally may indicate a non-specific affect.
While neuronal expression has not been reported in other studies examining Igf1, it may be that the relative expression level, when compared with that seen in macrophage-lineage cells, is simply too low to note unless specifically examining isolated, non-macrophage-lineage cell populations. Analysis of irg1 transcript levels by qPCR, using samples of dissected tissue from the forebrain of Tg(irg1:EGFP)nz4 larvae and tissues where irg1 expression has not been previously detected, may be more sensitive in assessing potential irg1 expression in these regions. It is also interesting to note that hindbrain injection of LPS into 2 dpf Tg(irg1:EGFP)nz4 larvae did not appear to stimulate an increase in expression of the -4.7irg1:EGFP transgene in the forebrain. This suggests that if the EGFP expression seen here is a faithful recapitulation of endogenous expression, induction of irg1 expression in the forebrain occurs by an unknown, non-infection-driven mechanism with the likely contribution of additional tissue-specific regulators. The necessity and function of low-level expression of irg1 in the forebrain, if supported, would require further characterisation.

Another possible explanation for the lack of previous detection of irg1 expression within the forebrain of larval zebrafish relates to the detection sensitivity and resolution of the methods used. Early analyses were done using colorimetric WMISH and light microscopy (Zimmerman, Peters, Altaras, & Berg, 2013). With this method, stain intensity increases over time proportionally to the level of target mRNA present in each tissue until the reaction is chemically halted. As such, tissue-level discrepancies in expression of the target mRNA within an embryo may lead to a reaction being stopped before lesser-expressing tissues reach the threshold of detection. If left to develop for longer periods of time it becomes more difficult to differentiate specific and non-specific (background) staining, while diffusion of the stain can also lead to reduced resolution.
Detection of fluorescent reporters via confocal microscopy, as used in later studies, has the potential for a much larger dynamic range and greater cellular resolution by comparison (Zimmerman, Peters, Altaras, & Berg, 2013). However, given that WMISH readout relies on the detection of transiently expressed, unstable RNA rather than highly stable fluorescent reporter proteins, it is more likely that initial irg1 expression analyses via WMISH were not temporally aligned with potential early expression events. As the half-life of GFP protein has been reported to be approximately 26 h (Corish & Tyler-Smith, 1999; Verkhusha et al., 2003), fluorescent reporter expression seen in transgenic embryos may represent a transcription event long passed. This may be assessed by performing WMISH on younger embryos, further exploring a potential developmental role for irg1 expression in the brain.

While the forebrain expression of the -4.7irg1:EGFP transgene seen in Tg(irg1:EGFP)nz4 larvae remains unexplained at present, it has served as a useful indicator of transgenesis and has not interfered with observation of irg1-expressing macrophage-lineage cells in the zebrafish hindbrain. It has also suggested a potential connection of irg1 to developmental processes not previously considered, but this requires further investigation to establish.

7.2.2 The Tg(irg1:EGFP)nz4 transgenic zebrafish line reports a subset of the Irg1 functions seen in mammals

The Tg(irg1:EGFP)nz4 transgenic line generated in this thesis is a valuable tool for the observation of irg1 expression in activated, macrophage-lineage cells. However, it is likely that this line does not fully report all instances of non-macrophage-specific IRG1 expression, as seen in the mouse and human, due to subfunctionalisation of the irg1 gene.
resulting from the genomic duplication event that occurred in the teleost lineage 350 million years ago (Postlethwait et al., 2004). In the instance of \textit{i}rg\textit{1}, a paralog has been identified in the zebrafish, \textit{i}rg\textit{1-like (irg1l)} (Hall et al., 2014). Similar to Irg1, Irg1l is also infection-responsive and functions to enhance fatty acid metabolism, albeit in keratinocytes rather than immune cells (Hall et al., 2014). This study further described the presence of murine IRG1 in the mitochondria of keratinocytes in a mouse model of atopic dermatitis. These data strongly suggest that zebrafish Irg1 and Irg1l represent subfunctionalisation of an ancestral Irg1 and observation of both of these genes together may be required to fully recapitulate the expression patterns and activities seen for mammalian IRG1 (Hall et al., 2014). The ability to observe subsets of gene function in isolation can be very powerful, though it also suggests that the \textit{Tg}(irg1:EGFP)\textsuperscript{nz4} reporter line may not report instances of non-macrophage-specific IRG1 expression as seen in the human and mouse. It would be of interest to assess the complementarity of \textit{i}rg\textit{1} and \textit{i}rg\textit{1l} through the development of a compound transgenic labelling expression of both of these genes under different fluorescent reporters. For example, developing a compound \textit{Tg}(irg1:EGFP)\textsuperscript{nz4}/\textit{Tg}(irg1l:mCherry) transgenic zebrafish would allow observation of the expression of these genes simultaneously in response to different microenvironments and may provide a more complete description of the roles of IRG1 in immune response and disease within humans.

### 7.3 The \textit{Tg}(irg1:EGFP)\textsuperscript{nz4} transgenic zebrafish line facilitates live imaging of activated macrophage-lineage cells during inflammatory immune response

This \textit{Tg}(irg1:EGFP)\textsuperscript{nz4} transgenic line has displayed \textit{i}rg\textit{1} expression in zebrafish macrophage-lineage cells, \textit{in vivo}, in response to a number of different stimuli in both the
hindbrain and peripheral tissues. Injections of transgene DNA into the single-cell embryo, LPS into the hindbrain or otic vesicle, and cancer cells into the perivitelline space have all demonstrated the ability to induce expression of the -4.7irg1:EGFP transgene in macrophage-lineage cells. Live confocal imaging of irg1 activation in each of these circumstances using Tg(irg1:EGFP)oz4/Tg(mpeg1:mCherry)gl23 double transgenic larvae, marking all macrophage-lineage cells with mCherry and co-labelling activated macrophage-lineage cells with both mCherry and EGPF, enables the observation of macrophage-lineage cells as they transition from a non-activated to activated state, as well as to differentiate the behaviours and phenotypes of macrophage-lineage cells that become activated following inflammatory stimulation from those that do not.

7.3.1 Injection of the -4.7irg1:EGFP transgene construct into single-cell embryos induces irg1 expression by 1 dpf

During the generation of Tg(irg1:EGFP)oz4, observation of embryos injected with transgene DNA and transposase mRNA revealed punctate transgene expression at 1 dpf in all areas of the body, despite a lack of LPS or bacterial stimulation. This was found to accurately report endogenous irg1 expression, supported by WMISH analysis of these embryos. As this expression is not seen in the progeny of germline transgenic fish, but only in the founders themselves, it indicates that irg1 expression is being induced by the injected transgene DNA or accompanying transposase mRNA. Considering that both foreign DNA and RNA can act as potent immunostimulatory compounds (Barbalat et al., 2011), an immune response stemming from their injection during transgenesis becomes less surprising. This does however, raise an important consideration for the generation of transgenic zebrafish lines that report immune cell responses, as this early, unanticipated stimulation may lead to unexpected expression patterns when screening
for founder lines, as observed during the generation of \( Tg(\text{irg1:EGFP})^{nz4} \), that should not necessarily be discarded as inaccurate representations of endogenous expression.

### 7.3.2 Live Imaging

Live imaging \( Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{\text{gl23}} \) larvae following LPS injection reveals rapid, robust activation of macrophage-lineage cells.

\( Tg(\text{irg1:EGFP})^{nz4} \) zebrafish larvae were observed from 2 dpf, following injection of LPS into the hindbrain. Zebrafish larvae develop both functional neutrophils and macrophage-lineage cells by 24 hpf, with seeding of the brain beginning by 35 hpf (Davidson & Zon, 2004; Herbomel et al., 2001). Markers for early, immature lymphocytes can be detected from 65 hpf, though full maturation of the adaptive immune system does not occur until 4-6 weeks of age (S. H. Lam et al., 2004; Willett, Cortes, Zuasti, & Zapata, 1999). Thus, observation of immune responses at 2 dpf allows examination of the contributions of innate immunity in isolation. Hindbrain injection with LPS or bacterial particles at this age is an established model for studying the responses of macrophage-lineage cells (Benard et al., 2012; Hall et al., 2013). As \textit{irg1} expression is anticipated to mark a specific population of inflammatory, activated macrophages and microglia, this study aimed to use the hindbrain stimulation of 2 dpf \( Tg(\text{irg1:EGFP})^{nz4}/Tg(\text{mpeg1:mCherry})^{\text{gl23}} \) larvae to compare the characteristics of macrophage-lineage cells that become activated following stimulation with those of macrophage-lineage cells that do not.

A caveat to interpreting \textit{irg1} expression using the \( Tg(\text{irg1:EGFP})^{nz4} \) transgenic reporter line is the fact that readout from stimulation of the -4.7\textit{irg1:EGFP} transgene relies on the production and maturation of the EGFP protein. While detection of EGFP fluorescence has been reported as early as 8 min following induction, reaching half-maximal intensity...
within 25 min (Cormack, Valdivia, & Falkow, 1996; Iizuka, Yamagishi-Shirasaki, & Funatsu, 2011), it is difficult to determine by how much detection of this fluorescence lags behind transcription of the irg1 gene. Additionally, translation of the EGFP mRNA into functional fluorescent protein does not necessarily correlate with translation of endogenous irg1 mRNA produced through the same induction. Similarly, no interpretations regarding the presence or stability of Irg1 protein can be made from observation of the EGFP protein, which has been reported to have a half-life near 26 h (Corish & Tyler-Smith, 1999; Verkhusha et al., 2003). However, these considerations also provide certain benefits. For example, as production of the EGFP protein is not coupled to translation of Irg1, EGFP expression can be used to mark cells that respond to stimuli with induction of irg1 expression but are selectively ablated of Irg1 activity, by specifically targeting irg1 transcript. This property was exploited for RNA-Seq analysis as discussed later. Similarly, when paired with an analysis tool detecting transcription, such as WMISH, discrepancies in expression patterns between the irg1 transcript and EGFP protein, driven from the -4.7irg1:EGFP transgene, may indicate post-transcriptional regulation of the irg1 mRNA. This proved useful in the analysis of small molecules identified through the drug screen performed in this thesis, as discussed in more detail in 7.5.1. Additionally, the long half-life of the EGFP protein (26h) facilitates the tracking of macrophage-lineage cells long after transcription of irg1 has ceased, allowing observation of these cells for potential lasting effects of activation on their lifespan, effector functions, and subsequent stimulation/reactivation potential.

