The promise of zebrafish: a novel resource to study the myeloid response *in vivo*



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Abstract

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Oncogenic transformation, local tissue damage at a site of injury, or invading pathogens all induce signals resulting in the recruitment of macrophages and neutrophils to the inciting stimulus. This study describes the development of an innovative binary approach in a zebrafish animal model that enables genome-wide profiling of specific cell types isolated directly from the *in vivo* context. The modular system harnesses the power of zebrafish genetics using an *in vivo* biotinylation system consisting of a zebrafish line ubiquitously expressing a nuclear envelope with an Avi-tag. The Avi-tag is biotinylated by the biotin ligase, BirA, specifically expressed by macrophages and neutrophils, and subsequently the nuclei of these cells types can be isolated by streptavidin affinity purification. The model was used to profile the active transcriptome of neutrophils in two disease pathologies, *Mycobacterium marinum* infection and the earliest stages of melanoma. For studies of the immune response to *M. marinum* infection, a granuloma-forming model was optimised, which recapitulates human tuberculosis. Profiling revealed a unique transcriptional signature for neutrophils, characterised by both factors that are host protective and those that may contribute to the pathogenesis of the disease. For studies of melanoma onset, we have generated a novel inducible model for melanocyte oncogenic transformation. The model for melanoma onset was crossed to the binary neutrophil-specific *in vivo* biotinylation model. Profiling of the neutrophils in response to oncogene-transformed melanocytes revealed a neutrophil transcriptional signature consistent with a pleiotropic role for neutrophils at the onset of melanoma. Not only does the model generated in this study prove to be an excellent resource for studying inflammation *in vivo*, but the data obtained provides novels insights into the role of neutrophils in mycobacterium infection and the onset of melanoma, that can be extended to human studies and harnessed for immunotherapy.

List of Abbreviations

atf3	Activating transcription factor
BAC	Bacterial artificial chromosome
BPI	Bactericidal permeability-increasing protein
CFU	Colony forming units
CR	Complement receptor
CRISP	Cysteine-rich secretory protein
CTLA	Cytotoxic T-lymphocyte-associated protein
DC	Dendritic cell
dpf	days post fertilisation
dpi	days post infection
dtc	Dopachrome tautomerase
DUOX	Dual oxidase
ECM	Extracellular matrix
Edu	5'-ethynyl-2'-deoxyuradine
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FACS	Fluorescent activated cell sorting
fMLP	Formyl-methionyl-leucyl-phenalalanine
Frt	Flippase recognition target
GDP	Guanosine diphosphate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPC-2	Granulocyte chemotactic protein-2
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HGF	Hepatocyte growth factor
HNP	Human neutrophil peptides
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
HOI	Hypoiodous acid
hpf	Hours post fertilisation
HRMA	High resolution melt analysis
ICAM	Intracellular adhesion molecule
ICM	Intermediate cell mass
IDO	Indoleamine dioxygenase
IFN	Inteferon
IL	Interleukin
ITGAM	Integrin Alpha M
LCM	Laser capture microdissection
lectl1	leukocyte cell-derived chemotaxin 2-like
LGP	Laboratory genetic physiology
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MDA	Melanoma differentiation-associated
MHC	Major histocompatibility complex

mitfa	Micropthalmia-associated transcription factor
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mxc	myxovirus
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NGAL	Neutrophil gelatinase associated lypocalin
NGS	Next Generation sequencing
NK	Natural Killer
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding-oligomerisation-domain
NOS	Nitric oxide synthase
OP	operon
ORF	Open reading frame
OSM	Oncostatin M
PaiI	Plasminogen activator inhibitor type I
PD	Programmed cell death
PDGF	Platelet-derived growth factor
PGE	Prostaglandin
PIGF	Placental growth factor
PRR	Pattern recognition receptor
RFP	Red fluorescent protein
RIG	Retanoic acid-inducible gene (RIG)
RLR	Rig-I-like receptor
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SA	Splice acceptor
sdf2l1	stromal cell-derived factor 2-like 1
sgRNA	Single guide RNA
SPARC	Secreted protein acidic and rich in cysteine
steap4	Six-transmembrane epithelial antigen of prostate
TAM	Tumour-associated macrophage
TANs	Tumour-associated neutrophils
TB	Tuberculosis
TCR	T cell receptor
TGF	Transforming growth factor
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tnip2	TNFAIP3 interacting protein
TP	Thymadine phosphorylase
UAS	upstream activating sequence
VEGF	Vascular endothelial growth factor
Wildtype	WT
YSL	Yolk syncytial layer

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Chapter 1

Introduction

1.1 The resolution of inflammation

Inflammation is vital for host defence against invading pathogens, for wound and tissue repair. Inflammation, can also be triggered by sterile stimuli which may be physical, chemical or metabolic[1]. Ideally, in a general response to inflammation, a cascade of signals leads to the recruitment of neutrophils to infected/damaged sites where they serve to neutralise and eliminate stimuli[2]. Early in the inflammatory response pro-inflammatory mediators are released including cytokines, chemokines and cell adhesion molecules[1]. Neutrophils are then disposed of by local death and phagocytosis by macrophages. Macrophages then subsequently leave the tissue via the draining lymphatics. As the inflammation progresses lymphocytes and other inflammatory cells are recruited and activated at the site of the inflammation and are all beneficial to the host in aiding tissue repair. Inflammatory signals and chemokine gradients subsequently revert back to normal, thereby preventing the further infiltration of immune cells. Ideally the resolution of inflammation ensues and normal tissue homeostasis returns[2].

Dysregulation of inflammation represents a central pathogenic feature of many life-threatening conditions including microbial diseases such as tuberculosis and diseases typical of sterile tissue damage such as atherosclerosis, obesity, cancer, asthma, inflammatory bowel disease and rheumatoid arthritis[3]. For example, in tuberculosis, the host inflammatory response often fails to clear the bacteria. Inflammation can stall for many years and immune cells not only provide a niche in which the bacteria can

proliferate and survive, but tissue damage from the host immune cells drives the pathogenesis of the disease[3]. In some cancers, an oncogenic change induces an inflammatory environment that may promote the development of cancers[4]. Thus, a large proportion of the global burden of disease involves non-resolving inflammation, exacerbated, at least in part, by the first responders, the myeloid component. It is thus essential to gain a full understanding of the mechanisms by which myeloid cells contribute to both sterile and non-sterile inflammatory pathologies.

1.2 Innate myeloid cells

Innate immunity is found in all classes of plants and animals and refers to the cells and mechanisms that provide the first line of defence from infection, occurring in a rapid and non-specific manner[5]. Traditionally, the innate immune system has been thought to control the inflammation until sufficient clonal expansion of the adaptive immune cells has occurred and they can eradicate the infection. However, it is becoming increasingly clear that the innate immune system plays a fundamental role in controlling inflammatory pathologies, irrespective of the contribution of the adaptive arm and that a dysfunctional innate immune system can contribute to disease[5]. In vertebrates innate immunity is largely dependent on the myeloid cells which include mononuclear phagocytes (monocytes, macrophages and dendritic cells) and polymorphonuclear phagocytes (neutrophils, basophils and eosinophils)[6]. This body of work focuses on neutrophils and macrophages, and their contribution to inflammatory pathologies.

1.2.1 Neutrophils: form and function

Neutrophils are polymorphonuclear leukocytes, released from the bone marrow following maturation in mice and humans[7]. Neutrophils are traditionally known for their role in host defence as they possess complex machinery to destroy invading microorganisms[8, 9]. Neutrophils use a vast array of effector host defence mechanisms (Figure 1.1) and can eliminate pathogens intracellularly and extracellularly, both through oxidative and non-oxidative activity[7]. As professional phagocytes, neutrophils can kill bacteria by nicotinamide adenine dinucleotide phosphate (NADPH) oxygenase-dependent mechanisms, through the generation of reactive oxygen species (ROS). They also possess granules which are filled with pro-inflammatory potent antimicrobial peptides and proteolytic enzymes for killing and digestion of microorganism and can release neutrophil extracellular traps (NETs) for the containment of infection and inflammation[7].

1.2.1.1 Neutrophil secretory vesicles

Neutrophils possess secretory vesicles, which contain membrane-associated receptors, required at the earliest phase of the inflammatory response for cell adhesion and sensing of pathogens[7, 10]. After exocytosis of secretory vesicles, their contents including the β2 integrin, the complement receptors (CR1/CR3), receptors for formylated bacterial peptides (formylmethionyl-leucyl-phenalalanine (fMLP) – receptors), the lipopolysaccharide (LPS)/lipoteichoic acid receptor (CD14) and the Fcγ receptor (CD16), are all localised to the plasma membrane[11]. The receptors mediate attachment to the activated

endothelium as well as control host-pathogen interactions[12]. Furthermore, the Fcγ receptor and complement receptors are essential for the coordination of the phagosome formation. Different signalling pathways are triggered by the different receptors that ultimately lead to the activation of PI3-kinase and Rho proteins and the formation of the phagocytic cup[11] (Figure 1.1 A).

1.2.1.2 Neutrophil respiratory burst

The first microbial pathway neutrophils employ is the oxidative response, also known as respiratory burst, in which ROS, which have microbicidal properties, are produced following the activation of NAPDH oxidase[11, 13]. The NADPH oxidase complex localises to the plasma membrane and the membrane of the forming phagosome[13]. The NADPH oxidase complex consists of cytosolic ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and membrane phox proteins ($gp91^{phox}$, $p22^{phox}$), which make up a heterodimer flavohemeprotein, termed cytochrome b558. Signalling inputs involving Rac2, RhoGDI and RapIA first lead to the activation and recruitment to the plasma membrane of the cytoplasmic components of the NADPH oxidase complex[13]. The NADPH oxidase complex is then able to generate the superoxide anion (O_2^-), which can be reduced to H_2O_2 and consequently the hydroxyl radical ($OH^\bullet$) is formed. Myeloperoxidase (MPO) then catalyses the production of reactive oxidants from H_2O_2, including hypochlorous acid and chloroamines which are highly bactericidal oxidants[14]. Similarly, nitric oxide (NO) synthases, calmodulin-dependent or calmodulin-containing cytochrome p450-like hemoproteins, catalyse the formation of toxic nitrogen intermediates[11] (Figure 1.1 B).

1.2.1.3 Neutrophil degranulation

Degranulation follows exocytosis of secretory vesicles[10]. Neutrophil granules are subdivided into peroxidase-positive granules, called primary or azuriphilic granules, secondary or specific granules and gelatinase or tertiary granules. Primary granules may contain bactericidal permeability-increasing protein (BPI), neutrophil elastase, cathepsin G, protease S, MPO, azurocidin, defensins and lysozyme. Secondary granules may contain collagenase, neutrophil gelatinase associated lipocalin (NGAL), gelatinase, cysteine-rich secretory protein 3 (CRISP3), β2-microglobulin, α1-anti-trypsin, cystatin and cathelicidin. Teritiary granules are known to contain gelatinase, cysteine-rich secretory protein 3 (CRISP3), β2-microglobulin, lysozyme, arginase I, matrix metalloproteinase (MMP)-9 and MMP-25, peptidoglycan recognition protein (PGLYRP), as well as Integrin Alpha M receptor (ITGAM)[15] (Figure 1.1 C). In response to pathogens, the granule proteins are released either into the phagosome or the extracellular space, by degranulation.

1.2.1.4 Neutrophil extracellular traps (NETs)

Neutrophils can also release NETs for the containment of infection and inflammation. NETs are composed of de-condensed chromatin and histones, as well as granule proteins (lactoferin, cathepsins, MPO and neutrophil elastases)[7]. NETs are thought to immobilize pathogens and thus prevent them from spreading. NETs may also aid in phagocytosis or kill pathogens directly by means of antimicrobial histones and proteases[16] (Figure 1.1 D).