In these studies, EGFP reporter protein was observed as early as 30 min following injection of Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae with LPS. This was followed by a gradual increase in fluorescence intensity, with the majority of macrophage-lineage cells expressing the -4.7irg1:EGFP transgene by 6 hpi. This left very few to no non-
activated macrophage-lineage cells with which to contrast population behaviours. The lack of a “non-activated” population in this model may speak to the developmental age of the macrophage-lineage cells in the hindbrain region at 2-3 dpf or the strength of LPS in inducing an inflammatory response.

7.3.3 Live imaging

Live imaging of Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae following LPS injection reveals two morphologically-distinct macrophage-lineage cell populations.

Closer examination of the macrophage-lineage cells present in the hindbrain of Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae following injection with LPS, as marked by expression of the mpeg1:mCherry transgene, revealed a range of cell morphologies and -4.7irg1:EGFP transgene expression intensities. Branched, highly motile macrophage-lineage cells were observed as early as 1 hpi along with more spherical cells that did not appear to migrate as rapidly. Assessing the depth of these spherical and branched cells within the hindbrain, relative to the epithelial surface, revealed that the branched cells tended to be more superficial, with the majority migrating within the first 20µm below the hindbrain epithelium. The more spherical cells tended to reside at least 40µm below the epithelial surface.

Further division of the branched and spherical cells by -4.7irg1:EGFP transgene expression intensity and assessing the velocity of their migration routes revealed that while branched cells did move more rapidly than those with more spherical morphology, transgene expression intensity did not significantly predict relative migration speed. One difficulty with assessing the velocity of the highly motile, branched cells was determining where within the cell to make these measurements, as it was not always clear where the main cell body was given the rapid extension and retraction of their
processes. Additionally, tracking the movement of the central cell body does not accurately reflect the motility of the processes.

A similar partitioning of phenotypes has been described in recent studies following the development of microglia in the optic tectum (Svahn et al., 2012). Svahn and colleagues reported early microglia from 3-6 dpf as being highly phagocytic, motile, and having a large cell body with short, transient processes. From 5-10 dpf these cells began taking on a more branched phenotype and demonstrated a gradual reduction in motility as they transitioned towards a more ramified and less phagocytic phenotype. Highly branched cells were also observed in the skin overlying this region from 8-9 dpf, which they suggested may be Langerhans cells (Svahn et al., 2012). While these observations were made in older larvae, Svahn and colleagues did not investigate earlier timepoints and did not assess the potential effect of immune stimulation on the progress of microglial development. The highly phagocytic early macrophages with large cell bodies observed by Svahn et al. appear to correspond with the more spherical cells described in this thesis, while the population of more branched macrophage-lineage cells described here may represent macrophage-lineage cells migrating through the skin, possibly early Langerhans cells or peripheral macrophages recruited to the inflammatory stimuli, which were not assessed in the studies by Svahn et al.

More recently, van Ham et al. investigated the microglial response to neuroinflammation in the forebrain of larval zebrafish at 3 and 5 dpf, induced through the genetically targeted ablation of neurons (van Ham et al., 2014). They described a staged response involving microglia and macrophages, but distinctly devoid of contributions from neutrophils. In this study, two populations of Apolipoprotein E (ApoE)-expressing macrophage-lineage cells were detected in the brain following neuron death: ApoE$^{\text{high}}$
microglia and ApoE\text{low} macrophages. The ApoE\text{low} cells were more amoeboid and generally appeared in closer proximity to the site of injury, consistent with a more phagocytic phenotype, while some ApoE\text{high} microglia remained at a distance from the site of injury, indicating lack of activation or delayed response to the stimulus. Interestingly, they also observed that, in comparison to control larvae with no neuronal degeneration, macrophage-lineage cells within the forebrain took on a more spherical morphology following neuronal degradation (van Ham et al., 2014). When compared to observations made in this thesis, the paper by van Ham \textit{et al.} seems to suggest that the more spherical cells described here are early, recruited macrophages, while the more branched cells are more likely to be early microglia. Given that Svahn \textit{et al.} investigated homeostatic development while van Ham and colleagues applied an inflammatory stressor, the observations and identifications made by these two groups are not necessarily mutually exclusive. Also, as both of these studies were performed using older larvae than those selected for the studies described in this thesis, neither the Svahn \textit{et al.} nor van Ham \textit{et al.} paper is better qualified to comment on the populations observed in this thesis. However, both do appear to support classification of the more spherical cells described in Chapter 4 as recruited macrophages, while the more branched cells may be of a more restricted lineage such as early microglia or Langerhans cells.

Studies observing the development of macrophage-lineage cells in steady state (Svahn \textit{et al.}, 2012), following non-infectious stimulation (van Ham \textit{et al.}, 2014), and during response to an injected, infectious stimulus, show that immune stimulation impacts the phenotype of macrophage-lineage cells within the larval zebrafish brain (Svahn \textit{et al.}, 2012; van Ham \textit{et al.}, 2014). They also indicate that subpopulations of macrophage-lineage cells do exist in young larvae prior to their terminal differentiation. This suggests
that while developmental age of the macrophage-lineage cells at 2 dpf may impact the phenotype of these cells, the lack of heterogeneity in activation phenotypes, as indicated by strong expression of the -4.7irg1:EGFP transgene, is more likely to be due to an overly-robust stimulation from the injected LPS, resulting in systemic signalling and activation of all macrophage-lineage cells. It is possible that by titrating the dose of LPS, a concentration may be found that only stimulates activation in a subset of the macrophage-lineage cells. Alternatively, other stimuli may be explored. For example, monosodium urate crystals, the causative agent in gout, have been reported to induce expression of Irg1 in murine macrophages in a dose-dependent manner (Pessler et al., 2008) and unpublished data from our research group indicates that they act similarly to stimulate irg1 in zebrafish. However, it is important to note that monosodium urate crystals model sterile inflammation, rather than the pathogenic/bacterial inflammation modelled by LPS, and thus macrophage responses and functions in these environments may be substantially different. Finally, these studies make apparent that the current methods of differentiating subpopulations of immune-responsive cells within the brain, often by morphology or phagocytic potential, are difficult to apply to studies examining different environments. In future, use of the Tg(irg1:EGFP)nz4 transgenic zebrafish may provide a more accurate delineation of these different immune responses.

7.3.4 High expression of the -4.7irg1:EGFP transgene correlates with high MMP activity

Given the connection of irg1l-driven mROS production to the transcription of pro-inflammatory genes, including matrix metalloproteinases (MMPs) (Hall et al., 2014; Sena & Chandel, 2012) it was investigated whether MMP activity within macrophage-lineage cells, as identified by expression of the mpeg1:mCherry transgene, could be used to
further differentiate subpopulations of activated macrophage-lineage cells within LPS-injected \( Tg(irg1:EGFP)^{nz4}/Tg(mpeg1:mCherry)^{gl23} \) larvae, as detected by coexpression of the \(-4.7irg1:EGFP\) transgene. High MMP activity did tend to correlate with increased expression of the \(-4.7irg1:EGFP\) transgene. However, a second population of cells with high transgene fluorescence yet no visible MMP activity was also observed. Whether this represents a true division of cell phenotypes is difficult to discern at present, as over the course of these experiments it became apparent that the MMPSense 645FAST reagent used to detect MMP activity did not readily diffuse through the blood brain barrier. This confounds observation of the MMP activity within cells in the process of migrating to and invading the hindbrain and those traveling through the epithelium, as they likely have reduced access to the reagent. Based on a low background fluorescence, it also appeared that the reagent became consumed over time, additionally limiting analysis to early events during the inflammatory response. In future, the MMP activity of these macrophage-lineage cell populations (peripheral/invading and intra-cerebral) may be more accurately assessed by immersing the larva in the MMP reagent and injecting it into the brain, or examining each system separately. These data do however, support a connection between infection-associated stimulation and the production of MMPs in activated, macrophage-lineage cells, as no MMP activity was registered in PBS-injected \( Tg(irg1:EGFP)^{nz4}/Tg(mpeg1:mCherry)^{gl23} \) larvae.

7.3.5 Live imaging \( Tg(irg1:EGFP)^{nz4}/Tg(mpeg1:mCherry)^{gl23} \) larvae following injection of a metastatic breast cancer xenograft reveals localised activation of macrophage-lineage cells

The association of macrophages with the tumour environment is complex. While pro-inflammatory, activated macrophages may present a substantial anti-tumour response, the tumour microenvironment is known to induce phenotype switching in recruited
macrophages, manipulating these cells into expressing a pro-tumoral phenotype (Chanmee et al., 2014). As such, current cancer therapies aim to reverse this process and reprogram tumour-associated macrophages (TAMs) back to an inflammatory phenotype (Cluff, 2010; Guiducci et al., 2005). However, TAMs are not explicitly anti-inflammatory, with certain pro-inflammatory characteristics also reported to aid in cancer growth and survival. For example, macrophage-produced MMPs have been reported to contribute to the degradation of the ECM and promote tumour metastasis (Chanmee et al., 2014).

Considering the role of the zebrafish Irg1 paralog, Irg1l, in driving mROS-dependent MMP production in epidermal cells following infection (Hall et al., 2014), it is of interest to determine whether Irg1 may act similarly within macrophages and, if so, whether the production of MMPs in activated macrophages contributes to a pro- or anti-tumoral effect in TAMs.

Live imaging Tg(irg1:EGFP)\textsuperscript{nz4}/Tg(mpeg1:mCherry)\textsuperscript{pl23} larvae following injection of a human breast cancer tumour xenograft into the perivitelline space has revealed that macrophages in close association with cancer cells do express irg1. These cells were also observed phagocytosing cancer cells at both the initial tumour site as well as at distal locations where the tumour cells had travelled through the vasculature. While phagocytosis of cancer cells by macrophages has been previously described in the larval zebrafish (Y. Feng et al., 2010) it has not previously been possible to observe the activation state of these cells \textit{in vivo}.

The MDA-MB-231 human breast cancer cell line was chosen for this study as it has been used extensively in the larval zebrafish to model cancer growth and immune response (Astin et al., 2014; Drabsch et al., 2013; Teng et al., 2013). This line has well-characterised metastatic and angiogenic properties (Teng et al., 2013), indicating that
the contribution or response of macrophage-lineage cells to these events may also be examined. As metastatic cancers have been reported to manipulate macrophages to take on a more M2-like phenotype in order to support tissue invasion (Chanmee et al., 2014), and given the association of irg1 expression with a more pro-inflammatory, potentially anti-tumour phenotype (Hall et al., 2013; H. Li et al., 2006), establishing the presence of irg1-expressing macrophage-lineage cells in the early inflammatory response to cancer cells suggests that observing behavioural and transcriptional changes in -4.7irg1:EGFP transgene-expressing macrophage-lineage cells over the course of tumourigenesis and invasion events may provide direct insight into the process of cancer-driven phenotype switching.

An advantage of grafting tumours is that the disease state is rapidly and precisely induced; specific cancer types can be studied in any genetic background, tissue, or age (Konantz et al., 2012). Grafting tumours into larval zebrafish allows observation of the relationship between innate immune cell populations and the tumour without contribution from the adaptive immune system; this provides an additional benefit in that immunosuppression prior to engraftment, a requirement for xenograft models in other animal systems, can be avoided (Konantz et al., 2012). However, graft cancer models allow little interpretation of the transformation events that precede the generation of a cancer. For this, cancer development has been studied in the zebrafish through both genetic models and induction via exposure to carcinogens (Langenau et al., 2003; Langenau et al., 2005; Patton et al., 2005; Spitsbergen et al., 2000). For example, expression of a mutant version of the serine/threonine kinase BRAF in a p53-deficient background in zebrafish generated invasive melanomas (Patton et al., 2005). Breeding zebrafish with this background to the Tg(irg1:EGFP)nz4 transgenic line would allow
observation of the response of activated macrophage-lineage cells to host-generated cancer at all stages of its development.

Recently, IRG1 expression has also been observed in human glioma cell lines and tissue samples (Pan et al., 2014). Enhanced IRG1 expression within glioma cell lines lead to increased growth and invasion, while depleting glioma cells of IRG1 activity slowed these oncogenic processes. These studies inferred that IRG1 expression within gliomas is a biomarker for poor prognosis and substantially contributed to cancer metastasis, in part through promotion of epithelial-mesenchyme transition. This study by Pan et al. further supports the possibility that irg1 expression in TAMs may similarly promote the epithelial-mesenchyme transition that facilitates cancer growth and invasion, through the mROS-driven production of MMPs. If true, considering that expression of irg1 has also been observed in this study in phagocytic, potentially anti-tumour macrophages, ablating Irg1 to reduce cancer invasion may be detrimental to the general innate immune response against the tumour. Inducing cancer development in Tg(irg1:EGFP)z4 zebrafish either chemically or genetically, by outcrossing this line to a genetically-susceptible transgenic line, would allow analysis of these scenarios with the additional benefit of exploring the potential expression and role of irg1 in the cancer itself. Understanding how macrophage phenotype is directed in the tumour environment and being able to observe and interrogate this process in vivo will provide valuable insight into potential routes of manipulation that may effectively promote tumour destruction while minimising its potential for invasion and metastasis.
7.3.6 The \textit{Tg(irg1:EGFP)nz4} transgenic zebrafish line holds potential for elucidating the role of macrophage-lineage cells in the progression of metabolic and neurodegenerative diseases

Though not examined in this thesis, development of the \textit{Tg(irg1:EGFP)nz4} transgenic reporter line has also opened the door for live imaging macrophage activation in the context of diseases where the presence of macrophage-lineage cells is either unexplained or has been associated with disease progression. For example, a model of diet-induced obesity has been developed in zebrafish and shown to exhibit common pathways and regulators with mammalian models, including dysregulated fatty acid metabolism, triglyceride metabolism, and cholesterol efflux, and regulatory involvement of the pro-inflammatory cytokines IL-6 and IL-1\(\beta\) (Oka et al., 2010). Given the relationship between increasing adiposity and development of a pro-inflammatory microenvironment that promotes macrophage phenotype switching from anti- to pro-inflammatory effector functions (Heilbronn & Campbell, 2008; Weisberg et al., 2003), examining this model in a \textit{Tg(irg1:EGFP)nz4} background would facilitate analysis of the contribution of \textit{irg1}-expressing, activated macrophage-lineage cells to the progression of obesity-associated inflammation. Expression of the -4.7\textit{irg1:EGFP} transgene may be used as a biomarker to assess the inflammatory status of the obese animal and as a readout for assessing the success of chemical interventions for the treatment of obesity associated inflammation, to prevent or reverse the development of obesity-associated co-morbidities.

\textit{IRG1} expression has also been previously described as an indicator of neurotoxicity within a population of murine microglia (H. Li et al., 2006) Observing the activation and behaviours of microglia within zebrafish models of neurodegenerative diseases is possible by pairing the genetics of the \textit{Tg(irg1:EGFP)nz4} transgenic reporter line with existing zebrafish models of tauopathies (Bai, Garver, Hukriede, & Burton, 2007; Paquet
et al., 2009; P. Song & Pimplikar, 2012), Parkinson's disease (McKinley et al., 2005), and Huntington's disease (Lumsden, Henshall, Dayan, Lardelli, & Richards, 2007). These models may provide greater insight into the roles of microglia in these diseases and the pathways present in these disorders that lead to microglial activation.

7.4 Irg1 activity promotes the transcription of inflammation-associated genes

RNA-Seq analysis, performed on isolated populations of Irg1-replete and Irg1-depleted activated macrophage-lineage cells during their response to LPS injection within Tg(irg1:EGFP)nz4/Tg(mpeg1:mCherry)gl23 larvae, has supported an integral role for Irg1 in the inflammatory response within these cells. In addition, these data further implicate it as a core regulator of obesity-associated-inflammation within adipose tissue macrophages, due to its effects on transcription of both tnfα and il1β, among others.

7.4.1 Depletion of Irg1 activity through injection of the splice-blocking morpholino IRG1SBMO3 reveals differential expression of key regulators of obesity-associated inflammation

Deep sequencing of macrophage-lineage cells depleted of irg1 expression through the use of a splice-blocking morpholino revealed differential expression of 99 genes following stimulation with LPS when compared with samples injected with a control, non-targeting morpholino. Among the identified genes were tnfα and il1β, two potent immune activators and among the first genes to link immunity to metabolism, through observation of their pro-inflammatory activities in adipose tissue macrophages that connect obesity to the propagation of insulin resistance (Hotamisligil et al., 1993; Hotamisligil et al., 1996; Lagathu et al., 2006)
The failure of a macrophage-lineage cell to induce \textit{tnfa} and \textit{il-1}\textbeta following inflammatory stimulation when depleted of \textit{Irg}1 indicates that \textit{Irg}1 acts upstream of these genes, regulating their expression as a means of propagating the pro-inflammatory phenotype associated with \textit{irg}1-expressing cells. This is anticipated to occur through \textit{Irg}1-driven production of mROS as a result of enhanced fatty acid β oxidation, given that a similar pathway has been seen with the \textit{irg}1 paralog \textit{irg1l} and mROS-driven production of MMPs (Hall et al., 2014; Sena & Chandel, 2012). TNFα, expressed from both hypertrophic adipocytes and inflammatory adipose associated macrophages, has been widely reported as a driver of obesity-associated inflammation (Guilherme et al., 2008). Similarly, IL-1β is a strongly inflammatory cytokine positioned to promote dysregulation of glucose metabolism leading to insulin resistance and the further propagation of inflammation (Lagathu et al., 2006). In addition, a third strong driver of adipose-associated inflammation is free fatty acids, which are known to promote pro-inflammatory activation of ATMs (Guilherme et al., 2008; Han et al., 2014). Zebrafish macrophages activated by way of LPS injection have shown enhanced fatty acid β oxidation and this metabolism is impeded when the expression of \textit{irg}1 is blocked (Hall et al., 2013). Together, these data suggest that \textit{Irg}1 may contribute to adipose-associated inflammation, possibly through the activation and presentation of recruited or resident macrophages. If so, \textit{Irg}1 may also present as a specific target for the modulation of macrophage phenotype to reduce inflammation in these tissues by dampening expression of these inflammatory mediators, thereby reducing the progression of disease and the development of obesity-associated disorders.
7.4.2 Depletion of Irg1 activity through injection of the splice-blocking morpholino IRG1SBMO3 reveals differential expression of targets of NFκB signalling as well as regulators of NFκB activity

Also of significance in the list of differentially regulated genes identified through transcriptome analysis and comparison of LPS-stimulated macrophage-lineage cells replete or depleted of Irg1 were a number of genes involved in the regulation of NFκB activity, such as bcl3, traf3, and bcl10. Bcl3 has varied functions in either promoting or inhibiting NFκB depending on environmental cues or its own post-translational modification, while Traf3 and Bcl10 both act to promote NFκB through associations with tumour necrosis factor receptor type 1-associated death domain (TRADD) (Bundy & McKeithan, 1997; Guiet & Vito, 2000; Michallet et al., 2008; Oeckinghaus & Ghosh, 2009; Richard et al., 1999). Possibly stemming from the resulting lower level of NFκB functionality due to reduced expression of these three genes, downstream targets of NFκB were also found to be significantly reduced in samples from Irg1-depleted macrophage-lineage cells, including aplp2, atp8b4, ctdsp1, gtpb4, il-1β, irf8, and traf3 again. NFκB signalling is another key pro-inflammatory process with relevance to pro-inflammatory immune cell effector functions and the propagation of metabolic disease (Baker et al., 2011; Carlsen et al., 2009).