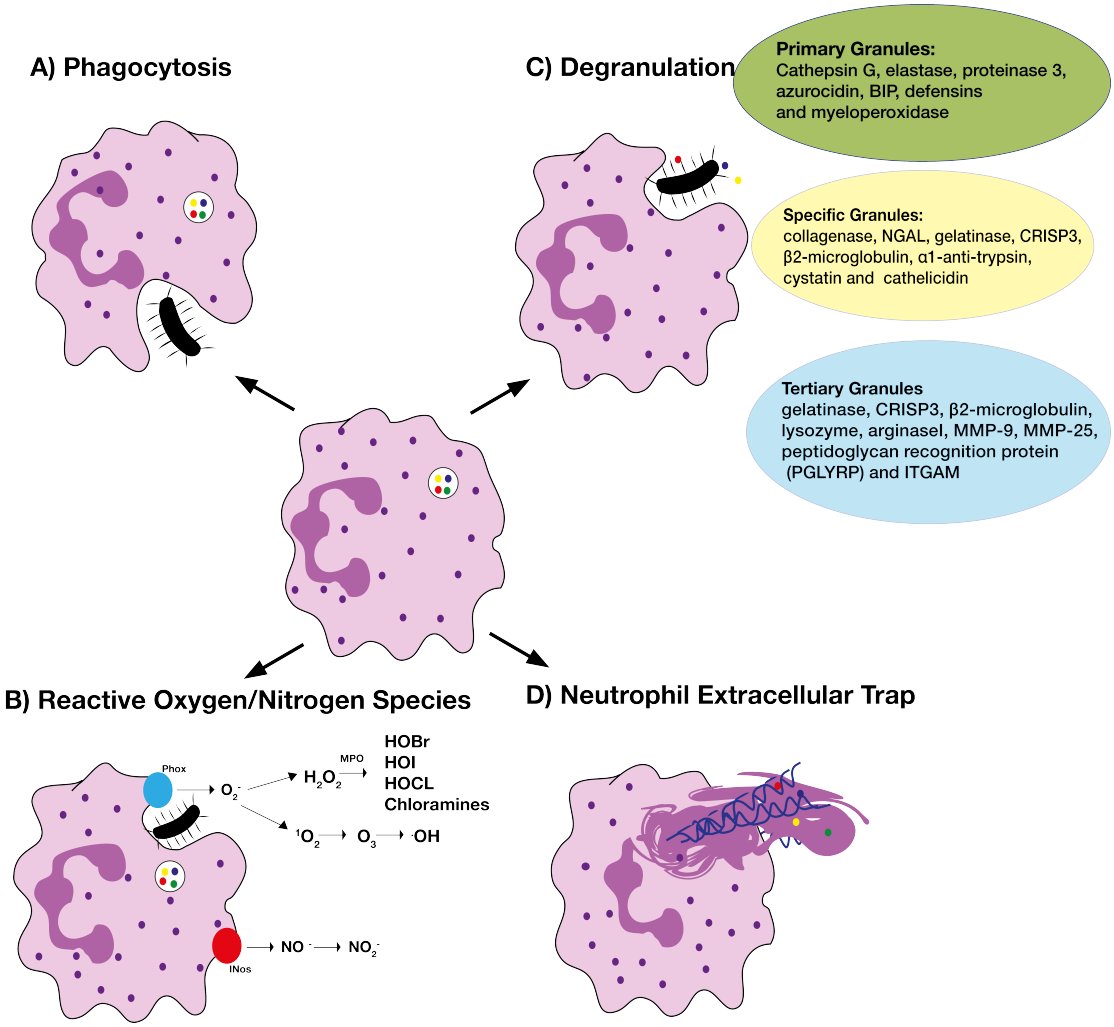


Figure 1.1 Neutrophil effector mechanisms in host defense. Neutrophils can eliminate pathogens by multiple means, both intra-and extracellularly. A) Phagocytosis of microorganism by neutrophils. B) Oxygen dependent response: reactive oxygen species including, superoxide anion (O_2 -), hydrogen peroxide (H_2O_2), and myeloperoxidase-catalysed hypochlorous acid (HOCl), hypobromous acid (HOBr), hypoiodous acid (HOI), and chloramines are produced. C) Non-oxygen–dependent: antibacterial proteins, are released from the primary, secondary and tertiary neutrophil granules into phagosomes or into the extracellular milieu, thus acting on either intra- or extracellular pathogens, respectively. D) Highly activated neutrophils can eliminate extracellular microorganisms by releasing neutrophil extracellular traps (NETs). NETs are composed of decondensed chromatin, attached to histones, granule proteins and enzymes. NETs may immobilise pathogens, preventing them from spreading as well as facilitating phagocytosis. They are also thought to directly kill pathogens by means of antimicrobial histones. Adapted from[7, 11]

1.2.1.5 Neutrophil regulation of the adaptive immune response

Neutrophils can serve to regulate the adaptive immune response, by both direct and indirect mechanisms. There is a growing body of evidence to suggest that neutrophils themselves can function as antigen presenting cells

and prime both Th1 and Th17 cells[17]. Neutrophils express major histocompatibility complex (MHC) Class II molecules when cultured in the presence of a number of cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and interferon (IFN)-γ[16, 18]. Neutrophils may also serve to activate macrophages and dendritic cells and in this capacity indirectly function to regulate the adaptive immune response in the fight against pathogens and tumour antigens[16]. Dendritic cells (DCs) are potent antigen producing cells and are involved in the T cell immune response against pathogens. Neutrophils serve to transport antigens to DCs for T cell activation. Furthermore, the release of certain proteins from neutrophil granules such as defensins, cathelicidins, high mobility group proteins and lactoferrin can lead to the maturation of DCs required for antigen presentation[16].

1.2.2 Macrophages: form and function

Macrophages are tissue-resident terminally differentiated myeloid cells that are derived from monocytes circulating in the peripheral blood[8]. In healthy individuals, macrophages function in host defence, wound healing and in regulating adaptive immunity[19]. Macrophages are generally involved in tissue homeostasis that occurs independently of immune cell signalling where they perform phagocytic clearance of dying cells[20]. In immunity, macrophages are professional phagocytes and essential for immune surveillance[21]. Macrophages recognise foreign particles, tissue damage, apoptotic cells and invading organisms which they phagocytose.

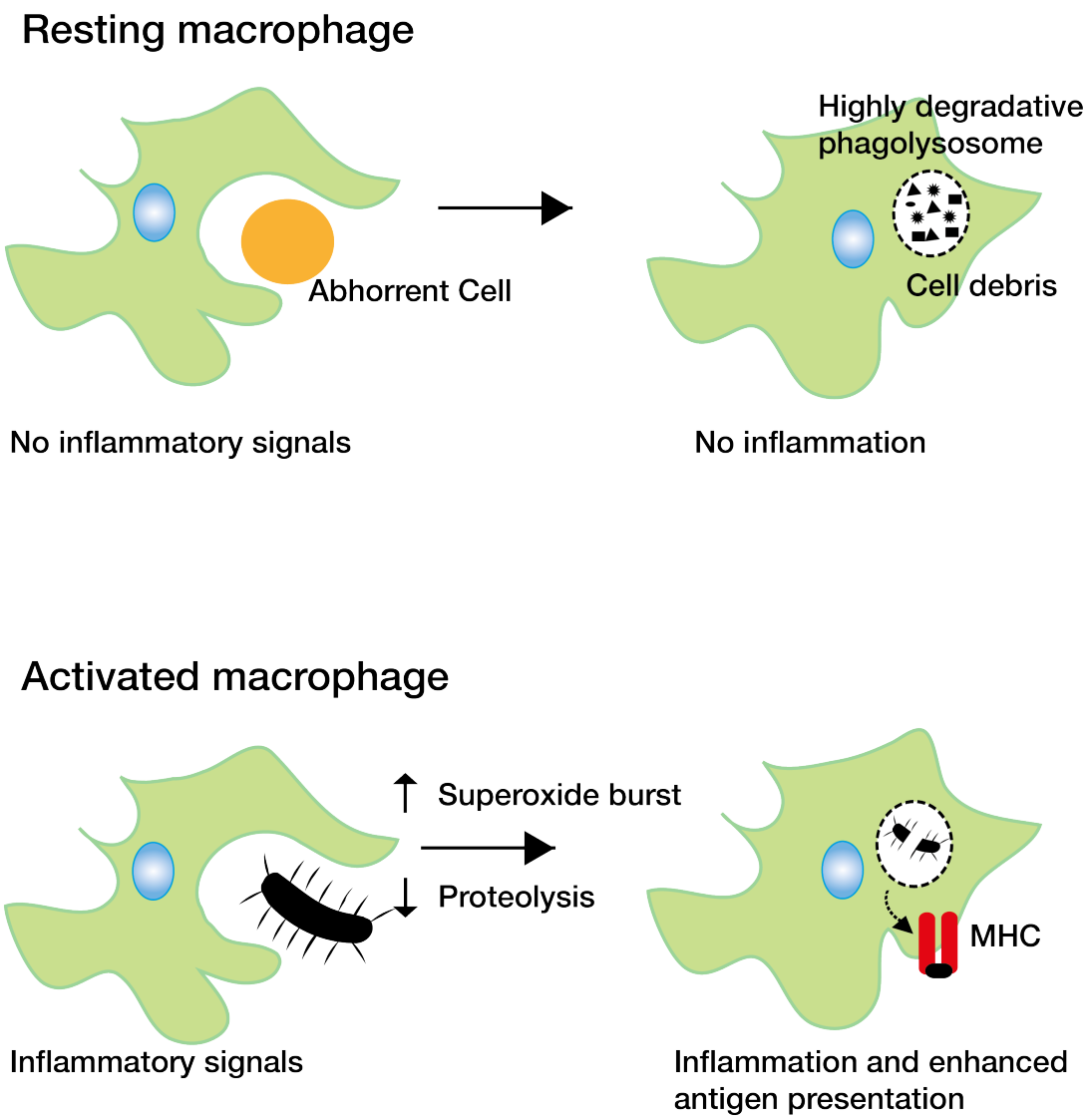


Figure 1.2 Phenotypic differences of macrophages mediating tissue homeostasis and immune surveillance. A) Resting macrophages remove cellular debris such as apoptotic cells, they do not require inflammatory signals and the phagosome is highly degradative for complete destruction of the material. B) Activated macrophages respond to inflammatory stimuli, have an enhanced superoxide burst, are less proteolytically active, thus enhancing antigen processing and presentation. Adapted from[22].

1.2.2.1 Macrophage pattern recognition receptors (PRRs)

PRRs allow macrophages to monitor and respond to changes in their environment. Macrophages via PRRs, must are be able to detect self from non-self or self from abhorrent and select the material that they phagocytose, as well as initiate and assist in the mechanisms of phagocytosis and hence

are important at different stages of phagocytosis. Some PRRs, such as the mannose receptor and macrophage receptor with collagenous structure (MARCO) function in pathogen binding and phagocytosis. PRRs, including the toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I like receptors (RLRs) and nucleotide-binding-oligomerization-domain (NOD)-like receptors (NLRs), sense microbial products and abhorrent cells, and in response unfold the transcriptional programmes that lead to phagocytosis. Others including the TLRs, C-type lectin receptors, scavenger receptors, RLRs and NLRs function during phagocytosis and recognise signals associated with pathogens, foreign substances, or dead or transformed cells. Macrophages also express Fc receptors and complement receptors that bind opsonin molecules, c3b and antibodies, resulting in the activation of the complement system, the tagging of foreign materials and their subsequent phagocytosis[21].

1.2.2.2 Macrophages effector mechanisms

Phagocytosis is governed by the activation of different intracellular signalling pathways that may involve the guanosine triphosphate (GTP)ases, Rho, Rac1/2 and cdc42, triggered by the PRRs, CR3 or FcRs, to subsequently activate Arp2/3 and induce the reorganisation of the actin cytoskeleton and the formation of a sealed intracellular compartment[23]. The phagosome matures and fuses with lysosomes to form a phagolysosome in which the phagocytosed material is degraded[24]. Following closure of the phagocytic cup, the phagosome becomes increasingly more acidic and hydrolytically active. Abhorrent cells or pathogens are degraded by proteolysis or free radicals generated by the superoxide burst that is mediated largely by

phagocyte oxidase and nitric oxide synthase (NOS)[25]. Phagosomes differ depending on the stimulus. In simple tissue homeostasis, macrophages function in the absence of inflammatory stimuli. In this case, the phagosome is highly degradative and relies heavily of proteolysis to destroy cellular debris. By contrast, macrophages activated by exposure to PRR agonists have an enhanced superoxide burst and rely on reactive oxygen and nitrogen intermediates and are less proteolitically active. Proteolytic antigen processing produces short peptides that bind MHC class II molecules present in the phagosome[26] (Figure 1.2).

1.2.2.3 Macrophage subsets

Macrophages can be classified based on their ontogeny and function. In mice, three definitive macrophage lineages can be defined; those that start to develop from the primitive ectoderm of the yolk sac, those that are formed during definitive haematopoiesis in the fetal liver and lastly, a bone marrow-derived lineage, which gives rise to the circulating monocytes and their progeny macrophages[27]. Functionally, macrophages have traditionally been classified into two subsets; M1 or classically activated macrophages (activated by TLR ligands such as LPS and IFNγ) and M2 or alternatively-activated macrophages (activated by interleukin (IL)-4 or IL-13)[8]. M1 macrophages express pro-inflammatory cytokines and inducible nitric oxide synthase (iNos) and mediate host defence from a variety of bacteria, protozoa and viruses, but also play roles in anti-tumour immunity. M2 macrophages express Arginase I, mannose receptor CD206 and IL-4 receptor α chain[19]. M2 macrophages are anti-inflammatory and regulate wound healing and promote

tumour progression. More recently, Mosser and Edwards[19], classified macrophages based on their function into three basic macrophage populations; classically-activated (and involved in host defence), wound healing and involved in immune regulation. Classically-activated macrophages are activated by IFNγ and bacterial products and express high levels of IL-12 and low levels of IL-10[19]. Regulatory macrophages are generated in response to various stimuli including immune complexes, prostaglandins, G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells and IL-10[19] and act to suppress immune response mechanisms. Wound-healing macrophages arise in response to IL-4 and have a role in tissue repair[19]. However both macrophage definitions are an oversimplification and it is more likely that macrophage subpopulations exist as a continuum, exhibiting characteristics of more than one subtype[19].

1.3 Zebrafish as a model to study innate immunity

Mammalian and murine models have demonstrated a complex inflammatory response modulated by macrophages and neutrophils that ensues various types of inflammation. In this study, the zebrafish, which offers unique advantages over other model organisms, is used to study inflammation.

1.3.1 Zebrafish for studying disease

Traditionally, the zebrafish was used to gain an understanding of early vertebrate development but the model has quickly expanded to study organogenesis and human disease[28]. Zebrafish share most of the same cell types, tissues, organs and biological circuits as humans, ensuring that the lessons from zebrafish can be directly applied to other vertebrate systems.

Furthermore, most zebrafish genes have mammalian orthologs and most epigenetic regulators, oncogenes and tumour suppressor genes are conserved[29]. The small size, shorter life cycle and ease of breeding make the zebrafish an appealing alternative to other vertebrate systems[30]. The zebrafish provides the potential for *in vivo* imaging and genetic approaches that are unparalleled to mammalian models[31]. With excellent tractability of zebrafish genetics, excellent advances in zebrafish transgenic models have been achieved, including the use of tissue specific promoters for studying specific cell populations, bacterial artificial chromosome (BAC) recombineering, the recent developments in inducible systems and rapid advances in genome editing techniques[32].