The possibility that Irg1 may be promoting intracellular changes within macrophages through the promotion of mROS, produced through enhanced fatty acid β oxidation, is supported by existing data connecting NFκB and ROS signalling. This crosstalk however, is complex, with ROS-mediated effects acting to inhibit or promote NFκB signalling in a variety of ways and NFκB similarly regulating the production of ROS (Morgan & Liu, 2011). For example, NFκB signalling has been shown to protect against ROS-mediated cellular damage by enhancing the production of cellular antioxidants such as manganese.
superoxide dismutase (Morgan & Liu, 2011). Conversely, during infection NFκB has been shown to promote expression of NADPH oxidase and cyclooxygenase 2, among others, with these enzymes functioning to increase cellular ROS either directly or indirectly (Anrather, Racchumi, & Iadecola, 2006; Morgan & Liu, 2011). With regards to ROS mediation of NFκB signalling, ROS have been demonstrated to promote the expression of a subset of NFκB target genes by driving the phosphorylation of NFκB-family member RelA, leading to the production of inflammatory mediators such as IL-8 (Nowak et al., 2008). There is also evidence that ROS promote cytosolic NFκB signalling cascades and nuclear translocation during inflammatory response, while concurrently inhibiting its nuclear DNA binding efficiency (Kabe, Ando, Hirao, Yoshida, & Handa, 2005). The abundant cross-regulation of ROS and NFκB signalling suggest that activities resulting in a shift in ROS abundance would be expected to have effects on NFκB signalling. In light of the research presented in this thesis, it seems likely that ROS generated through increased Irg1-driven fatty-acid β oxidation in activated macrophages responding to LPS would act on NFκB to promote the transcription of pro-inflammatory mediators. A similar mechanism is anticipated to be at play in adipose tissue macrophages, where NFκB activity was found to be significantly increased in a murine model of diet-induced obesity (FIG 7.1) (Carlsen et al., 2009).
FIGURE 7.1: Proposed model for Irg1-dependent propagation of inflammation in adipose tissue macrophages

Inflammatory cytokines and free fatty acids produced by hypertrophic adipocytes are hypothesised to activate expression of \( \text{irg1} \) and promote increased fatty acid \( \beta \) oxidation, leading to increased levels of \( \text{mROS} \). \( \text{mROS} \) is then anticipated to promote the expression of inflammatory cytokines through direct regulation of transcription and by promoting inflammatory NF\( \kappa \)B signaling. Together, these lead to propagation of an inflammatory environment within adipose tissue. Abbrev. CAC, citric acid cycle; ETC, electron transport chain; FFA, free fatty acids.

These data strongly support the positioning of Irg1 as a driver of an inflammatory phenotype within macrophage-lineage cells and indicate that this gene may be responsible, at least in part, for the chronic inflammation that underlies the propagation of obesity associated disorders. The specificity of Irg1 expression to macrophage-lineage cells under many circumstances suggest that targeting Irg1 may be a highly specific method of treating the inflammation associated with metabolic disease.
7.4.3 Depletion of Irg1 activity in macrophage-lineage cells responding to hindbrain injection of LPS reveals global trends towards impaired expression of genes related to the inflammatory response

While strong indications of deregulated NFκB signalling were identified through examination of genes exhibiting significant differential expression, gene enrichment analysis investigating the expression trends of all genes, whether differentially expressed or not, did not reveal any significantly enriched pathways or gene sets, though it did suggest a global trend towards impaired expression of inflammatory response genes. The lack of significance in this analysis may be reflective of the relatively poor genome annotation, as compared with other model systems such as the mouse or human, rather than poor strength of data. Additionally, the current existing annotation appears to carry a bias towards developmental pathways, introducing the possibility of overlooking key pathways.

Due to concerns over cell survival and the effect of extended stress resulting from a second sort, samples were only purified by a single pass through the FACS machine. While double-sorting is frequently performed to enhance the purity of samples collected by FACS, it is unlikely that single sorting would have contributed to significant contamination of the collected populations in this experiment, given that cells were collected from LPS-injected, Tg(irg1:EGFP)\textsuperscript{uz4}/Tg(mpeg1:mCherry)\textsuperscript{023} larvae based on expression of both the macrophage-specific mpeg1:mCherry transgene and activation-specific -4.7irg1:EGFP transgene. In the unlikely event of the presence of contaminating cells, it is not anticipated that they would contribute to the false identification of differentially-expressed sequences during transcriptome analysis, as the presence of irg1-targeting morpholinos in non-macrophage-lineage cells is not anticipated to have
any effect in these cells. These considerations support high confidence in the accuracy and significance of differentially-regulated sequences identified in this analysis.

The RNA-Seq experiment had been designed with two different irg1-targeting morpholino treatment groups as an additional control against the possibility of significant off-target morpholino effects. Upon analysis, larvae injected with IRG1SBMO1 produced no significantly altered transcripts when compared to the control group, while IRG1SBMO3-mediated depletion of Irg1 elicited changes in the expression of 99 different genes. This suggests that the IRG1SBMO1 splice-blocking morpholino did not sufficiently abrogate Irg1 activity in these samples. As the predicted protein from IRG1SBMO1-induced alternate splicing of the irg1 transcript is more truncated than that predicted from IRG1SBMO3 treatment, the production of truncated Irg1 containing a functional protein domain cannot account for the similarity of the control- and IRG1SBMO1-injected samples.

Upon closer examination of genes with significantly affected expression resulting from injection with IRG1SBMO3, the IRG1SBMO1-injected samples appeared to very closely mirror the expression levels seen in the control morpholino group in many instances, indicating no change. These data suggest that treatment with IRG1SBMO1 did not effectively deplete Irg1 function. As morpholinos act on a 1:1 molecular ratio (Moulton & Yan, 2008) it is unlikely that morpholino treatment will ever achieve total ablation of a targeted transcript due to variable gene expression levels or duration, potential feedback mechanisms that may lead to increased transcription during times of scarcity, and morpholino dilution over time as cells multiply. While morpholino dosage had been titrated earlier for toxicity and efficacy, as efficacy had been determine by RT-PCR, it is difficult to determine what is happening at the protein level. It may be that the
IRG1SBMO3 morpholino is simply more potent in vivo than IRG1SBMO1. Alternatively, rather than a simple loss of function, the unique composition of the truncated protein produced by IRG1SBMO3 could possibly confer a dominant negative effect. If true, this could result in an exaggeration of the effects of irg1 expression ablation.

It may be argued that, in light of the increasing accessibility of creating zebrafish knock-out or targeted mutant lines, morpholino treatment is outdated or potentially less valid in exploring gene functions (Schulte-Merker & Stainier, 2014). However, in this instance, for the examination of a gene induced in a highly specific population of cells within young larvae, it seems less likely for morpholino treatment to present with off-target effects or developmental aberrations.

7.4.4 Assessing similarly-regulated transcripts between IRG1SBMO1 and IRG1SBMO3-injected larvae, as compared to steady state response, following injection with LPS suggests a potential role for Irg1 in regulating expression of glycine N-methyltransferase

Examining the possibility that a lesser reduction in irg1 may still suggest downstream mediators, the most differentially-expressed genes between the IRG1SBMO1-treated and control-treated group were compared to those identified by comparison to the IRG1SBMO3 treatment. This revealed one gene, gnmt, which was not quite at the level of significance in the IRG1SBMO1 group (q value = 0.0508), but was significantly affected in the IRG1SBMO3 group (q value = 0.0098). This gene encodes glycine N-methyltransferase, an enzyme involved in cholesterol metabolism (C. Y. Chen et al., 2012). In both treatment groups, gnmt was more highly represented than in the control group. Loss of gnmt expression in macrophages has previously been linked to cholesterol accumulation, exacerbation of inflammation, and the progression of
atherosclerosis (C. Y. Chen et al., 2012). While it is challenging to interpret how a lack of irg1 induction may lead to increased gnmt expression, this does suggest that downregulation of gnmt may be a mechanism of reinforcing pro-inflammatory phenotype switching in macrophage-lineage cells.

7.5 A small molecule drug screen has identified compounds that may be used in the modulation of irg1 expression for therapeutic benefit

Assessing the effects of exogenously applied drug treatments on the expression of irg1 explored the possibility of using small molecules to modulate its expression in instances of disease where irg1 is known, or suspected, to contribute to pathogenesis.