1.3.2 Zebrafish and immunity

The zebrafish has proven to be a powerful tool in studying human immunity[33]. There is clear temporal separation between the innate and adaptive immune responses, with only the innate immune system present until roughly three weeks after fertilization[34]. This makes the zebrafish a unique model to study components of the innate immune system in isolation. Recent studies using transparent zebrafish larvae have proved invaluable in modelling host-pathogen interactions using transgenic lines with fluorophores labelling distinct innate immune cell lineages[31]. The contribution of the innate immune system to oncogenesis is now well-documented[35]. Zebrafish proved to be a suitable model in cancer studies, with the use of tissue-specific promoters to achieve expression of human chimeric oncogenes and fluorescent reporters expression to help monitor the tumour initiation and progression. Additionally,

the transparency of zebrafish larvae allows live imaging of the earliest dynamic interactions between the host innate immune cells and oncogene-transformed cell clones as they first establish themselves as the precursors of cancer[32, 36].

1.3.3 Innate immunity in zebrafish

1.3.3.1 Myelopoiesis in zebrafish

The myeloid component of zebrafish larvae is comprised mainly of macrophages and neutrophils[37]. In zebrafish, the transcription factor Pu.1 or Spi1 regulates myelopoiesis[38]. Pu.1-expressing myeloid precursors arise as early as 12 hours post fertilization (hpf) and give rise to both macrophages and neutrophils[39]. Haematopoiesis in the larval zebrafish occurs in two waves, primitive and definitive. Myeloid precursors emerge from the anterior lateral plate mesoderm, migrate over the yolk sac where they differentiate into primitive macrophages by 22 hpf and then further differentiate into primitive neutrophils by 33 hpf[40, 41]. However neutrophils are first evident as early as 18 hpf in the posterior intermediate cells mass which is the site of definitive haematopoiesis[42]. By 4 days post fertilization (dpf), the kidney marrow starts to mature and will become the site of definitive haematopoiesis in the adult fish[43]. Macrophages and neutrophils can be recruited to sites of infection and exhibit phagocytic behaviour from 30 hpf and 52 hpf, respectively[42].

1.3.3.2 Transgenic models of myeloid cells

Transgenic zebrafish lines with fluorescent reporter expression in macrophages and neutrophils already exist. The macrophage specific mpeg-1

promoter was used to drive the expression of enhanced green fluorescent protein (EGFP) and mCherry fluorophores[37] and the neutrophil-specific MPO promoter was used to drive the expression of GFP[34] in cell-specific fashion. These transgenic zebrafish lines can be used as imaging tools to study the recruitment of macrophages and neutrophils to inflamed sites and are discussed in more detail in chapter 3[44].

1.3.4 Characterisation of the zebrafish innate immune response

To render the zebrafish a useful model to study human immunity, there must be sufficient overlap between the zebrafish and human genomes. To date, many genes relevant to the immune system response have been characterised in zebrafish, including those coding for cytokines, the complement system, lectins, immunoglobins and cell surface molecules[44]. (Table 1.0)

1.3.4.1 Activation of the innate immune response

As the first line of defence against infection, the main role of the innate immune system is to recognise invading pathogens and trigger an appropriate pro-inflammatory response. In response to inflammation, infection or cellular stress PRRs located on the cells surface, the surface of endosomal compartments and in the cytoplasm recognize microbes and pathogens and initiate phagocytosis, the upregulation of pro-inflammatory signalling pathways including a range of cytokines and antimicrobial peptides[45]. Several classes of PRRs have been identified in fish including toll-like receptors (TLRs), Rig-I like receptors (RLRs) and nucleotide-binding-oligomerization-domain (NOD)-like

receptors (NLRs)[44]. Nineteen putative TLRs have been identified in zebrafish, including orthologs of the TLR 2-5 and TLR 7-9 in humans[46]. Similarly, TLR signalling pathways are conserved[47]. TLRs transduce signals intracellularly via the recruitment of adaptor molecules (MyD88, MAL, TRIF and SARM), which in turn act through the central intermediate TRAF 6, NFκB and the mitogen-activated protein kinase (MAPK) pathways, resulting in the transcription of genes involved in the immune response[48, 49]. Interestingly, unlike in humans, zebrafish TLR4 cannot be stimulated by LPS, inferring that an alternative signalling pathway predominates in fish[50].

The NLR family is comprised of both NODs and NALPs, which are cytosolic proteins present in immune cells where they recognise bacteria and may contribute to autoimmune disease[51]. Three NLR subfamilies have been identified in zebrafish, NLR-A (which resemble the mammalian NODs), NLR-B (mammalian NALPs) and NLR-C (unique to teleost fish)[51]. NOD-like receptors may initiate or inhibit NF-κB signalling as well as MAPK pathways. NALPs are fundamental for inflammasome activation where they activate caspase-1, which ultimately leads to the production and processing of important inflammatory cytokines, including IL-1, IL-18 and IL-33[31]. Although NLRs have not been widely studied in zebrafish, as yet, their antibacterial role in a model of *Salmonella enterica* was confirmed[52]. Interestingly, knockdown of nod1/2 in zebrafish depleted dual oxidase (duox), necessary for reactive oxygen species formation[52]. The RIG-I-like receptor family is comprised of three members all of which are conserved in zebrafish; RIG-1, melanoma

differentiation-associated gene 5 (MDA5) and laboratory genetics physiology (LGP2) that can all detect viral RNA[31, 53].

1.3.4.2 Effector mechanisms of the innate immune response in zebrafish

The complement system consists of a number of different serum and membrane-bound proteins that are involved in fighting pathogens. Different pathways are triggered by pathogens to activate the complements system; the classical pathway (activated by the binding of the first component of the complement, C1 to antibodies (IgG or IgM) at the surface of a microbe), the lectin pathway (activated by mannose-binding lectin), and the alternative pathway (activated when C3b covalently binds to hydroxyl or amino groups on the pathogen surface)[54, 55]. Irrespective of the mechanism of activation, the three pathways all merge at a common amplification step resulting in the formation of the C3-covertase protease complex (C3bBb in the alternative pathway and C4bC2a in the classical and lectin binding pathway) and subsequent assembly of C5, C6, C7, C8, C9 forming a membrane attack complex for lysis of invading pathogens[55]. Members of the complement system can also promote pathogen phagocytosis and recruitment of phagocytic cells to sites of inflammation[55]. Phylogenetic analysis revealed that all of the fundamental components of the mammalian complement system are present in zebrafish including C1q-A, C1q-B, C1q-C, C1r/S, C3, C4, C5, C6, C7, C8 and C9[54].

1.3.4.3 Zebrafish cytokines and chemokines

Although the main innate immune signalling pathways and the downstream components are highly conserved in zebrafish[44], it is generally agreed that class II cytokines and their receptors have diverged[44]. However, a number of functionally active cytokines have been identified in zebrafish including the pro-inflammatory cytokines IL-1b, tumour necrosis factor (TNF)-α and IL-6, transforming growth factor (TGF)-β, IL-12, interferon regulatory factor, type I IFNs and a putative IFN receptor[44].

Chemokines are a family of proteins, primarily known for their role in activating and inducing migration in leukocyte populations[56]. They signal through chemokine receptors which make use of a complex network of intracellular signalling machinery[56]. Chemokines are divided into CR, CCR, CXCR and CXCR3 subfamilies. Consequently chemokine receptors are similarly classified according to the chemokine ligands that they bind[53, 56]. Both chemokines and chemokine receptors have been characterized in bony fish. At least 17 genes coding for CC-chemokine receptors are present in zebrafish including orthologs of human CCR6, 7, 8, 9 and CCRL1[56]. Orthologs for all 7 CXC receptors known in mammals, except CXCR6, have also been identified in fish[53].

1.3.5 Adaptive Immune system in zebrafish

Although not the focus of this study, zebrafish also have a fully functional rag-dependent adaptive immune system including a thymus, T cells and B cells. The T cell receptor (TCR) locus was cloned and showed mammalian-like

variable segments with rag-dependent V(D)J recombination[57]. Zebrafish T-cell specific genes such as ikaros, lck, GATA-3, rag-1 and rag-2 have been cloned and shown to exhibit a strong functional similarity to their mammalian counterparts as well as similar gene expression profiles[33]. Similarly, MHC class I, class II and class III genes have also been identified in zebrafish[58, 59].

	Human	Zebrafish
General		
Sites of early hematopoiesis	Yolk sac	ICM
	AGM	Dorsal aorta
	Fetal liver	CHT
Site of adult hematopoiesis	Bone marrow	Kidney marrow
Lymphatic system	ZF has no lymph nodes but does have putative lymphatic vessels including a thoracic	
Complement system	Well developed in ZF with shared human elements	
Inflammatory proteins	Well conserved between human and ZF, i.e., TNFα, NF-κB, COX2, IL-1, IL-8 and other C-C and C-X-	
MHC	Class I, II, III all present in ZF	
Myeloid		
Neutrophils		
Nuclear lobes	4–5	2–3
Cytoplasm	Heterophilic with azurophilic and non-azurophilic granules similar to human	
Motility	+	+
Phagocytic activity	+	+
Myeloperoxidase	+	+
Acid phosphatase	+	+
Periodic acid Schiff	+	–
Respiratory burst	+	+
Eosinophils		
Morphology	Morphologically distinct from human counterpart	
Monocytes/macrophages		
Motility	+	+
Phagocytic activity	+	+
Respiratory burst	+	+
Ability to activate T/B cells	+	+
Mast cells and basophils	Currently not characterized in ZF	
Lymphoid		
Thymus		
Anatomy	Multilobulated midline	Bilateral single-lobed twin structures which remain attached to pharyngeal endoderm
Histology	Demarcated cortex and medulla in human and ZF	
T cells		
Site of initial development	Bone marrow	Kidney marrow
Educated in thymus	+	+
TCR	ZF TCRα, β, δ, γ chains identified	
Rag-dependent V(D)J	+	+
Gene expression	Similarities include ikaros, lck, GATA-3, rag	
B cells		
Embryonic development	Initially develop in ZF pancreas	
Ig subtypes	A,D,G,E, M	D, M, Z
Rag-dependent V(D)J	+	+
AID	+	+
Class switch recombination	+	–
Somatic hypermutation	+	Inefficient
Antibody response to	+	+
NK cells		
Receptor	Classical NK receptor	Putative NITRs

Abbreviations: AGM ¼ aorta, gonad, and mesonephros; ICM ¼ intermediate cell mass; CHT ¼caudal hematopoietic tissue; NITR ¼

Table 1.0 Comparison of the human and zebrafish innate immune systems. Adopted from[33].

1.4 The contribution of the myeloid response to sterile and non-sterile disease pathologies

The zebrafish model created in this study provides extensive possibilities to study the contribution of the myeloid component of the innate immune system to inflammation. This study focuses on the response of the myeloid cells to Mycobacterium Tuberculosis (*M. marinum* in the case of the zebrafish) and melanoma. Although, very different disease pathologies, they both feature non-resolving inflammation. Furthermore, in both pathologies, the contribution of the innate immune response, may either be protective to the host, or may contribute to the pathogenesis of the disease.

1.4.1 Mycobacterium Tuberculosis

1.4.1.1 *Mycobacterium Tuberculosis* (TB) and granuloma development

9 million people were estimated to have developed Tuberculosis in 2013 and 1.5 million people died from the disease[60]. Although, an appropriate immune response generally follows infection, these responses often fail to clear the bacteria. The outcome of *M. tuberculosis* infection ranges from early asymptomatic clearance to latent disease, with 10% of these patients developing active disease[61]. Despite decades of research, the determinants of disease outcome remain poorly understood[62].

The immune response to TB is a multi-staged process. Hence defining the immune response at specific stages could provide a clearer understanding of the correlates of immunity that impact disease progression[63]. *M. Tuberculosis*

is transmitted by the inhalation of aerosol droplets that contain bacteria. The innate immune phase is initiated by the phagocytosis of bacteria in the lung by macrophages, followed by the accumulation of macrophages, neutrophils, DCs and innate lymphoid cells[63]. As they are recruited, some get infected by the expanding population of mycobacterium and early granulomas start to form[63]. The granuloma is the hallmark lesion of tuberculosis, an organised structure, in which bacteria may persist[64, 65] (Figure 1.3). Large macrophages, termed epitheliod macrophages, with interdigitated cell membranes linking adjacent cells together form the basic structure of the granuloma[65]. Infected macrophages become activated and subsequently recruit other immune cell types including neutrophils, dendritic cells, B cells and T cells to the granuloma[64]. This marks the onset of the adaptive immune response. Granulomas can also contain extracellular matrix components and have central necrotic and hypoxic cores. Epitheloid macrophages may differentiate into lipid-filled foam cells, visible at the rim of the necrotic core[66]. The central core is filled with a lipid-rich debris termed caseum[64]. Caseating granulomas are a signature of active tuberculosis and are implicated in the transmission of the disease. Yet, such a concentrated immune response does not eradicate infection. Much controversy exists as to whether the granuloma is a host protective structure, or rather a niche in which the mycobacteria can proliferate, survive and later disseminate to form active disease[66].

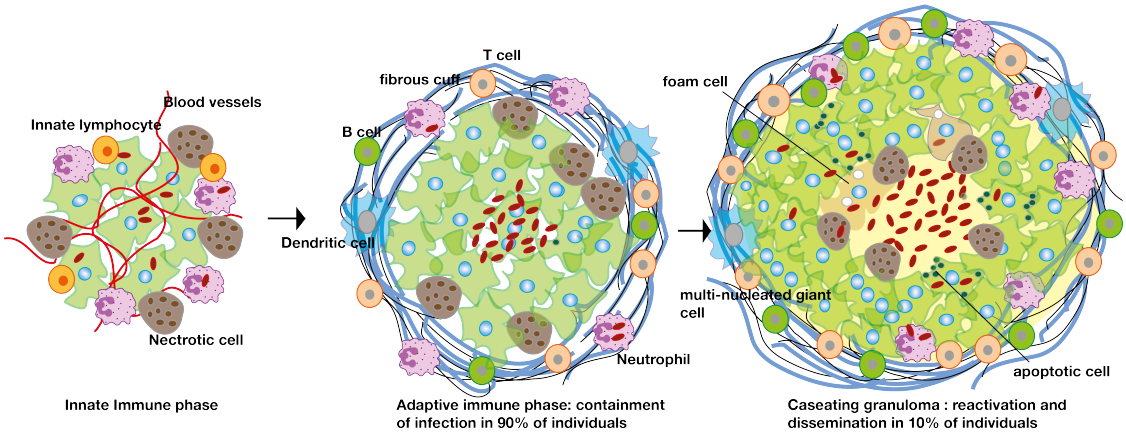


Figure 1.3 Granuloma composition from TB onset to active disease. A) Innate immune phase: the initial stages of infection are characterized by innate immune responses that involve the recruitment of inflammatory cells to the lung. Granulomas are comprised of mainly of infected macrophages, neutrophils, innate lymphocytes and some necrotic cells. B) Adaptive immune phase: granuloma is composed of compact, organized aggregate of epithelioid macrophages, neutrophils, dendritic cells, B and T cells, surrounded by a fibrous cuff. C) Active disease: Granuloma macrophages can also fuse into multinucleated giant cells or differentiate into foam cells, which are characterized by lipid accumulation. Foam cells have been noted to be most frequently located at the rim of the necrotic center of a mature tuberculous granuloma. Bacteria are most commonly present in the central caseating, necrotic areas in which necrotic and apoptotic macrophages can be seen. Adapted from[66, 67].

1.4.1.2 Innate immune cells in early Tuberculosis infection

The outcome of infection can, at least partially, be attributed to the efficiency of various innate host defence mechanisms including, immune recognition phagocytosis, cytokine production and downstream effector mechanisms that may result in bacterial killing and the initiation and direction of T cell immunity by presentation of bacterial antigens[68]. Phagocytosis of *M. tuberculosis* by both macrophages and neutrophils is initiated by receptors (complement receptors, scavenger receptors, the mannose receptor) on the surface of the phagocyte. This coupled to PRR signalling, including TLR and the C-type lectin family member activation, results in immune activation. Recognition of *M. tuberculosis* leads to the production of cytokines, which in itself induces further cytokine production resulting in a complex immune network[68]. For example, TNFα, produced mainly by neutrophils, monocytes, macrophages

and DCs, plays a role in granuloma formation and induces macrophage activation[67]. Furthermore, TNFα also exhibits immunosuppressive properties, by inhibition of TH2 cell responses, therefore controlling IL-10 levels[67]. One study reported that neutrophils in the presence of elevated levels of TNFα had a higher killing efficiency than in the absence of the cytokine[69]. IL-1β is expressed at high levels at the site of infection and plays a role in controlling mycobacterial burden in the lungs and distant organs. In the absence of IL-1β, granuloma formation is impaired, with granulomas containing fewer macrophages and lymphocytes but more granulocytes[70]. IL-6, also produced in high amounts early in infection, has both pro- and anti-inflammatory properties. IL-6 inhibits TNF-α and IL-1β production and hence promotes bacterial growth[68]. However, other reports suggest that IL-6 may play a protective role[68]. IL-12 is thought to play a protective role, largely because it links the innate and adaptive immune responses by inducing IFN-γ production from CD4+ T cells and natural killer (NK) cells at the site of infection. Furthermore, IL-12 induces the differentiation of CD4+ T cells into Th1 cells, necessary for fighting bacterial infection[71]. The pro-inflammatory response is subsequently halted by the production of IL-4, IL-10 and TGF-β, mainly by macrophages[67, 68]. While anti-inflammatory signalling may favour bacterial growth, excessive pro-inflammatory signalling may result in tissue damage.

Macrophages are predominantly responsible for killing of the mycobacteria following phagocytosis of the bacteria. Within the phagolysosome, macrophages may mediate bacterial killing by lyosomal enzymes, ROI and RNI[68]. A few studies have suggested a similar role for neutrophils in mycobacterial killing by ROI[72]. However, there is evidence to suggest that

mycobacterial products are able to scavenge free radicals and limit neutrophil effectiveness[68]. Other mechanisms suggested for bacterial killing by neutrophils include the granule proteins human neutrophil peptides (HNP), cathelicidin and lipocalin[73]. Following initiation of the adaptive immune system, MHC class I and II antigen presentation occurs. While pro-inflammatory cytokines stimulate the expression of MHC, anti-inflammatory cytokines inhibit it[68].

After the uptake of *M. tuberculosis* by macrophages, the innate immune system may destroy the bacteria immediately. However, should infection be established, the innate immune response is activated and an orchestrated response of pro- and anti-inflammatory cytokines follows. Innate immune cells also serve to initiate T cell-mediated immunity, both by the cytokines they release and by antigen presentation. Therefore, the innate immune response plays an important role in determining both the outcome of the infection and later the containment of infection. Disease progression, characterised largely by changes in the granuloma composition (Figure 1.4), is a stepwise process, involving both the innate and adaptive immune system. Thus dissecting granuloma assembly and the individual contribution by different cell types, to both host's and pathogen's advantage would provide the opportunity to modulate the granuloma to be a truly host protective entity.

1.4.2 Melanoma

Melanoma is the most aggressive form of skin cancer with approximately 10% lethality in all patients diagnosed with this malignancy[74]. Melanoma progression reflects a stepwise transformation that is initiated by genetic

alterations that transform a normal melanocyte or nevus into a primary and then metastatic melanoma, including the activation of oncogenes and the inactivation of tumour suppressor genes[75, 76, 77]. The Ras/MAPK pathway is universally activated and critical for melanoma growth. Growth factors activate Ras proteins localised to the plasma membrane, which in turn activates a downstream effector Raf, followed by sequential activation of MEK and extracellular-signal-regulated kinase (ERK) and resulting in the regulation of transcription in the nucleus. The most commonly mutated members of the Ras family are NRas, KRas and HRas, harbouring activating mutations that render them constitutively expressed[78]. The Ras proteins are small GTPases that transduce signals from receptors activated by extracellular cues. They cycle between a guanosine diphosphate (GDP)-bound inactive and a GTP-bound active state in which they can transduce signals that control cell differentiation, proliferation and survival[78]. NRas is the most relevant to human melanoma as the mutations yielding constitutively active form of NRas are present in 20-30% of melanoma patients[79].

1.4.2.1 Melanoma and immunity

It is now appreciated that all cancers including melanoma are immunogenic and thus are composed of various cell types in addition to tumour cells including innate (macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells and natural killer cells) and adaptive (T and B lymphocytes) immune cells[76]. In a paradoxical manner, although the immune system may provide protection against cancer by immune elimination of

transformed cells, it may also aid tumour development by immunosuppressive mechanisms triggered by tumours. Different cytokines present in the tumour microenvironment can either promote or inhibit tumour development through activation of various downstream transcription factors that control the immune and inflammatory milieu[80].

1.4.2.2 The role of myeloid cells in tumours

1.4.2.2.1 Dual role for macrophages in tumours

In response to IFNγ, macrophages, referred to in the field as M1, express high levels of IL-12 and low levels of IL-10 and are tumouricidal[8]. This can be directly achieved by the release of free radicals (reactive oxygen and nitrogen intermediates) or indirectly by regulating the immune system by cytokine secretion (IL-1β, TNF, IL-6), the release of Th1-attracting chemokines (CXCL9 and CXL10) and by controlling antigen processing and presentation[81]. However, classically activated macrophages can also contribute to tumour progression primarily because the free radical production can result in DNA damage[81]. It appears that as a result of the tumour microenvironment, macrophages with immunosuppressive properties (M2 polarisation) prevail in cancers, and are more generally termed tumour-associated macrophages (TAMs)[4, 8]. TAMs are activated by IL-4, IL-10, IL-13 and glucocorticoid hormones and express high levels of IL-10 and low levels of IL-12[8]. TAMs facilitate tumour progression by promoting tumour growth, tissue and matrix remodelling, stimulating angiogenesis and by developing immunosuppressive mechanisms that dampen the adaptive immune response[82].

TAMs can produce a number of proteolytic molecules including plasmin, urokinase-type plasminogen activator, cathepsin β and MMPs, which directly serve to promote ECM remodelling, invasion and metastasis. TAMs regulate angiogenesis because they express factors that are either directly or indirectly involved in new vessel formation and sprouting including, TGFβ, vascular endothelial growth factor (VEGF)A, VEGFC, platelet-derived growth factor (PDGF), MMP9, thymidine phosphorylase (TP) and IL-8[82]. For example, TAMs may produce MMP9, which enhances the association of VEGF with its receptor present on angiogenic endothelial cells[83]. Similarly, placental growth factor (PIGF), a homolog of VEGFA, was also shown to stimulate angiogenesis in tumours[84]. Tumour-derived signals appear to recruit a subset of macrophages that express tyrosine kinase with immunoglobulin-like and EGF-like domains (Tie)2, which is normally restricted to endothelial cells. Tie2 is responsible for neoangiogenesis[85] (Figure 1.4).

TAMs mediate immunosupression by producing factors such as IL-10, iNos, prostaglandins (PGE)-2, TGFβ, indoleamine dioxiginase (IDO) metabolites and Arginase 1[8]. IDO is an enzyme that catalyses the oxidative catabolism of tryptophan[8]. IDO activity limits T cell growth by depleting L-tryptophan and promotes T cell apoptosis through the generation of tryptophan metabolites. Similarly, Arginase 1 deprives T cells of L-arginine necessary for their growth[86]. TAMs release CCL17, CCL22 and CCL24 that are involved in the recruitment of Tregs, Th2 cells, eosinophils and basophils, thus further contributing to immunosuppression (Figure 1.4).

TAMs are ineffective antigen presenting cells. Tumour cells not only have cancer-driving mutations but passenger mutations that drive the formation of neoantigens which are presented by MHC molecules on the cells surface and are recognised by T-cells as foreign, resulting in an immune response against the tumour[87]. However, T cells express two cell surface receptors, cytotoxic T-lymphocyte-associated protein (CTLA)-4 and programme cell death (PD)-1, which when bound by their ligands, expressed on the cancer cells, suppresses the expansion of cytotoxic T cells[87]. In the case of PD-1, the ligand, PD-L1, is also expressed on the surface of macrophages, and engagement of the ligand with PD-1 on T cells can directly lead to apoptosis[88].

1.4.2.2.2 Dual role for neutrophils in tumours

Like macrophages, tumour-associated neutrophils (TANs) also exhibit different states of activation, crudely referred to as N1 and N2[89]. Although there is a growing body of evidence for their role in cancer, the mechanisms responsible for this activity have not yet been elucidated. TANs are known to be involved in angiogenesis, immune surveillance, as well as the secretion of chemokines, cytokines and reactive oxygen species[82].

TGF-β blockade has previously been shown to slow down tumour growth. A recent study revealed how TGF-β blockade not only increased the infiltration of neutrophils into the tumour but it can also switch neutrophils from an N2 to N1 neutrophils that are cytotoxic and express high levels of pro-inflammatory cytokines. Depletion of neutrophils following TGF-β blockade reduces the

anti-tumour effect of the TGF-β treatment. This attributes the mechanistic role in anti-tumour activity, at least in part, to cytotoxic tumour-associated neutrophils. In comparison, when TGF-β signalling was not inhibited and neutrophils were depleted, tumour growth decreased[89].

It is likely that neutrophils can facilitate an angiogenic switch. In models of subcutaneous melanoma or fibrosarcoma, TANs regulate angiogenesis by expressing high levels of VEGF and MMP-9[90]. Neutrophil elastase has also been shown to play a role in cancer progression. In a lung cancer model, neutrophil elastase was taken up by cancer cells into endosomal compartments and found to degrade the insulin receptor substrate (IRS)-1, which usually blocks PI3K and PDGFR interactions. This enhanced PI3K and PDGFR signalling, resulting in increased proliferation[91]. However, in breast cancer cells, neutrophil elastase triggers the adaptive immune response. Neutrophil elastase cleaves cyclin E to form an HLA-A2 restricted peptide which is presented to cytotoxic T cells on the surface of the breast cancer (Figure 1.4)[92].

Neutrophils can also contribute to metastatic disease. GM-CSF derived from breast cancer cells induced the production of oncostatin M (OSM) in neutrophils. In turn, OSM derived from the neutrophils stimulated the production of VEGF in breast cancer cells, which subsequently decreased their cell detachment and proliferative capacity[93]. TNF-α and GM-CSF derived from bronchoalveolar carcinoma induced the release of hepatocyte growth factor (HGF) by neutrophils, leading to increased tumour cell invasion[94].

Melanoma cells express CXCL8, which promotes the infiltration of neutrophils into the melanoma and leads to an increase in expression levels of the β2 integrin on their cell surface. β2 integrin signalling with ICAM-1 on the melanoma surface facilitated lung metastasis (Figure 1.4)[95].

This prompts the need for us to better understand how cancer cells and the tumour microenvironment affect gene expression in immune cells and to understand the mechanisms that prevent innate immune cells from killing tumour cells.

In this study a transgenic zebrafish model for investigating the myeloid response (macrophages and neutrophils) under different conditions of inflammation has been established. The model allows us to profile of the active transcriptomes of pure cell populations and hence to elucidate the regulatory networks that govern the immune cell responses in inflammatory disease. The versatility of this model is then demonstrated under conditions of sterile inflammation, in a transgenic model of melanoma, also established here, as well as under a condition of non-sterile inflammation, in a granuloma-forming infection model of *M. marinum*.

1.5 Objectives

1 Establish a zebrafish model system to study the myeloid (specifically macrophages and neutrophils) response under conditions of sterile and non-sterile inflammation.