7.5.1 Identification of compounds affecting irg1 expression within macrophage-lineage cells following stimulation with LPS reveals enrichment for compounds with anti-inflammatory, antifungal, and anti-bacterial properties

In this study, 20 compounds from a library of 1120 FDA-approved drugs were determined to have significant effects on irg1 expression following infection. Utilising a stepwise approach of increasing stringency, these 1120 drugs were first narrowed to a “first-pass” selection of the 146 most promising candidates. These were then further narrowed to the top 20 “second-pass” hits, and then the most promising four. This saw a proportional increase in the representation of anti-bacterial compounds (from 15-25%), anti-inflammatory compounds (from 8.1-15%), and antifungal compounds (from 2.9-15%) from the original library of 1120 to the 20 second pass hits. Given the infection-inducible nature of irg1, it is not unexpected that compounds with anti-inflammatory
properties may reduce its expression. While supporting the use of anti-inflammatory treatments in reducing \textit{irg1} expression, this trend also indicates that other compounds selected in the second pass hits may have unrecognised anti-inflammatory properties. With reference to the increasing proportion of anti-bacterial and anti-fungal compounds observed, growing data endorse a range of immunomodulating effects within these compounds (Ben-Ami et al., 2008; Kwiatkowska et al., 2013; Yamaguchi et al., 1993). Frequently, these effects are immunostimulatory, enhancing the intrinsic anti-microbial properties of the compound by improving recognition of the pathogen by immune effector cell.

The validity of inferring mechanisms of action from a group of drugs is strengthened by assessing structural similarities and determining the success rate of selecting multiple compounds from the same chemical family from those available within the library (Zon & Peterson, 2005). Compound clustering using structure-activity relationship mapping revealed that the top hit identified from this screen, chrysin, an antifungal, 5-lipoxygenase inhibitor, had similarities with six other compounds within the library. Of these, kaempferol, an anti-inflammatory topoisomerase I inhibitor, was also selected in the top four candidates, with a probability less than 0.02% of this selection occurring randomly when assessed using a hypergeometric distribution. These data suggest that further analysis of all the compounds contained within this cluster may indicate mechanisms of action through which chrysin and kaempferol may be acting to effect \textit{irg1} expression.
7.5.2 Post-transcriptional regulation is a potential mode of action used by a subset of top drug candidates to reduce \textit{irg1} expression

As reported previously, \textit{irg1} transcript stability varies in different circumstances, such as during the response to LPS as compared with the response to the macrophage-targeting pathogen \textit{Mycobacterium avium} subspecies \textit{paratuberculosis}, and likely acts as an additional regulator of \textit{Irg1} expression (Basler et al., 2006; Lee et al., 1995). Further evidence of post-transcriptional regulation of \textit{irg1} was observed through the small molecule screen performed as part of this thesis. The readout obtained by WMISH of drug-treated larvae two hours post-injection with LPS was compared with that of expression of the -4.7\textit{irg1}:EGFP transgene reporter at 16 hpi following drug treatment and LPS-injection. This revealed three compounds that displayed reduction or ablation of \textit{irg1} by ISH, yet distinct cellular expression of EGFP in \textit{Tg(irg1:EGFP)}\textit{nz4} transgenic larvae, suggesting that they affect Irg1 activity by reducing the stability of \textit{irg1} transcript. These compounds were chrysin, an anti-fungal and 5-lipoxygenase inhibitor; azlocillin sodium salt, an anti-bacterial transpeptidase inhibitor; and alclocetasone dipropionate, an anti-inflammatory glucocorticoid (Prestwick Chemical).

7.5.3 Chrysin is a strong candidate for the treatment of macrophage-driven inflammatory diseases

The drug screen performed in this thesis was designed with the hypothesis that compounds found to significantly reduce expression of \textit{irg1} would likely have promise in the treatment of disorders where macrophage activation contributes to chronic inflammation, as is seen in the adipose tissue macrophages of obese individuals. The top candidate identified from this screen was chrysin (5,7-dihydroxyflavone), which had been annotated by Prestwick Chemical as a 5-lipoxygenase inhibitor. Chrysin is a
naturally-occurring flavone that has received wide attention in recent years for its repeated identification as a potent anti-inflammatory compound acting through modification of macrophage effector functions (X. Feng et al., 2014; Ha et al., 2010). Most recently, Feng and colleagues assessed chrysin efficacy in the treatment of obese and high-fat feeding mice. They observed that chrysin reduced inflammation, reversed steatosis, limited macrophage infiltration into adipose tissue, and drove macrophages to adopt a more anti-inflammatory, M2-type phenotype (X. Feng et al., 2014). These data again support a role for Irg1 in the propagation of obesity-associated inflammation, while indicating the strength of chrysin in inhibiting this response and the significant effects on disease prognosis that may be made by targeting Irg1 for therapeutic modulation of macrophage phenotype. In their study, Feng et al. attributed this phenotype switching to increased expression of PPARγ, a transcription factor known to negatively regulate inflammatory cytokine production and mediate lipid storage (Siersbaek et al., 2010). Chrysin has also demonstrated potency in protecting neurons in a model of neuroinflammation (Gressa-Arribas 2010). Resulting again in inhibition of anti-inflammatory cytokine production, the researchers examining this model correlated chrysin’s effects to reduced expression and functionality of the C/ebpδ gene and protein. C/EBPδ is a transcription factor that is highly induced in microglia following exposure to inflammatory stimuli and has been implicated in the production of inflammatory compounds such as TNFα, IL-6, and IL-1β (Valente et al., 2013). There is also evidence that C/EBPδ and C/EBPβ, previously shown to be necessary for irg1 expression (Hall et al., 2013), compensate for each other in some environments (Tsukada et al., 2011). These data suggest that C/EBPδ may have a role in the induction of irg1 expression and that the anti-inflammatory effects of chrysin seen in a number of environments may be attributed, at least in part, to the prevention of irg1 expression.
The considerable array of research supporting chrysin’s anti-inflammatory effectivity in treating obesity-associated inflammation strengthens confidence in the design of this screen and the potential therapeutic uses of other top compounds. Of note among these, kaempferol, categorised by Prestwick Chemical as an anti-inflammatory with inhibitory activities against topoisomerase I, has also shown promise in reducing lipid accumulation within macrophages, suggesting applications to atherosclerosis where lipid-filled “foam” cells predominate (X. Y. Li et al., 2013), and the antifungal piperlongumine has demonstrated selective anti-cancer activities mediated by inducing oxidative stress within these cells (T. H. Kim et al., 2014).

7.6 Future directions

The key strength of the \textit{Tg(irg1:EGFP)nz4} transgenic zebrafish reporter line lies in its ability to report activation of macrophage-lineage cells in the context of many different immune responses and disease states. Thus, the production of a second line utilising either the mCherry or DsRed2 red fluorescent reporter driven by the -4.7\textit{irg1} promoter region would increase compatibility for compounding the observation of \textit{irg1} expression within existing transgenic lines, such as the \textit{Tg(Apo-E:GFP)} line that specifically marks microglia (Peri & Nusslein-Volhard, 2008).

The importance of observing \textit{irg1} in these contexts is to inform on the potential involvement of an Irg1-driven inflammatory macrophage phenotype in the propagation of disease. In the instance that Irg1 is subsequently identified as a contributor or driver of the disease phenotype, the next questions become how may Irg1 be reduced in this environment, and what are the consequences of this reduction? While the
*Tg(irg1:EGFP)* reporter line displays rapid induction of the -4.7irg1:EGFP transgene following promoter stimulation, its utility in reporting effects on *irg1* transcription beyond this first induction is limited by the ~26 h half-life of the EGFP reporter protein (Corish & Tyler-Smith, 1999; Verkhusha et al., 2003). Greater temporal resolution may be attained through the generation of a transgenic line driving a destabilised fluorescent reporter from the -4.7irg1 promoter. For example, the ZsGreen-Dr1 destabilised *Zoanthus sp.* green fluorescent protein is reported to have a half-life of only two hours (Clontech) (Corish & Tyler-Smith, 1999; Gubbels, Woessner, Mitchell, Ricci, & Brigande, 2008; X. Li et al., 1998). Use of a *Tg(irg1:ZsGreen-Dr1)* transgenic to explore disease environments where targeting transcription of *irg1* is an attractive therapeutic aim will allow *in vivo* observation of drug treatment effects on *irg1* expression and direct observation of the consequences of these treatments on the behaviours and phenotype of macrophage-lineage cells responding to the treatment.

A destabilized *irg1* reporter line would also allow the observation of priming and re-stimulation challenges and investigation into the potential roles Irg1 may have in the development of “trained memory” in macrophage-lineage cells. The possibility of such a role has been suggested by the observation that IRG1-driven mROS expression in mice following priming and re-challenge with LPS is correlated with a paradoxical reduction in inflammatory cytokine production and the development of endotoxin tolerance (Y. Li et al., 2013). The concept of innate immune memory is relatively recent and has reported observations of learned behaviours in macrophages that are induced through priming events leading to epigenetic changes and altered subsequent response, as when compared to naive cells (Saeed et al., 2014). Considering the dramatic changes in Irg1 associations when murine macrophages were rechallenged with LPS (Y. Li et al., 2013), it would be of interest to assess the effects on macrophage behaviours and “memory” when
expression of *irg1* has been reduced or ablated. In addition, the simple *in vivo* observation of macrophage behaviours following successive stimulations may provide unique insights, especially when performed in a compound *Tg(irg1:ZsGreen-Dr1)/Tg(irg1:DsRed2)* transgenic, for example, which allows *in vivo*, live-imaging analysis of the effects of priming events on macrophage phenotypes by monitoring changes in the expression of ZsGreen-Dr1. Following an initial LPS stimulation, activated macrophage-lineage cells will express both DsRed2 and ZsGreen-Dr1. The rapid degradation of the ZsGreen-Dr1 protein will lead to DsRed2+ZsGreen-Dr1- macrophage-lineage cells, indicating these cells have been primed. It is expected that this would accurately reflect the *irg1* transcript degradation seen in LPS-injected larvae, as 2 hpi has been determined as the point of peak *irg1* transcript expression, after which transcript levels drop sharply, returning to steady state levels by 4 hpi. Primed macrophage-lineage cells can then be observed during a second stimulation event to determine whether or not they all re-initiate *irg1* expression, indicated by induction of ZsGreen-Dr1 within DsRed2+ cells, whether or not they respond more rapidly to the stimulus as compared with cells activating for the first time, and whether they display unique behaviours. These different populations may also be collected by FACS for genetic analysis to determine evidence of epigenetic regulation or transcriptome differences within each of these scenarios.

This thesis has also reported strong evidence placing Irg1 as a key driver of obesity-associated inflammation in adipose tissue macrophages (ATMs) by regulating the expression of the adipokines TNFα and IL-1β. Crucial to solidifying this connection is the direct observation of *irg1* expression in adipose tissue macrophages and assessing the effect of blocking *irg1* on the phenotype of these cells and the progression of disease. This is possible *in vivo* by applying the diet-induced-obesity (DIO) protocol to
Tg(irg1:EGFP)/Tg(mpeg1:mCherry) double transgenic zebrafish developed in a casper (natural albino mutant) background (Oka et al., 2010; L. Zhang et al., 2012). As the DIO protocol develops obesity in adult zebrafish, the transparency of the casper albino mutant is required to facilitate in vivo imaging at this late stage. Use of both the -4.7irg1:EGFP and mpeg1:mCherry transgenes would allow observation of all macrophage-lineage cells in the body to determine the proportion of macrophage-lineage cells affected by the developing adiposity over time and for comparison of the transcriptomes of irg1-expressing with irg1 non-expressing macrophages from dissected adipose tissues. A second approach to studying the role of irg1 in ATMs would be to apply the DIO protocol to a zebrafish irg1 mutant line and determine effects on the development of obesity. Irg1-modulating small molecules may then be applied to these models to determine their therapeutic potential, should Irg1 be confirmed as a driver of obesity associated inflammation. The anticipated relevance to human health of discoveries made in these models would then necessitate extrapolation to mammalian cell lines and disease models.

Further validation of results obtained through the RNA-Seq experiment described in this thesis are also required. The development of a loss-of-function irg1 mutant zebrafish line could provide a more robust means of knocking down Irg1 activity than the morpholino-based approach used in this study. Assessing the transcription of key genes identified through the RNA-Seq analysis via qPCR of macrophage-lineage cells from an irg1 mutant line is expected to strongly recapitulate the results reported here, such as reduced expression of TNFα and IL-1β and impaired NFκB activity. An irg1 mutant may also provide insight into the potential developmental role of irg1 in the neural development of zebrafish larvae, building from the observation of weak -4.7irg1:EGFP transgene expression in non-stimulated larvae at 2 dpf. A loss-of-function irg1 mutant
line may result in defective embryo development. If this proves true, alternate approaches to the knockdown/knockout of \textit{irg1} expression may need to be considered in order to validate RNA-Seq data. For example, a conditional knockout using cre-lox technology may be developed to selectively ablate Irg1 activity from macrophage-lineage cells, leaving the potential developmental roles of \textit{irg1} intact.

The \textit{Tg(irg1:EGFP)nz4} transgenic zebrafish line has demonstrated the ability to live image macrophage activation in response to multiple infection-associated stimuli. In order to reach the full potential of this model in comparing the functions of activated with non-activated macrophage-lineage cells during an immune response, optimised conditions for the differentiation of these populations must be further explored. These include assessing a wider range of immunogens for modelling infection, methods for attenuating dose, and models/genetic backgrounds for further delineating cell types. Also, as these studies were performed in larval zebrafish beginning from 2 dpf, characterisation in older larvae is required to determine how activated populations may segregate once macrophage-lineage cells have fully matured. This would also allow more accurate dissection of the functional responses of tissue-resident versus recruited, monocyte-derived macrophages, especially in combination with zebrafish lines like the \textit{Tg(Apo-E:GFP)} transgenic reporter line labelling microglia or the xpr1b mutant, carrying a loss-of-function mutation in the xenotropic polytropic virus receptor 1 phosphate exporter, that fails to develop tissue-resident macrophage populations (Meireles et al., 2014; Peri & Nusslein-Volhard, 2008). Studies in the mouse have reported that the Irg1\textsuperscript{+} macrophages that invade the brain contribute to neurotoxicity, compared with the neuroprotective phenotype of the Irg1\textsuperscript{-} resident microglia (H. Li et al., 2006). If the \textit{Tg(irg1:EGFP)nz4} transgenic zebrafish supports these findings, fluorescent labelling will allow the isolation and analysis of these cells as well as \textit{in vivo} observation of their
relative invasive, phagocytic, and migratory behaviours. Assessing treatments for the selective reduction of \textit{irg1} expression in invading macrophages or attenuation of the invasive abilities of this population may present potential options for preventing the initiation of neuroinflammation in some instances.
8 Appendices

Appendix 1: Protocol for WMISH on 2 dpf zebrafish larvae using irg1 antisense probe in the Intavis InsituPro VSI Robot

********** method-listing: WISH zebrafish Crosier.IPM **********
********** path: C:\INTAVIS\InsituPro VSI 3.1\Methods\Methods Whole mounts & vibratome sections\Medium baskets\
** 14.08.2014 - 14:19:20
** Software : InsituProVSI
** Version : 08.10.2012
********** configuration: insituPro VSI-VT.IPC **********
********** path: C:\INTAVIS\InsituPro VSI 3.1

********** ROBOTER : 223v4.2.1 , ID: 10
Rinse
Home : X:260 Y:205 Z:0 F:0  0.1mm
Drain : X:260 Y:205 Z:300 F:300  0.1mm
Rinse : X:260 Y:550 Z:1080 F:1080 0.1mm

********** DILUTOR : 402 , ID: 0
Configuration
dilutor syringe : 10000
transfurttubes : 10100 ul
transfurttube - 2 by valve at ->
roboter output nr : 3
nr
Pipeting
reservoir aspiration speed : 30 ml/min
prime (dispense) speed : 72 ml/min
aspiration speed LOW : 10 ml/min
aspiration speed MEDIUM : 15 ml/min
aspiration speed HIGH : 20 ml/min
dispense speed LOW : 10 ml/min
dispense speed MEDIUM : 15 ml/min
dispense speed HIGH : 20 ml/min

inside rinse vol. NORMAL : 700 µl
inside rinse vol. INTENSIVE : 1400 µl
outside rinse vol. NORMAL : 700 µl
outside rinse vol. INTENSIVE : 1400 µl
rinse dispense speed LOW : 20 ml/min
rinse dispense speed HIGH : 30 ml/min
(System: Baskets M)

********** THERMOCONTR 610 , ID: 1
Contacts
output cool-fan : 4 1.4

********** method

1 Module Starting Method
1.1 XYZCheck
1.2 SetTempReg to : OFF
1.3 PrimaNeedle 12000
1.4 PrimePort 10000 Port 1->Drain
...
2 Module Rehydration
2.1 IncubateVT 00:05 500µl 50% MeOH/PBST->Specimen
2.2 IncubateVT 00:05 500µl PBST->Specimen
...
3 Module Prot.K max. 24h max.batch:28
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<th>Module</th>
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<th>Duration/Condition</th>
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</tr>
<tr>
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<td></td>
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********** end of method *** ***************
Appendix 2: pTol2linkerswitch plasmid map
Appendix 3: pTol2linkerswitch-irg1 plasmid map
Appendix 4: Macrophages within Tg(mpeg1:EGFP)µZ2 zebrafish larvae respond to cancer xenograft by expressing irg1 and engulfing cancer cells, as observed using WMISH

A. MDA-MB-231 metastatic breast cancer cells were stained with CellTracker Green, to enable fluorescent imaging, prior to resuspension in matrigel and injection into the perivitelline space of 2 dpf Tg(mpeg1:EGFP)µZ2 larvae. B. Larvae injected with matrigel alone demonstrated no expression of irg1. C. By 4 hpi, strong irg1 expression was seen in the perivetelline space and caudal haematopoietic tissue region, with expression noted in the head and trunk as well. D. By 8 hpi, irg1 expression had restricted to the perivitelline space. E-E". Fluorescent WMISH using antisense probe to detect irg1 (AlexaFluor 555) and anti-GFP antibodies (AlexaFluor 488, psuedo coloured in E") to detect mpeg1:EGFP transgene-expressing macrophages demonstrated colocalisation of irg1 within Mpeg1+ macrophages that surrounded the MDA-MB-231 cancer cells, as identified by staining with CellTracker Green, suggesting phagocytosis of the cancer cells by activated macrophages at 4 hpi. Abbrev. hpi, hours post-injection. Larvae oriented anterior to left. Scale bar 100 μm in B. Numbers indicate the proportions of larvae that displayed the presented phenotype. (Christopher Hall, unpublished data)
Appendix 5: Differentially expressed transcripts in activated macrophages following injection with IRG1SBMO3 and stimulation with hindbrain injection of LPS, as compared with similarly stimulated macrophages from CTRLMO-injected larvae

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<th>q value</th>
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Inf = infinite. Infinite values indicate a transcript that was not detected in one of the treatment groups.
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Inf = infinite. Infinite values indicate a transcript that was not detected in one of the treatment groups.
## Appendix 6: Drug screen timeline