2 Optimise a granuloma infection model in zebrafish

   i. Carry out a genome-wide analysis of macrophages or neutrophils' response to *M. marinum*.

   ii. Validate genome-wide analysis using genome editing technology

3 Establish a melanoma model in zebrafish

   i. Characterise the interaction of neutrophils and macrophages with oncogenic melanocytes *in vivo*.

   ii. Carry out a genome-wide analysis of macrophages or neutrophils response to melanoma compared to control melanocytes early in cancer progression.

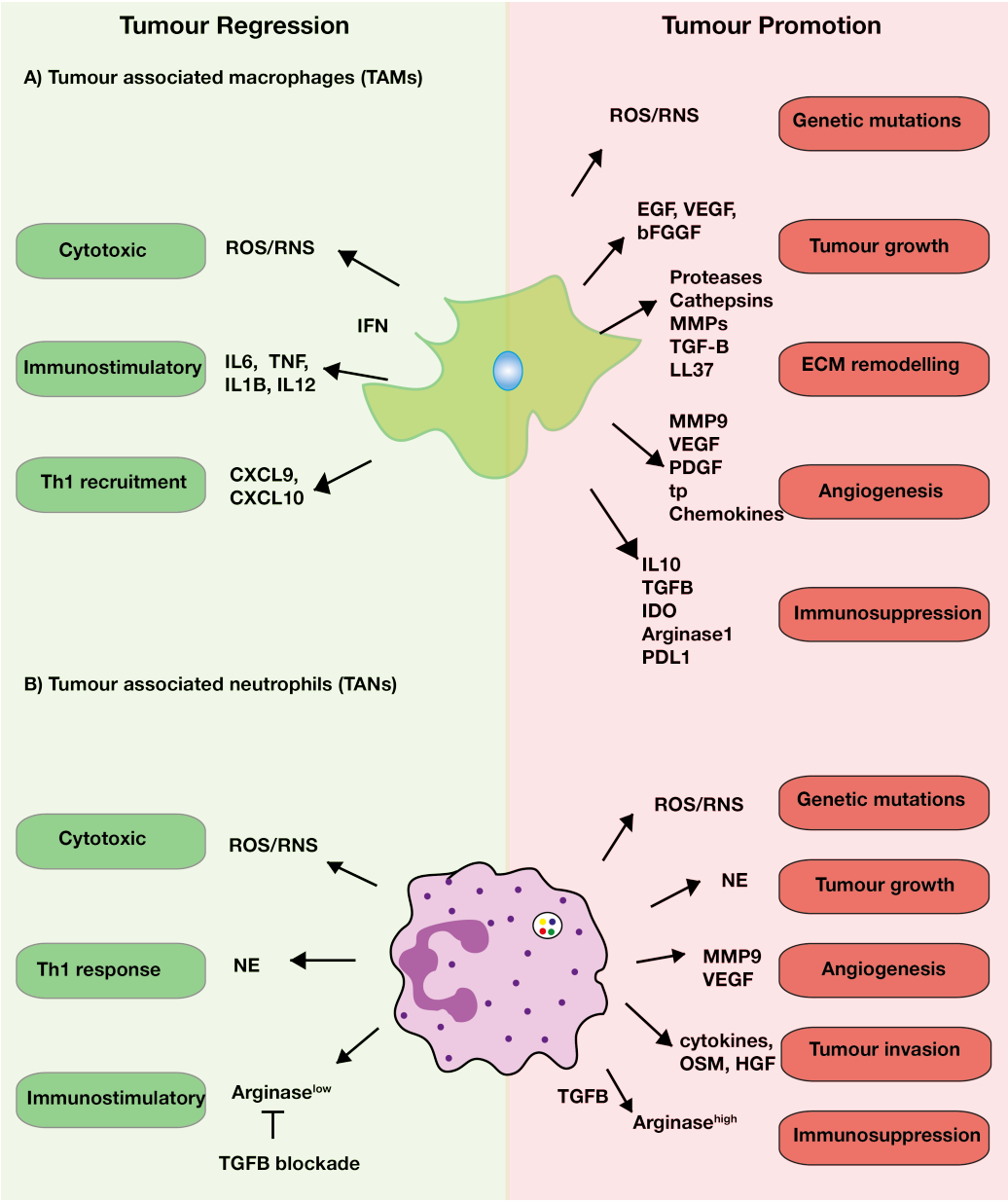


Figure 1.4 Conflicting roles of macrophages and neutrophils in tumourigenesis. A) TAMs can elicit tumoricidal properties by the release of ROS/RNS, indirectly by regulating the immune system by cytokine secretion (IL-1β, TNF, IL-6), the release of Th1 attracting chemokines (CXCL9 and CXL10) and by controlling antigen processing and presentation. TAMs can increase genetic instability through the release of ROS. TAMs promote tumor growth through the production of growth factors (EGF, VEGF, bFGF), are involved in the remodeling of the ECM (cathepsins, MMP-2, MMP-9, TGF-β), favour angiogenesis (MMP-9, VEGFs, PDGF, thymidine phosphorylase (tp), CXCL8) and suppress the anti-tumoral immune response through the release of soluble mediators (IL-10, TGF-β, IDO, Arginase 1, PD-L1). B) TANs can elicit tumouricidal properties by the release of ROS/RNS and neutrophil elastase, and promote an anti-tumoral T cell responses after inhibition of TGF-β. In contrast, TANs can increase genetic instability through the release of ROS, promote tumour cell proliferation through elastase, sustain angiogenesis (VEGF, MMP-9), enhance neoplastic cell invasiveness (OSM and HGF) and suppress effective anti-tumoural CD8+ T-cell immunity through the expression of arginase 1 driven by TGF-β[82].

Chapter 2

Materials and Methods

2.1 Expression construct cloning

2.1.1 mCherry-Ras Effector construct

The constructs mEGFP-HRasG12V and pBabe-NRasQ61K were obtained from addgene. pEFm.6:HA-KRas was a gift from Dr Xin Lu. pDs (cry:ECFP-LexOP:Cherry) was a kind gift from Dr Alexander Emelyanov and Dr Sergey Parinov[96]. pDs (cry:ECFP-LexOP:Cherry) was digested with SacII and NotI to remove Cherry. mCherry-HRasG12V, mCherry-NRasK61Q, mCherry-KRasG12V, generated by fusion PCR, were amplified with primers, listed below, that overlapped with +/-20 bp (shown in blue) upstream and downstream of the SacII and NotI sites respectively (shown in red) and the effector constructs subsequently cloned by infusion cloning (Clontech, cat # 638911).

Primer Name	**Sequence**
5'Cherry for LexOP effector construct	CTGCAGTCGACGGTACCGCGGGCCCGGGATCCCGCCACCATG GTGAGCAAGGGCGAGGAG
3'HRasG12V for LexOP effector construct	TATGATCTAGAGTCGCGGCCGCTTTATCAGGAGAGCACACACTT GC
3'NRasK61Q for LexOP effector construct	TATGATCTAGAGTCGCGGCCGCTTTACATCACCACACATGGCAA TC
3'KRasG12V for LexOP effector construct	TATGATCTAGAGTCGCGGCCGCTTTATTACATAAA

2.1.2 BirA-driver construct

Citrine-SV40-FRT-kan-FRT was made by replacing EGFP in EGFP-sv40-FRT-kan-FRT with Citrine from pGEMteasy-Citrine-FRT-kan-FRT by digestion with SacII and BsrGI. A splice acceptor (SA) site in SV40 was removed by site directed mutagenesis using primers listed below (mutated nucleotide in red). BirA-2A-Citrine was generated by fusion PCR and inserted into the Citrine-SV40-FRT-kan-FRT construct digested with SacII and BsrgI (shown in red).

Primer Name	Sequence
SA mutated a49gsense	TGCATTCATTTTATGTTTCGGGTTCAGGGGGAGGTG
SA mutated a49g_antisense	CACCTCCCCCTGAACCGAAACATAAAATGAATGCA
5'-BirA-for citrine fusion	TTTTCCGCGGATGGCCACCTATGACGTCCCAGATTAG
3'-BirA-for citrine fusion	CCCTTGCTCACCATAGATCTGGGCCCGGGATTT
5'-citrine for BirA fusion	CCCAGATCTATGGTGAGCAAGGGCGAGGA
3'-citrine for BirA fusion	TTTACTTGTACAGCTCGTCCATGC

2.1.3 LexPR driver construct

Cerulean-SV40-FRT-kan-FRT was made by replacing Citrine in Citrine-SV40-FRT-kan-FRT with Cerulean by digestion with SacII and BsrGI. The LexPR transactivator was synthesised based on the sequence obtained from Dr Alexander Emelyanov and Dr Sergey Parinov as six 400 bp G-blocks (Integrated DNA Technologies) with 20 bp overlaps, with the potential splice donor and splice acceptor sites, that could interfere with proper production of the LexPR transcript, had been removed in the synthesis. The LexPR transactivator was assembled by two rounds of fusion PCR using primers A and B to assemble 3 G-blocks, primers C and D to assemble the following 3 G-blocks and primers A and D to assemble the final fusion product. Cerulean-SV40-FRT-kan-FRT plasmid was linearized with SacII and LexPR-Cerulean-SV40- FRT-kan-FRT construct made by Infusion cloning using primers E and F, listed below, where the overlap with the effector construct is shown in blue.

PRIMER NAME	SEQUENCE
A) 5' flanking primer driver	CGAATGGCACCCAAGAAGAAGAG
B) 3 primer fusion primer driver	GACTGGATAAATGTATTCAAGCAGTACAGA
C) 5' fusion primer driver	TCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGTGTT
D) 3' flanking primer driver	TCCGGGCCCGGGATTTTCCTCCAC
E) 5' infusion primer driver	TCCCGGCCGCCATGGCGGCCGCATGGCACCCAAGAAGAAGAG
F) 3' infusion primer driver	CTTGCTCACCGATCGCGACCGGGCCCGGGATTTTCCTCCAC

2.2 Bacterial Artificial Chromosome (BAC) Recombination

BAC DNA was obtained from BACPAC Resources Centre. BAC recombination was achieved as previously described[97]. Briefly, BAC clones containing the neutrophil-specific myeloperoxidase (mpo) gene, macrophage-specific macrophage expressed gene 1 (mpeg1) and the melanocyte-specific micropthalmia-assocaited transcription factor (mitfa) and kita were identified using Ensembl[34, 37]. For BAC manipulation, the purified mpeg1 (73k14), mpo (CH211 1F11), mitfa (CH73 334I17) and kita (CH211 179C6) BAC DNA was introduced into competent SW105 E.coli cells by electroporation (1600 V, ± 4 ms).

The BirA-2A-Citrine-sv40-FRT-kan-FRT cassette was amplified with gene specific primers (listed below) homologous to 50 bp (shown in red) upstream and downstream of the first exon of mpo. Plasmid DNA was removed by digesting the PCR product with DpnI (3 µL, 37 °C, overnight). For mpeg1, oligos were ordered that were homologous to 100 – 200 bp upstream and downstream of the first exon of mpeg1 and fusion PCR carried out with the BirA-2A-Citrine-SV40-FRT-kan-FRT cassette followed by subcloning into the pGEM –T easy vector (Promega) and the plasmid digested with NdeI (shown in blue) and KpnI to yield

the BirA-2A-Citrine-sv40-FRT-kan-FRT cassette with 100 – 200 bp arms homologous to upstream and downstream of the first exon of mpeg (shown in red). Similarly, oligos were ordered that were homologous to 100–200 bp upstream and downstream of the first exon of kita and mitfa and fusion PCR carried out with the LexPR-Cerulean-SV40-FRT-kan-FRT cassette followed by subcloning into the pGEM –T easy vector (Promega). Digestion with SnaBI (shown in blue) similarly yielded the LexPR-Cerulean-SV40-FRT-kan-FRT cassette with 100 – 200 bp arms that were homologous to upstream and downstream of the first exon of either kita or mitfa. This was followed by electroporation into SW105 cells with mitfa or kita BAC DNA.

Primer Name	Sequence
5' mpo recombination primer	AATGCATGTTATGTGTTTACTCTATAAATGTCTCAATATGTGTTTTTAGAGATGGCCACCTATGACGTCCCAGATTACG
3' mpo recombination primer	ATTGTTAAGCATTACCTGTTTTAATTTTGTTGGCATAAAACATAAACTTACTGATTGAGCTTTTCCAGAAGTAG
5' mpeg recombination oligo	CAAAATCTATAATCACCATATTTGATTGAAAATCACATTAGTGCAGGAGACAGCAAAACACCCATCTGGCGATCATGGCCACCTATGACGTCCCAGATTA
3’ mpeg recombination oligo	GCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGTAACTTCTCCATGGTAGCCTCCAAAAAAGCCTCCTCACTACTTCTGGAAAAGCTCAATCAGTAAGTACAAAATAATGATAATGAAAAAAAGAGCTATATGTATATGATAGTTATACATATGTAGGCCCGCACGGAATAATCACTAGTTTTT
3’mitfa recombination oligo	GGAAAAGCTCAATCAGTGAGATGGGATTTTGTGTGTTAACCTTGAAGAAAAATATTCTTTAACACATATTAAGACGTAAGCAGATTAGAATATTCATTTAATCTTCTGTATGTTTGCATGCTTGCATATTTATAGACTGAGCGGTTATAAAATACGTA
5’ mitfa recombination oligo	TACGTAAAAAAAACAATATGAGAAGAGCAATCCCCTCCCACCCCCTTAAATACATTTTGTGTCTTTTCTTTGGCATTGACATCTTCAGCTGGCCAAGACGACTGGTCAGTTCTTGCACATCACCTAAATTCACAAAGGGAATACTTTTTTGAAGCTAACACATAGTTGAACATGGCACCCAAGAAG
5’ kita recombination oligo	TACGTAGAAAACAAGGCTGGCCCTTTAAGACGGCAGTGATCGCTCCGCAGGCGACCGTTCGGCGTTATTATCGAGCTCCAAGACTATACAACACACATGAGATAGATGGACTTATAACAATGGCACCCAAGAAG
3’ kita recombination oligo	GGAAAAGCTCAATCAGTAGGGCGGCTGTTATCTTTTTTTTGCTTTTTTTATGGTCTTACTTTGTTCCCCAAGCTTAAAGATAAACTAGAAGTTGTTCAGTGAGGAGTAGGGATGTGCACTTGCTGAGAAGAGAGCCTTGCTGAGTTACGTA

The BirA-2A-citrine-sv40-FRT-kan-FRT and LexPR-Cerulean-SV40-FRT-kan-FRT cassettes with homologous arms were electroporated into SW105 cells containing mpo, mpeg1, kita and mitfa BAC DNA respectively following heat activation of the λ prophage homologous recombination system. The SW105 strain contains the Flp recombinase expression cassette under the control of arabinose inducible promoter. Activation of flippase removes the kanamycin cassette embedded between short flippase recognition target (Frt) sites from the modified BAC[97, 98]. In a second recombination step, an iTol2-Ampicillin cassette (provided by Prof Kawakami) was introduced into the BAC backbone as previously[99, 100]. The iTol2 casette was amplified by PCR using primers. 500 ng of the PCR product was electroporated into the SW105 cells containing the mpeg1, mpo, kita or mitfa BAC DNA, and harbouring BirA-2A-citrine-SV40-FRT or LexPR-Cerulean-SV40-FRT accordingly.

2.3 Zebrafish Care and Strains

Zebrafish lines were handled in compliance with local ethical approval from the University of Oxford and in accordance with UK Home office regulations.
Zebrafish were maintained according to standard protocols (zfin.org).

2.4 Wholemount *in situ* hybridisation

Wholemount *in situ* hybridization was performed as previously described with minor modifications[101]. The template for mitfa, mpo, mpeg1, atf3, mmp13a, gpr55a, cxcl-c1c, sdf-2l1 antisense probes was generated by PCR amplification of cDNA from 3 days post fertilization (dpf) wildtype zebrafish, using the following primers:

Primer Name	Sequence
5' mpeg1 sp6 sense	GATTTAGGTGACACTATAG**CTGCAACCTGCATCCACTTA**
3' mpeg1 t7 antisense	TAATACGACTCACTATAGGG**CTGTTGGTGTTGGGAGGTCT**
5' mpo sp6 sense	GATTTAGGTGACACTATAG**GCTGCTGTTGTGCTCTTTCA**
3' mpo t7 antisense	TAATACGACTCACTATAGGG**CGCAGTTCCTTTGCTAGTCC**
5' cxcl-c1c sp6 sense	GATTTAGGTGACACTATAG**CACCGCAGCATTTCCTACAT**
3' cxcl-c1c t7 antisense	TAATACGACTCACTATAGGG**ATCCGGGTGTTTTCAGGTCT**
5' GPR55a sp6 sense	GATTTAGGTGACACTATAG**AGCCTGCTCTACGTCAACAT**
3' GPRR55a t7 antisense	TAATACGACTCACTATAGGG**TGGTTCAATTCCATGTCTACAGC**
5' atf3 sp6 sense	GATTTAGGTGACACTATAG**TCGAGGAGCTGAAGAACCAA**
3' atf3 t7 antisense	TAATACGACTCACTATAGGG**TCCTGGGAGATTAGTAGCAGC**
5' mmp13a sp6 sense	GATTTAGGTGACACTATAG**CCCAATGGATTTCTGGCTCA**
3' mmp13a t7 antisense	TAATACGACTCACTATAGGG**AAATACTGATGGCTCGGATTCT**
5' sdf2l1 sp6 sense	GATTTAGGTGACACTATAG**CAGTCAGTAACGGGTGTGGA**
3' sdf2l1 t7 antisense	TAATACGACTCACTATAGGG**ACTAAGGCGTTCATTCATCAAC**

The PCR product was gel extracted and purified by phenol chloroform extraction. 1 µg of template DNA was transcribed using T7 RNA polymerase (Promega, #P207B) in the presence of digoxygenin (DIG)-labelled dNTPs (Roche, #12430721). Probes were purified by two passes through G-50 micro-columns (GE Healthcare, #28-9034-08). Before in situ hybridization, fixed embryos (stored at -20 °C in methanol) were rehydrated in PBST. Embryos older than 24 hours post fertilization (hpf) were permeabilised with proteinase K in phosphate buffered saline Tween-20 (PBST)/diethylpyrocarbonate (DEPC) (10 *µ*g/mL) for 30 minutes, followed by glycine solution in PBST/DEPC (2 mg/mL) for 10 minutes and then fixed with paraformaldehyde (PFA) (20 minutes at RT). 24 hpf embryos were permeabilised with 1% H_2O_2. Embryos were then equilibrated with hybridization buffer (50% Formamide, 1.3 × SSC pH 5.0, 5 mM EDTA pH 8.0, 200 µg/mL Baker's yeast tRNA, 0.2% Tween-20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 µg/mL Heparin) and incubated overnight with DIG-labeled probes at 68 °C. Embryos

were blocked with 20% sheep serum+2% Boehringer Blocking Reagent (Roche, cat # 11096176001). Probes were detected with an anti-DIG-alkaline phosphatase antibody (Roche, cat # 11093274910) and the signal was developed using BM Purple AP (Roche, cat # 11442074001).

2.5 Generation of transgenic fish lines

2.5.1 Transposase RNA synthesis

Tol-2 and pAc transposase RNA was synthesised in vitro using pMTB2-tol2 plasmid kindly provided by Dr. Le Trinh and pAc-transposase plasmid, kindly provided by Drs. Emelynov and Parinov. DNA was linearised and capped mRNA was synthesised using the mMESSAGE mMachine SP6 kit (Ambion, cat # AM1340M). RNA was purified using the RNeasy mini kit (Qiagen, cat # 74104).

2.5.2 Microinjections

For pCrysB:ECFP-LexOP-mCherry-Ras, 5-10 pg of plasmid DNA was co-injected with 25-50 pg of in vitro synthesised transposase mRNA of the maize dissociation element[96]. For BAC transgenesis, BAC DNA was purified with the GenElute Endotoxin-free Plasmid Midiprep kit (Sigma, cat # PLED35). Wiltdtype embryos were injected at the one-cell stage with 200 ng/µL purified BAC DNA and 100 ng/µL tol2 transposase mRNA. All microinjections were carried out using a nanoject II (Drummond scientific company, cat # 3-000-206AO).

2.5.3 Transgenic Zebrafish Genotyping

The injected fish were raised and out-crossed to the wildtype, and the resulting embryos were screened for the fluorescent transgene on an Olympus MVX10

stereomicroscope. For *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129,ox130} and *TgBAC(mpeg1:BirA-2a-Citrine)*^{ox122}, three month old zebrafish were outcrossed to wildtype, DNA isolated using the Purelink Genomic DNA minikit (Invitrogen) and the clutch screened for expression of the transgene by PCR. Offspring of positive founders were then grown to three months, anaesthetised (0.1 mg/mL tricaine), and a small portion of the caudal fin removed and genomic DNA isolated using the Purelink Genomic DNA minikit (Invitrogen). Transgene expression in the F1 generation was similarly identified by PCR amplification of the transgene.

2.6 Tail transection

3 days post fertilisation (dpf) larvae were anaesthetised with 0.4 % 3-aminobenzoic aid ethyl ester (tricaine) in E3 and tail transection was performed with a sterile scalpel as previously described[34].

2.7 Whole-mount immunofluorescence and imaging

Embryos/larvae were fixed in 4% PFA in Phosphate buffered saline (PBS) (0.1 M, pH 7.4) for 2 hours at RT. Embryos were then rinsed 3X15 minutes in PBT (2% DMSO, 0.5% Triton in PBS), incubated in block (10% donkey serum in PBT) for 2 hours at RT, followed by incubation with primary antibody (1:200 in block) O/N at 4 °C. Embryos/larvae were then washed 3-5 X 1hour at RT + O/N at 4 °C in PBT and then incubated with secondary antibody (1:500 in PBT) followed by a final wash step of 6-8 X 1 hour at RT + O/N at 4 °C in PBT. Primary antibodies used in this study were, mpeg1 (GeneTex, cat # GTX54246), mpx (GeneTex, cat # GTX128379), and the conjugate αGFP-Alexa488. The secondary antibody used was AlexaFluor-488 IgG (Invitrogen). mCherry signal was boosted using the RFP-booster as per the manufacturers instructions (Chromotek, rba594). Images were

taken on a Zeiss 780 confocal microscope with either a 10x or a 25x objective and × 0.7 digital zoom.

2.8 Edu labelling of proliferating melanocytes

At 96 hours post fertilization 5 nL 5'-ethynyl-2'-deoxyuradine (Edu) (150 μM in 2% DMSO, 0.1% phenol red) was injected into the pericardium of zebrafish embryos. The embryos were incubated overnight and labelled with the Click-iT Edu Alexa Fluor Imaging kit (Invitrogen) as per the manufacturers instructions. Fluorescence was visualised with a Zeiss 780 inverted confocal microscope with a 25x objective and × 0.7 digital zoom.

2.9 *M. marinum* Culture Preparation

M. marinum (ATCC # BAA-535) was grown at 30 ^{o}C in Middlebrook 7H9 broth (Difco) containing 0.5% BSA, 0.2% glucose, 0.2% glycerol, 0.085% NaCl, catalase and 0.05 % Tween80. Cultures were grown to an optical density at 600 nm and maintained at -80 °C in freezing medium (30% glycerol, 0.05% Tween 80). A M. marinum glycerol stock was transformed by electroporation (2.5 kV, 4.5 ms pulse) with a pTEC27 expression vector (Addgene) and plated on 7H10 agar, supplemented with 100 μg/mL hygromycin. Transformed colonies were picked and a single cell suspension prepared as previously described[102]. Briefly, a single colony was inoculated in 10 mL of 7H9C in a 50 mL flask, incubated at 33 °C for 10 days, the optical density was measured at 600 nm, and 50 mL of 7H9C inoculated in a 250 mL flask. The flask was incubated at 33 °C for 8 days until an OD_{600} of 0.5 was reached. The culture was then pelleted by centrifugation (4000 g, at RT, 15 mins), the supernatant removed and the pellet resuspended in 1 mL

of 7H9OADC (4.7% w/v 7H9 broth base, 2% glycerol, 10% OADC where OADC is 5% w/v BSA, 2% w/v glucose, 8.5% w/v NaCl, 2% of 2.5% w/v oleic acid in 0.2 N NaOH) and then aliquoted into 200 µL aliquots. Using a 27-gauge tuberculin syringe, the bacteria was aspirated and ejected 10 times. 1 mL of 7H9OADC was added to each 200 µL aliquot and then centrifuged (100g, 1 min). The supernatant was collected and transferred to a 15 mL centrifuge tube and the process repeated with the remaining 200 µL twice more. The final collected syringed culture was then process through a 5 µL sterile filter to isolate single bacteria. The filtrate was then centrifuged (20000 g, 5 mins, RT) and the supernatant discarded. The pellet was resuspended in 200 µL 7H9OADC and diluted until 100 bacteria/nL was reached. Bacteria was stored at -80 °C.

2.10 *M. marinum* injections

Embryos were injected at the 32-cell stage (20 CFU *M. marinum* in PBS, 0.1% phenol red). Embryos were maintained under standard conditions.

2.11 *In vivo* 'biotagging'

2.11.1 Nuclei Isolation

Zebrafish embryos were anaesthetised with Tricaine (0.01%) and washed in hypotonic buffer H (20 mM HEPES (pH 7.9), 15 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 1 X Complete protease inhibitor) and subsequently resuspended in buffer H (500 µL/50 embryos). Embryos were transferred to a Dounce homogenizer (2 mL Kontes Glass Co, Vineland, NJ) and dissociated by 10 strokes with the loose fitting pestal A and incubated on ice for 5 minutes. Further dissociation was carried out by 10 strokes with tight fitting pestal B, every 5

minutes for 15 minutes. Nuclei were collected by centrifugation (2000 g, 4 °C) and resuspended in 1 mL buffer NDP (10 mM HEPES (pH 7.9), 40 mM NaCl, 90 mM KCl, 0.5 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol and 1 X Complete protease inhibitor). For nuclei purification, nuclei were incubated with 5 mg of M-280 streptavidin-coated dynabeads prepared for RNA procedures (2.11.2) with rotation for 30 minutes at 4 °C. A flow-based system based on the previously published protocol by Deal and Henikoff[103] was used to capture the nuclei bound on the streptavidin beads. A 10 mL serological pipette (VWR, cat # 8913-898) attached to a 1 mL micropipette tip (Rainin reach pipet tip, cat # RT-L1000S), both pre-treated with NPB/BSA for 30 minutes, and added to a MiniMACS separator magnet (OctoMACS Separator, Miltenyl Biotec, cat # 130-042-109). A two-way stopcock (Biorad cat # 732-8102) was connected to the end of the 1mL micropipette tip via a piece of Tygon tubing (Fisher Scientific, cat # 8220) and the flow-rate set to ~0.75 mL/min. The nuclei beads suspension was diluted by addition of 9 mLs of nuclei pulldown buffer with 0.01 % Triton X-100 (NPBt) and added to the slow-flow setup. The tip was subsequently removed from the stand and the nuclei-beads released from the tip by slowly pipetting 1 mL of NPBt in and out of the tip. The solution was then diluted again to 10 mL with NPBt and added again to the slow-flow setup. Nuclei-beads were eluted in 1 mL of NPBt as described above and the NPBt removed using a magnetic stand (DynaMag ^{TM}-2 magnet, Invitrogen, cat # 12321D). Nuclei-beads were then processed for RNA-seq analysis (2.12).