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<th>Drugs Weeks</th>
<th>Workflow</th>
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<td>1 set of ctrls/25 drugs</td>
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<tr>
<td>1 ctrl/drug</td>
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### Appendix 7: 1st pass hits from irg1 drug screen

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<td>Dorzolamide hydrochloride</td>
<td></td>
<td>Antiglaucoma agent</td>
</tr>
<tr>
<td>Reduce</td>
<td>Trichloron</td>
<td>Cholinesterase inhibitor</td>
<td>Anticholinergic, insecticide</td>
</tr>
<tr>
<td>Reduce</td>
<td>Brompheniramine maleate</td>
<td>H1 Antagonist</td>
<td>Antihistaminic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Chlorpheniramine maleate</td>
<td>H1 antagonist, Anticholinergic</td>
<td>Antihistaminic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Hydralazine hydrochloride</td>
<td>Adrenergic antagonist</td>
<td>Antihypertensive</td>
</tr>
<tr>
<td>Reduce</td>
<td>Pindolol</td>
<td>K+ channel Ca++ dependent activator</td>
<td>Antihyperpertensive</td>
</tr>
<tr>
<td>Reduce</td>
<td>Alprazolam hydrochloride</td>
<td>Beta2 antagonist, beta2 antagonist</td>
<td>Antihyperpertensive, antianginal, antiarrhythmic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Oxyazolol hydrochloride</td>
<td>Blocking beta-adrenergic receptors</td>
<td>Antihyperpertensive, Antianginal, Antiarrhythmic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Hydroflumethalide</td>
<td>Norepinephrine transport inhibitor</td>
<td>Antihyperpertensive, Diuretic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Timolol maleate salt</td>
<td></td>
<td>Antihyperpertensive, Antianginal agent</td>
</tr>
<tr>
<td>Reduce</td>
<td>Propyphenazone</td>
<td>Anorexiant</td>
<td>Antihyperthyroid</td>
</tr>
<tr>
<td>Reduce</td>
<td>Acetylsalicylic acid</td>
<td>Cyclooxygenase inhibitor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Acetylsalicylic acid</td>
<td>Cyclooxygenase inhibitor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Aldometaplatinid brosopionate</td>
<td>Corticoid</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Amiopine</td>
<td>ACTH secretor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Ajagmin</td>
<td>MAP kinase inhibitor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Buseonide</td>
<td>-</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Dexamethasone acetate</td>
<td></td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Harpagozide</td>
<td></td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Mometasone furoate</td>
<td></td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Pataniban</td>
<td>MAP kinase inhibitor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Taprotenic acid</td>
<td>Cyclooxygenase inhibitor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Reduce</td>
<td>Trimetolone</td>
<td></td>
<td>Antiinflammatory</td>
</tr>
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# Appendix 7: 1st pass hits from irg1 drug screen, continued

<table>
<thead>
<tr>
<th>Reduce</th>
<th>Kaempferol</th>
<th>Topoisomerase I inhibitor, Tyrosin kinase, Xanthin oxidase</th>
<th>Antinflammatory, diuretic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduce</td>
<td>Artemisinin</td>
<td>Oxidant</td>
<td>Antimalarial, immunosuppressive, Anticancer</td>
</tr>
<tr>
<td>Reduce</td>
<td>Rondarone</td>
<td>Antimicrobial</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Letrozole</td>
<td>Antineoplastic</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Betulin</td>
<td>Antineoplastic, Antinflammatory</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Carcinine</td>
<td>Antioxidant, Vasodilator</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Cotrimoxan</td>
<td>Antipyrin (VET)</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Brompropindol</td>
<td>Dopamine antagonist</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Holperidol</td>
<td>Dopamine antagonist, dopamine antagonist</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Promazine hydrochloride</td>
<td>Dopamine receptor antagonist</td>
<td>Antipsychotic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Sulpiride</td>
<td>D2 antagonist</td>
<td>Antipsychotic, antidepressant, antiepileptic</td>
</tr>
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<td>Reduce</td>
<td>Acetaminophen</td>
<td>Cyclooxygenase inhibitor</td>
<td>Antipyretic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Lycorene hydrochloride</td>
<td>Inhibitor of protein translation</td>
<td>Antitumoral, antiviral</td>
</tr>
<tr>
<td>Reduce</td>
<td>Norcyclobenzprine</td>
<td>-</td>
<td>Antilucenecive</td>
</tr>
<tr>
<td>Reduce</td>
<td>Propylthiouracil</td>
<td>-</td>
<td>Antiviral</td>
</tr>
<tr>
<td>Reduce</td>
<td>Saquinavir mesylate</td>
<td>Protease inhibitor</td>
<td>Artiviral (HIV)</td>
</tr>
<tr>
<td>Reduce</td>
<td>Lovopine succinate</td>
<td>Dopamine antagonist</td>
<td>Anxiolytic, antipsychotic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Bucladesine sodium salt</td>
<td>Adenylate cyclase modulator</td>
<td>Carotidion</td>
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<td>Reduce</td>
<td>Tacrine hydrochloride hydrate</td>
<td>Cholinesterase inhibitor</td>
<td>Cognition enhancer</td>
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<tr>
<td>Reduce</td>
<td>Iodixanol</td>
<td>Na+ Cl- transport inhibitor</td>
<td>Diagnostic aid (radioopaque medium)</td>
</tr>
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<td>Reduce</td>
<td>Trimetholmethadine</td>
<td>-</td>
<td>Diuretic, antihypertensive</td>
</tr>
<tr>
<td>Reduce</td>
<td>Equin</td>
<td>-</td>
<td>Estrogen</td>
</tr>
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<td>Reduce</td>
<td>Ethylnestradiol 3-methyl ether</td>
<td>-</td>
<td>Estrogen</td>
</tr>
<tr>
<td>Reduce</td>
<td>Hexestrol</td>
<td>Nuclear receptor ligand</td>
<td>Estrogen antihepatic</td>
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<tr>
<td>Reduce</td>
<td>Betazole hydrochloride</td>
<td>Histamine analog</td>
<td>Gastric secretion stimulant</td>
</tr>
<tr>
<td>Reduce</td>
<td>Corticosterone</td>
<td>Glucocorticoid</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Humethasone</td>
<td>Glucocorticoid</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Methylenebisolone, 6-alpha</td>
<td>-</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>Reduce</td>
<td>Prenolone</td>
<td>Glucocorticoid</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Aminopenic acid</td>
<td>-</td>
<td>Hemostatic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Piriloco mesylate</td>
<td>Highly selective reversible inhibitor of monoamine oxidase type A</td>
<td>Highly selective reversible inhibitor of monoamine oxidase type A, Antidepressant</td>
</tr>
<tr>
<td>Reduce</td>
<td>Methyletoe nitrate</td>
<td>-</td>
<td>In presenile medication, Myoclonic, Antipsomodic, Antidote to organophosphorus insecticides (VET)</td>
</tr>
<tr>
<td>Reduce</td>
<td>Methanethol bromide</td>
<td>-</td>
<td>In treatment of urinary incontinence, Antipsomodic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Retinol acid</td>
<td>Keratolytic</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Benoxate hydrochloride</td>
<td>Na+ channel blocker</td>
<td>Local anesthetic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Oxythasine</td>
<td>Na+ channel blocker</td>
<td>Local anesthetic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Chlorotransanine</td>
<td>Nuclear receptor ligand</td>
<td>Non-steroidal estrogen</td>
</tr>
<tr>
<td>Reduce</td>
<td>Pantoprazol calcium salt</td>
<td>-</td>
<td>Nutritional factor</td>
</tr>
<tr>
<td>Reduce</td>
<td>Gabexate mesilate</td>
<td>Enzyme inhibitor (proteinase)</td>
<td>Severe acute pancreatitis, Anticoagulant</td>
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<tr>
<td>Reduce</td>
<td>Isometheptene mucate</td>
<td>Adrenergic</td>
<td>Symptommetric (VET), Antispasmodic for gut and urinary tract (VET)</td>
</tr>
<tr>
<td>Reduce</td>
<td>Lymecycline</td>
<td>-</td>
<td>Tetracycline antibiotic</td>
</tr>
<tr>
<td>Reduce</td>
<td>Natropodi dimydrochloride</td>
<td>Alpha agonist</td>
<td>Treatment of symptomatic benign prostate hypertrophy, Antihypertensive, Hypertension screen</td>
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<tr>
<td>Reduce</td>
<td>Dimobenzene</td>
<td>-</td>
<td>Ultraviolet screen</td>
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<tr>
<td>Reduce</td>
<td>Aminopenylline</td>
<td>Mastocytes degranulation inhibitor,-Benzodiazepines agonist</td>
<td>Vasodilator</td>
</tr>
<tr>
<td>Reduce</td>
<td>Isoxsuprine hydrochloride</td>
<td>beta adrenergic agonist</td>
<td>Vasodilator</td>
</tr>
<tr>
<td>Reduce</td>
<td>Piribedil hydrochloride</td>
<td>Vasodilator</td>
<td>Vasodilator</td>
</tr>
<tr>
<td>Reduce</td>
<td>Albendazole</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reduce</td>
<td>Benperidol</td>
<td>5-HT antagonist</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Hydroxyacrine malene (R,S)</td>
<td>Acetylcholine external inhibitor</td>
<td></td>
</tr>
<tr>
<td>Reduce</td>
<td>Methylnitratein-5-(D)</td>
<td>-</td>
<td></td>
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<tr>
<td>Increase</td>
<td>Antrodarone hydrochloride</td>
<td>Na+ channel blocker, K+ channel blocker, Non-competitive beta-adrenergic blocker</td>
<td>Antidysrhythm</td>
</tr>
<tr>
<td>Increase</td>
<td>Benzopenicillin sodium</td>
<td>-</td>
<td>Antibacterial</td>
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<tr>
<td>Increase</td>
<td>Cycloheximide</td>
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<td>Antibacterial</td>
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<tr>
<td>Increase</td>
<td>Josamincin</td>
<td>Ribosomal protein synthase inhibitor</td>
<td>Antibacterial</td>
</tr>
<tr>
<td>Increase</td>
<td>Methacycline hydrochloride</td>
<td>-</td>
<td>Antibacterial</td>
</tr>
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### Appendix 7: 1st pass hits from *irg1* drug screen, continued