2.11.2 Processing of streptavidin beads in RNasefree conditions

M-280 streptavidin-coated beads (Invitrogen, cat # 11205D) were vortexed (2

minutes), transferred to a microcentrifuge tube and washed twice with 1 mL of Solution A (DEPC-treated 0.1 M NaOH, DEPC-treated 0.05 M NaCl) on a nutator (2-3 minutes, 4 °C) followed by separation using a magnetic stand stand for 3 minutes or until the solution was clear (DynaMag™-2 magnet from Invitrogen, catalogue # 12321D). Beads were then washed in Solution B (DEPC-treated 0.1 M NaCl), separated as before on a magnetic stand and then transferred to 50 µL of NBP until the nuclei suspensions had been processed.

2.12 RNA Extraction, library preparation

RNA extraction and DNAse treatment were carried out using the RNAqueous®-Micro Kit (Life Technologies, cat # AM1931). RNA integrity was checked with a RNA pico chip (Agilent Technologies, cat # 5067-1513) using the Agilent 2100 Bioanalyzer.

cDNA was synthesized and amplified from 100 pg – 1 ng of input RNA using SMART-seq™v4 ultra low input kit for RNA (Clontech laboratories, cat #'s 634888, 634889, 634890, 634891, 634892, 634893, and 634894). For cDNA libraries of transcriptional profiling of neutrophils in response to *M. marinum*, sequencing libraries were prepared using NEBNext® Ultra™ RNA Library Prep Kit and Illumina Single Read 75 bp and sequencing was performed on a HiSeq2000 Illumina platform. For cDNA libraries for transcriptional profiling of neutrophils in response to oncogene-transformed melanonocytes sequencing libraries were prepared using Nextera XT DNA library preparation kits and sequencing was performed on a NextSeq platform using a NextSeq™ 500 High

Output Kit for the generation of 80 base pair paired end reads (150 cycles) (Illumina, cat # FC-404-1002).

2.13 RNA-seq analysis

Read quality was evaluated using FastQC[104]. Adapter contamination was removed using Scythe[105] and reads further trimmed based on base quality using Sickle[106]. Adapter-removed and quality trimmed reads were mapped to the (Jul. 2010 Zv9/danRer7 assembly) version of the zebrafish genome using STAR (v.2.4) splice-aware aligner[107]. Count tables were generated using subread FeatureCounts, using standard parameters[108]. For *M. marinum* analysis, differential expression analysis was carried out in two biological replicates. For oncogene-transformed melanocyte anaylsis, the analysis focuses only on the tail samples and hence was carried out for biological duplicates (tail control 1, tail control 2, tail $NRas^+$ 1, tail $NRas^+$ 2), and the second experimental replicate (tail $NRas^+$ 2) eliminated because the read depth and quality was not equivalent to the other two samples. Differential expression was carried out using DESeq2 R package[109]. Genes that were found to be differentially expressed with an adjusted p-value of < 0.05 were analysed further using gene ontology (GO) annotation[110, 111].

2.14 sgRNA mediated knockdown

2.14.1 sgRNA synthesis

Two different sgRNA template DNAs were generated with a unique oligonucleotide encoding the T7 polymerase-binding site (underlined) and the sgRNA target sequence and the overlap with the tracrRNA (blue) (cxcl-c1c_1

forward: GAAATTAATACGACTCACTATAGGGCTGTTATTCCATTACAGTTC GTTTTAGAGCTAGAAATAGC and cxcl-c1c_2 – forward: GAAATTAATACGACTCACTATAGGGCTCGCAGGGACACCATTA GTTTTA GAGCTAGAAATAGC) and a second oligonucleotide with the tracrRNA (overlap in blue) (AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTG ATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC). Oligos were annealed and amplified by PCR using Pfu Ultra II Hotstart PCR Master Mix (Agilent, cat # 600850) with cycles of (95 °C 5 mins, 35 cycles of (95 °C 30 s, 60 °C 30 s, 72 °C 15 s), 72 °C 10 min, 10 °C ∞) and purified by gel purification, followed by phenol chloroform extraction. *In vitro* transcription was performed with 500-1000 ng purified DNA template using the T7 RNA polyemerase kit (NEB) for 4 h at 37 °C, and sgRNA purified using the Megaclear kit (Ambion, cat # AM1908).

2.14.2 Cas9 mRNA

Cas9 mRNA was *in vitro* transcribed from plasmid MLM3613 (Addgene, cat # 42251) using the mMESSAGE mMACHINE T7 kit (Ambion, cat # AM1344).

2.14.3 HRMA analysis

gDNA was extracted from single embryos using the Purelink Genomic DNA minikit (Invitrogen). Primers, for sgRNA: cxcl-c1c_1 (5': CAGCAGGGAGAAGGTAAGACA; 3': TGACTCTTCACACTGGCACC) and sgRNA: cxcl-c1c_2 (5': GACCGGTGCCAGTGTGAAG; 3': ACTTTGTCGCAGTTTGGTCTTT) were designed to generate a ~ 200 bp product spanning the cut site. Hotshot Diamond PCR mastermix (Client Lifescience, #HS002-TS) was used to perform PCR with LC

Green Plus dye (BioFire Diagnostics, #BCHM-ASY-0005). Reaction solutions were cycled on a C1000 Touch™ Bio-Rad thermal cycler using (95 °C – 5mins, 49 cycles: 95 °C – 20 secs, 60 °C – 20 secs, 72 °C – 30 secs, followed by 72 °C – 2 mins, 95 °C – 30 secs, 25 °C – 30 secs, 95 °C – 30 secs) and the final melt curve (65 °C – 95 °C with increments of 0.2 °C for 0.02 secs) and then plate reading for melt analysis.

Chapter 3

Establishment of a binary transgenic zebrafish system for exploring the myeloid compartment at a gene regulatory level

3.1 Introduction

3.1.1 Zebrafish as a model for inflammation

Myeloid-mediated inflammation, a vital component of host defence, occurs in response to wounding and infection by pathogens[112]. In response to pathogen infection, a cascade of signals leads to the influx of neutrophils, followed by monocytes, which mature into inflammatory macrophages and exert effector functions by the production of cytokines and chemokines, phagocytosing of infectious agents and the activation of the adaptive immune response[113]. However, inflammation can also occur in the absence of pathogens as in the case of trauma- or chemically-induced tissue injury. As with pathogen-induced inflammation, sterile inflammation results in the recruitment of macrophages and neutrophils to the inciting stimulus and the production of cytokines and chemokines[1]. An example of immune cell infiltration into a tissue in the absence of a microorganism, is in cancer, where immune cells invade the tumour and affect tumour progression[1]. Previous transgenic zebrafish models have provided useful tools for modelling leukocyte behaviour in wound healing, response to pathogens and tumourigenesis, with coordinated responses of cell influx and effector functions observed *in vivo*[34, 36].

To date both larval and adult zebrafish have been used to study the myeloid response under different conditions of inflammation. Previous models for studying macrophage and neutrophil function in zebrafish have revealed how neutrophils rapidly accumulate at wounds and bacterial foci and allow for the visualisation of primitive macrophages as they phagocytose particles and bacteria[44]. Studies

using zebrafish have focused on the response of macrophages and neutrophils to intracellular pathogens including bacterial infection (*Mycobacterium marinum*[65], *Streptacoccus iniae*[114], and *Salmonella enterica serovar typhimurinum*[49]), fungal infection (*Candida albicans*[115]) and viral disease[44]. More recently, the zebrafish has emerged as a new model organism in cancer biology, with different models being developed to study aspects of oncogenesis in zebrafish, including immunity[116]. Recruitment of macrophages and neutrophils to oncogene transformed cells in a larval melanoma model, as well as the interaction and ultimate engulfment of transformed cells by macrophages was observed by 48 hours post fertilization (hpf)[36]. Thus, the zebrafish has proved to be a useful tool to model both sterile and non-sterile inflammation. To fully understand the inflammatory response under conditions of sterile and non-sterile inflammation, we need to be able to dissect the mechanisms that govern these interactions at a gene regulatory level. To do so, we need to isolate pure populations of effector cells and carry out genome-wide profiling at both transcriptional and epigenomic level under different conditions of inflammation.

3.1.2 Generating a zebrafish model to study the myeloid response to inflammation at systems level

3.1.2.1 Myelopoiesis in zebrafish

Zebrafish have a multi-lineage myeloid compartment comprising of granulocytes (neutrophils, eosinophils and circulating basophils/tissue-resident mast cells), circulating monocytes/tissue-resident macrophages[117] and dendritic cells, making up the innate immune system of the developing embryo[37, 118, 119]. In this study we have chosen to focus on macrophages and neutrophils. Primitive myeloid

precursors arise in the anterior lateral mesoderm and migrate to the yolk where they differentiate into primitive macrophages by 22 hpf and primitive neutrophils by 33 hpf. Macrophages and neutrophils can be recruited to sites of infection and exhibit phagocytic behaviour from 30 hpf and 52 hpf, respectively[42].

3.1.2.2 Macrophage and neutrophil-specific markers

A key step in making a cell-specific transgenic line is identifying a gene specifically expressed in a given cell type and subsequently using its regulatory elements to drive tissue specificity. The neutrophil is the most abundant zebrafish granulocyte, and as with its human counterpart, this cell has a multi-lobed nucleus and heterophillic cytoplasm filled with granules containing various enzymes necessary for its function[117]. The primary granule protein, myeloperoxidase, is specifically expressed in neutrophils[117]. A mpo-driven GFP reporter line where transgene expression is seen specifically in the neutrophils already exists[34].

Multiple genes have been suggested as markers for the monocyte/macrophage lineage but have subsequently been found expressed in other immune cells. L-plastin is an actin-binding protein that is present in human peripheral blood leukocytes, monocytes/macrophages, B lymphocytes, T lymphocytes and granulocytes[42]. However, in zebrafish L-plastin is a well-described myeloid lineage marker, with early studies showing L-plastin expression in macrophages migrating along the yolk of early embryos[40]. Previous studies have identified macrophages based on elimination using L-plastin and neutrophil-specific expression of mpo, where cells not expressing mpo were presumed to be macrophages[37]. Colony stimulating factor 1 receptor (CSF1R, or c-fms), was

shown to be co-expressed with L-plastin but not mpo and hence thought to be a suitable marker for macrophages. However, CSF1R is also expressed in neural crest derivatives[120]. PU.1/Spi1 is a transcription factor expressed specifically in cells of the hematopoietic lineage[121]. Knockdown and gene expression analysis revealed Cxcr3.2, mpeg1, ptpn6 and mfap4, targets of PU.1/Spi1, show expression specifically in early macrophages[121]. Mpeg1 expression has been tightly restricted to human and murine macrophages[37]. In a previous study, Ellet *et al.,* generated transgenic zebrafish lines based on the mpeg1 promoter and showed there was no overlap between mpeg1- and mpo-reported cells[37]. In light of this, the transgenic models generated here for studying macrophage and neutrophils make use of the mpeg1 and mpo reporters, respectively.

3.1.3 *In vivo* biotagging in zebrafish – affinity purification of nuclei from specific cell types

Genome-wide analysis of the regulatory networks that govern the inflammatory response *in vivo* is complicated by the fact that relatively small number of responding cells are present within complex tissues[103]. The current zebrafish lines for studying macrophage and neutrophil function utilise the mpeg1 and mpo promoters respectively, and make use of fluorescent reporters[34, 37]. These lines have largely been used for functional studies at the cellular level, allowing for a detailed examination of host-pathogen interactions, a comparative analysis of macrophage and neutrophil behaviours as they respond to inflammation and the dynamic interaction between the two cell types[34, 37]. To study the gene regulatory landscape of macrophages and neutrophils *in vivo* using the current transgenic lines would require either laser capture microdissection (LCM) of sectioned tissues or fluorescent activated cell sorting (FACs). Both techniques, however,

require extensive tissue manipulation and extended processing times in which cell state and gene expression may change. This coupled to a relatively low yield obtained by these techniques, makes transcriptional and epigenomic profiling of individually isolated cells difficult. The Sauka-Spengler laboratory has developed a genetic system for tissue-specific *in vivo* biotinylation of target proteins in zebrafish termed 'biotagging'. The *in vivo* biotinylation approach involves the co-expression of a biotin acceptor peptide (Avi-tag) and bacterial biotin ligase, BirA in the same cells, allowing labelling of the Avi-tagged protein with single biotin residue for single-step streptavidin-based affinity purification. Biotagging is a binary zebrafish genetic system that consists of stable transgenic 'effector' lines that express Avi-tagged proteins of interest, and biotinylation 'driver' lines, that express bacterial biotin ligase, BirA, in tissue-specific fashion. Embryos issued from crosses between tissue-specific biotinylation drivers and effector lines will have the Avi-tagged target protein biotinylated only in the specific group of cells that express both biotagging alleles. These embryos can then be used in affinity purification approaches, to isolate specifically biotinylated target proteins together with molecular and cellular components associated with them, including other protein complexes, cross-linked chromatin and different subcellular compartments such as nuclei and polysomes[103]. One of the biotagging effector lines generated in the lab, when crossed to tissue-specific BirA driver, allows for a high stringency affinity purification of biotinylated nuclei from selected cell types, using INTACT approach (Isolation of Nuclei Tagged in specific Cell Types) method, previously developed in plants[103]. This biotagging effector line (*Tg(bactin:Avi-Cerulean-RanGap)*^{ct700a}) features ubiquitous expression of a zebrafish-compatible version of Avi-tagged nuclear envelope-associated fusion protein, containing the outer

nuclear envelope-targeting sequence (carboxyl-terminal domain of Ran GTPase-activating protein1, RanGap1), fused to Cerulean and Avi-tag (Figure 3.1 B). This fusion protein, localizes to the outer nuclear envelope (Figure 3.1 B) and acts as a substrate for the cell-specific *E. coli* biotin ligase BirA (Figure 3.1 A) which results in deposition of biotin to the Avi-tag and "biotinylation" of nuclei in a tissue-specific fashion[122] (Figure 3.1 C). Using streptavidin-coated magnetic beads, the nuclei from the specific cell types, can be isolated and their contents analysed genome-wide using RNA-seq, chromatin immunoprecipitation (ChIP)-seq and assay for transposase-accessible chromatin (ATAC)-seq approaches. The mRNA levels within the nucleus represent the newly transcribed RNA and give an accurate picture of the actively transcribed regions of the genome, hence providing insight into the current transcriptional program of the cell[123].