<table>
<thead>
<tr>
<th>Increase</th>
<th>Drug Name</th>
<th>Effect</th>
<th>Category</th>
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</thead>
<tbody>
<tr>
<td>Increase</td>
<td>Novobiocin sodium salt</td>
<td>DNA topoisomerase IV inhibitor</td>
<td>Antibacterial</td>
</tr>
<tr>
<td>Increase</td>
<td>Piperacillin sodium salt</td>
<td>Bacterial transpeptidase inhibitor</td>
<td>Antibacterial</td>
</tr>
<tr>
<td>Increase</td>
<td>Sulfamethazine sodium salt</td>
<td>Inhibitor of folic acid synthesis</td>
<td>Antibacterial</td>
</tr>
<tr>
<td>Increase</td>
<td>(S)-(-)-Cycloserine</td>
<td></td>
<td>Antibacterial, Tuberculosis</td>
</tr>
<tr>
<td>Increase</td>
<td>Econazole nitrate</td>
<td>Ergosterol synthesis inhibition</td>
<td>Antifungal</td>
</tr>
<tr>
<td>Increase</td>
<td>Orphenadrine hydrochloride</td>
<td>H1 antagonist</td>
<td>Antihistaminic</td>
</tr>
<tr>
<td>Increase</td>
<td>Orphenadrine hydrochloride</td>
<td>H1 antagonist</td>
<td>Antihistaminic, muscle relaxant</td>
</tr>
<tr>
<td>Increase</td>
<td>Lovastatin</td>
<td>HMG CoA reductase inhibitor</td>
<td>Antihypercholesterolemic</td>
</tr>
<tr>
<td>Increase</td>
<td>Simvastatin</td>
<td>HMG-CoA reductase inhibitor</td>
<td>Antihyperlipoproteinemic</td>
</tr>
<tr>
<td>Increase</td>
<td>Fluvastatin sodium salt</td>
<td>HMG CoA reductase inhibitor</td>
<td>Antihyperlipoproteinemic</td>
</tr>
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<td>Increase</td>
<td>(S)-propranolol hydrochloride</td>
<td>Beta-adrenergic blocking agent</td>
<td>Antihypertensive</td>
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<td>Increase</td>
<td>(S)-Naproxen sodium salt</td>
<td>Cyclooxygenase inhibitor</td>
<td>Antiinflammatory</td>
</tr>
<tr>
<td>Increase</td>
<td>Finasteride</td>
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<td>Antiinflammatory</td>
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<tr>
<td>Increase</td>
<td>Suprofen</td>
<td>Cyclooxygenase inhibitor</td>
<td>Antimicrobial</td>
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<td>Increase</td>
<td>Cloperastine hydrochloride</td>
<td>Histamine antagonist</td>
<td>Antitussive</td>
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<tr>
<td>Increase</td>
<td>Omeprazole</td>
<td>Non competitive ATPase H+ pump inhibitor</td>
<td>Antulcerative</td>
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<tr>
<td>Increase</td>
<td>Digoxin</td>
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<td>Cardiotonic</td>
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<tr>
<td>Increase</td>
<td>Antrimone</td>
<td>Phosphodiesterase inhibitor III, Inotropic, TNF production inhibitor</td>
<td>Cardiotonic, Antischemic</td>
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<tr>
<td>Increase</td>
<td>Demecarium bromide</td>
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<td>Cholinergic (ophtalmic)</td>
</tr>
<tr>
<td>Increase</td>
<td>Guafenesin</td>
<td></td>
<td>Expectorant, Bronchodilator</td>
</tr>
<tr>
<td>Increase</td>
<td>Hydrocotamine hydrobromide</td>
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<td>Hemostatic</td>
</tr>
<tr>
<td>Increase</td>
<td>Tetrahydroxy-3,4-quinone monohydrate</td>
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<td>Keratolytic</td>
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<tr>
<td>Increase</td>
<td>(-)-Eseroline fumarate salt</td>
<td>Anti-acetylcholinesterase activity, Opiate agonist activity</td>
<td>Potent analgesic</td>
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<tr>
<td>Increase</td>
<td>Tribenoside</td>
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<td>Sclerosis agent</td>
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<tr>
<td>Increase</td>
<td>Phthalylsulfathiazole</td>
<td>Inhibitor of folic acid synthesis</td>
<td>Sulfonamide antibacterial</td>
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<td>Increase</td>
<td>Nitrofurantoin</td>
<td>Bacterial DNA damage</td>
<td>Urinary antiseptic</td>
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<tr>
<td>Increase</td>
<td>Alprostadil</td>
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<td>Vasodilator</td>
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<tr>
<td>Increase</td>
<td>Yohimbine hydrochloride</td>
<td>Alpha antagonist</td>
<td>Vasodilator</td>
</tr>
<tr>
<td>Increase</td>
<td>Arcaine sulphate</td>
<td>Lowers blood sugar</td>
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<tr>
<td>Increase</td>
<td>Tropine</td>
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References


doi:10.1242/dev.012385


doi:10.1016/j.cmet.2012.05.008


dysfunction in alzheimer's disease and related neurodegenerative disorders. *Current
Alzheimer Research, 9*(1), 5-17.

obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults.
*The New England Journal of Medicine, 348*(17), 1625-1638.
doi:10.1056/NEJMoa021423

mediate tissue-specific and microbial control of angptl4/fiaf transcription. *PLoS
Genetics, 8*(3), e1002585. doi:10.1371/journal.pgen.1002585

Carlsen, H., Haugen, F., Zadelaar, S., Kleemann, R., Kooistra, T., Drevon, C. A., & Blomhoff,

Cavalla, D. (2013). Predictive methods in drug repurposing: Gold mine or just a bigger

Chanmee, T., Ontong, P., Konno, K., & Itano, N. (2014). Tumor-associated macrophages as
major players in the tumor microenvironment. *Cancers, 6*(3), 1670-1690.
doi:10.3390/cancers6031670

doi:10.1038/nri3071


susceptibility to infection in the brain by positive-stranded RNA viruses. *Nature Medicine, 19*(4), 458-464. doi:10.1038/nm.3108


266


Joung, J. K., & Sander, J. D. (2013). TALENs: A widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology, 14*(1), 49-55. doi:10.1038/nrm3486

kappaB activation: Distinct redox regulation between the cytoplasm and the nucleus. 
Antioxidants & Redox Signaling, 7(3-4), 395-403. doi:10.1089/ars.2005.7.395

Kalderon, B., Mayorek, N., Berry, E., Zevit, N., & Bar-Tana, J. (2000). Fatty acid cycling in 
the fasting rat. American Journal of Physiology, Endocrinology and Metabolism, 
279(1), E221-7.

species promote TNFalpha-induced death and sustained JNK activation by inhibiting 

simulated blast overpressure: Possible role of pulse duration. Neuroscience Letters, 
522(1), 47-51. doi:10.1016/j.neulet.2012.06.012

F. (2011). Microbial colonization induces dynamic temporal and spatial patterns of 
NF-kappaB activation in the zebrafish digestive tract. Gastroenterology, 141(1), 197- 
207. doi:10.1053/j.gastro.2011.03.042

Kawakami, K., Takeda, H., Kawakami, N., Kobayashi, M., Matsuda, N., & Mishina, M. 
(2004). A transposon-mediated gene trap approach identifies developmentally 
regulated genes in zebrafish. Developmental Cell, 7(1), 133-144. 
doi:10.1016/j.devcel.2004.06.005


factor/osteoprotegerin. Biochemical and Biophysical Research Communications, 247(3), 610-615. doi:S0006291X98986971


doi:10.1128/MCB.01152-07

doi:10.1101/cshperspect.a000034

doi:10.1002/dvdy.22519


Orkin, S. H. (2000). Diversification of haematopoietic stem cells to specific lineages. *Nature Reviews Genetics, 1*(1), 57-64. doi:10.1038/35049577


inflammation. *Cell Motility and the Cytoskeleton, 63*(7), 415-422.
doi:10.1002/cm.20133

Reed-Inderbitzin, E., Moreno-Miralles, I., Vanden-Eynden, S. K., Xie, J., Lutterbach, B.,
deacetylases and SUV39H1 to repress transcription. *Oncogene, 25*(42), 5777-5786.
doi:1209591

3976-3978. doi:blood-2006-05-024075


Richendrfer, H., & Creton, R. (2013). Automated high-throughput behavioral analyses in
zebrafish larvae. *Journal of Visualized Experiments, (77)*:e50622. doi:10.3791/50622

Zebrafish screen identifies novel compound with selective toxicity against leukemia.
*Blood, 119*(24), 5621-5631. doi:10.1182/blood-2011-12-398818

(2010). Zebrafish behavioral profiling links drugs to biological targets and
rest/wake regulation. *Science, 327*(5963), 348-351. doi:10.1126/science.1183090

factor cyclic AMP response element binding protein (CREB) in macrophages
following infection with pathogenic and nonpathogenic mycobacteria and role for


*Nature Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019