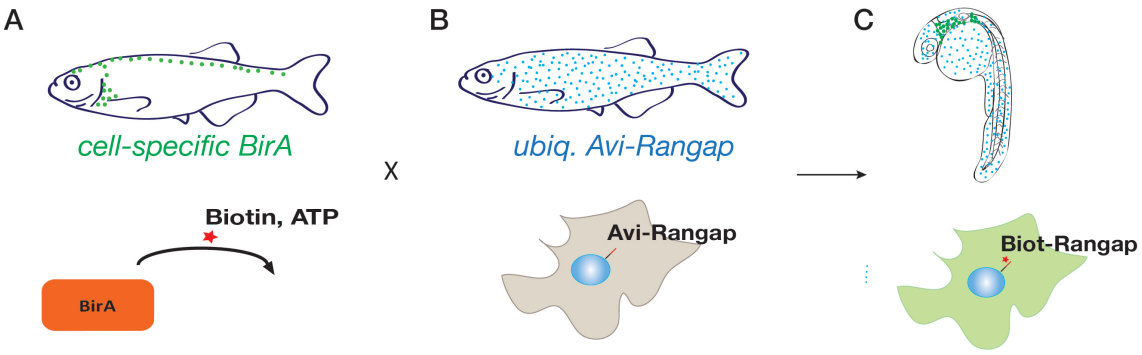


Figure 3.1 Schematic of binary transgenic system for *in vivo* biotagging of nuclei. Driver line expressing BirA in specific cell populations (A) is crossed with effector transgenic fish line ubiquitously expressing Avi-tagged Rangap (B) for biotinylation of nuclei. In double transgenic fish, only the nuclei of the cell type of interest will be biotinylated (C).

3.1.4 Bacterial artificial chromosome (BAC)-mediated transgenesis

The generation of transgenic zebrafish lines that express a transgene or a fluorescent reporter in a cell- or tissue-specific manner relies on the identification of promoter fragments that recapitulate endogenous expression patterns and ideally physiological levels[99, 100]. Simply selecting and cloning functional promoter fragments containing all the regulatory elements of the transgene of choice is

difficult because of the technical challenges which may arise when cloning a transgene of this size[99]. BACs are DNA constructs that contain up to 300 kb of genomic DNA. Because of their large size, BACs can contain the complete regulatory setup of a gene required for cell type-specific and temporal expression, encompassing all or majority of long-range *cis*-regulatory elements. Transgenic lines that are generated using BAC-mediated transgenesis are more resistant to positional effects because of the large size of the BAC DNA that is integrated into the genome. BAC-mediated transgenic lines were originally made by microinjection of naked DNA into the fertilized zebrafish egg, with less than 1-3% germline transmission[99]. Similarly, linearization of the DNA results in low integration rates. The use of meganucleases and retroviral vectors have been shown to increase integration rates in transgenesis, but have a low DNA carrying capacity, ~ 5 kb and < 8 kb respectively[99]. Therefore, Suster *et al*[99]*,* adapted the use of *Tol2,* a transposase, which results in a high rate of integration into the genome (5 – 20%) and a higher carrying capacity (<160kb). The medaka Tol2 element is a DNA-type transposase. When *cis*-sequences from the left and right ends of Tol2 are placed on either side of a DNA insert, the insert is integrated into the genome via a transposase cut and paste mechanism. By inverting these minimal Tol2 ends and placing them 1 kb apart on the BAC plasmid backbone, the insert can be efficiently excised by the Tol2 transposase and inserted into the genome when microinjecting into fertilized zebrafish eggs[99].

Here I describe a new transgenic model for studying pure populations of the myeloid compartment, at a transcriptional and epigenomic level, in both larval and adult zebrafish, under different conditions of inflammation.

3.2 Results

3.2.1 Generation of BirA driver lines by BAC-mediated transgenesis

3.2.1.1 BAC recombination

To characterise the myeloid-mediated inflammatory response by *in vivo* biotagging, we generated macrophage (mpeg1) and neutrophil (mpo) BirA transgenic zebrafish driver lines by BAC-mediated transgenesis. *In situ* hybridisation for mpeg1 and mpo reveal macrophage and neutrophil expression patterns, respectively consistent with those reported by other groups[42]. At 24 hpf mpo expression was detected anteriorly over the yolk sac and in the posterior intermediate cell mass (ICM) (Figure 3.2 Ai). By 3 dpf, mpo expression was found over the anterior yolk, in the posterior ICM and in the posterior blood island (Figure 3.2 Aii). Mpeg1 transcripts were distributed predominantly over the yolk sac at 24 hpf (Figure 3.2 Aiii). By 3 dpf mpeg1 expression could be seen over the yolk sac and in the posterior ICM (Figure 3.2 Aiv).

Mpeg1- and mpo-specific driver lines were generated by Tol2 mediated transgenesis of recombineered BACs. BACs containing the entire locus of the mpeg1 and mpo genes were identified using Ensemble. A construct harbouring the open reading frame (ORF), coding for bacterial biotin ligase BirA, a viral self-cleaving peptide 2A and a Citrine fluorescent protein, followed by a SV40 polyA tail and a kanamycin selection cassette (BirA-2A-Citrine-pA-Frt-kan-Frt) were cloned using several rounds of fusion PCR (Figure 3.2 Bi). The BirA-2A-Citrine-pA-Frt-kan-Frt fragment was recombined into the mpeg1 or mpo BAC by

replacing the first exon of the corresponding gene using the bacterial recombination strain (SW105) of *E.coli*[97, 98] (Figure 3.2 Bi-ii). The SW105 strain also carries the Flippase (Flp) expression cassette under the control of arabinose inducible promoter. Activation of Flp recombinase was used to remove the kanamycin cassette from the modified BAC[97, 98] (Figure 3.2 Biii). An iTol2-Ampicillin cassette (kindly provided by K. Kawakami) was also inserted into the BAC backbone to increase the efficiency of integration into the zebrafish genome[100] (Figure 3.2 Biv-vi).

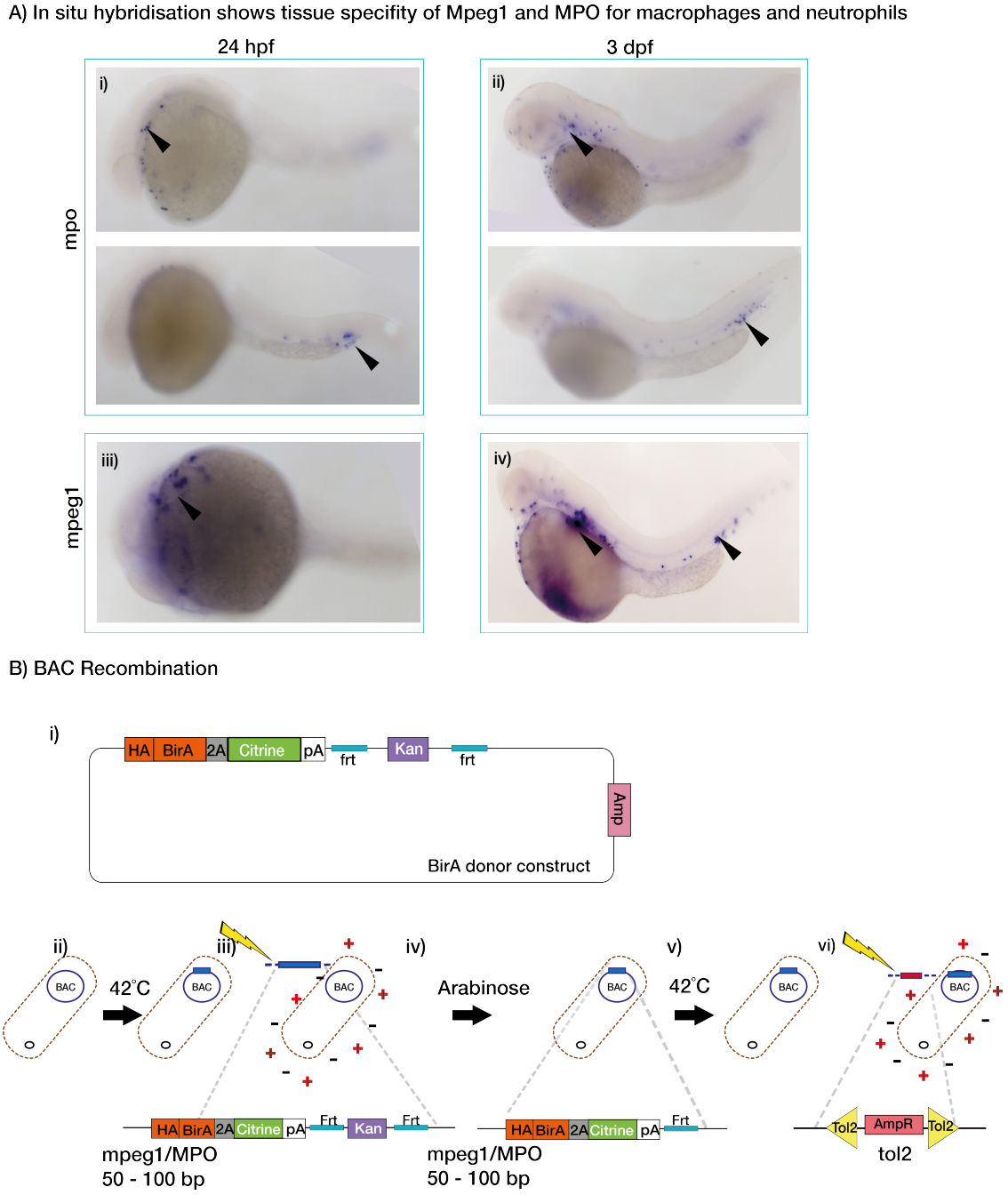


Figure 3.2 BAC recombination for macrophage and neutrophil BirA driver lines. A) *In situ* hybridisation using a digoxigenin-labelled RNA probe shows mpo expression at 24 hpf (i) and 3 dpf (ii) and mpeg expression at 24 hpf (iii) and 3dpf (iv) as indicated by the black arrows. B) Schematic of BAC recombination. BirA driver construct for BAC recombination containing hemagglutinin (HA)-tagged BirA (orange), a viral self-cleaving peptide (2A, grey) and a fluorescent reporter (Citrine, green), followed by a polyA tail (pA, white) and a kanamycin selection cassette (kan, purple) embedded between Frt sites (turquoise) (i). Heat activation (42 °C) of the λ prophage homologous recombination system in SW105 competent cells containing MPO or mpeg1 BAC DNA (ii). Electroporation of the BirA-2A-Citrine-pA-Frt-kanamycin-Frt cassette with 100–200 bp arms homologous to upstream and downstream of the first exon of either mpeg1 or mpo into SW105 cells (iii). Activation of flippase removes the kanamycin selection marker embedded between the Frt sites of the modified BAC (iv). Heat activation (42 °C) of the λ prophage homologous recombination system (v). Electroporation of iTol-2-Ampicillin cassette into the BAC backbone (vi).

3.2.1.2 BAC-mediated transgenesis

For generation of transgenic lines, modified BAC DNA (Figure 3.3 A) was co-injected with tol2 mRNA into a fertilized zebrafish embryo at the single cell stage to facilitate germline integration of the construct[100]. Injected parental embryos (F_0) were raised to reproductive age. Neutrophil and macrophage (BirA) founders were identified by outcrossing F_0 fish to wildtype fish and screening for fluorescence. However, in the macrophage BirA driver lines, the endogenous levels of mpeg1 were too low to drive sufficient Citrine expression needed for effective screening on a stereomicroscope (Figure 3.3 Bii). It should be noted, that Citrine expression in the mpeg1 line can be visualised by confocal microscopy. In this line, founder fish were screened for by genomic DNA isolation and PCR amplification of the transgene. In *TgBAC(mpo:BirA-2a-Citrine)*^{ox121} founder fish, Citrine expression is visible in circulating cells and over in the intermediate cell mass (Figure 3.3 Bi). In *TgBAC(mpeg1:BirA-2a-Citrine)*^{ox122} founder fish, staining for GFP shows expression in circulating cells and over the anterior yolk sac. The F_1 colonies of *TgBAC(mpo:BirA-2a-Citrine)*^{ox121} and *TgBAC(mpeg:BirA-2a-Citrine)*^{ox122} transgenic lines were subsequently expanded for future experiments.

The Sauka-Spengler laboratory has previously generated a ubiquitous effector line using the zebrafish beta-actin promoter to drive the expression of Avi-Cerulean-Rangap *Tg(bactin:Avi-Cerulean-RanGap)*^{ct700a} (Figure 3.3 C). Cerulean expression can be seen distributed throughout the embryo (Figure 3.3 D). To generate the binary transgenic fish lines, myeloid BirA driver lines, *TgBAC(mpo:BirA-2a-Citrine)*^{ox121} and *TgBAC(mpeg1:BirA-2a-Citrine)*^{ox122}, were

crossed to the ubiquitously expressing Avi-Rangap effector line, *Tg(bactin:Avi-Cerulean-RanGap)*^{ct700a}, and F_2 offspring, harbouring both alleles, were expanded for future experiments.

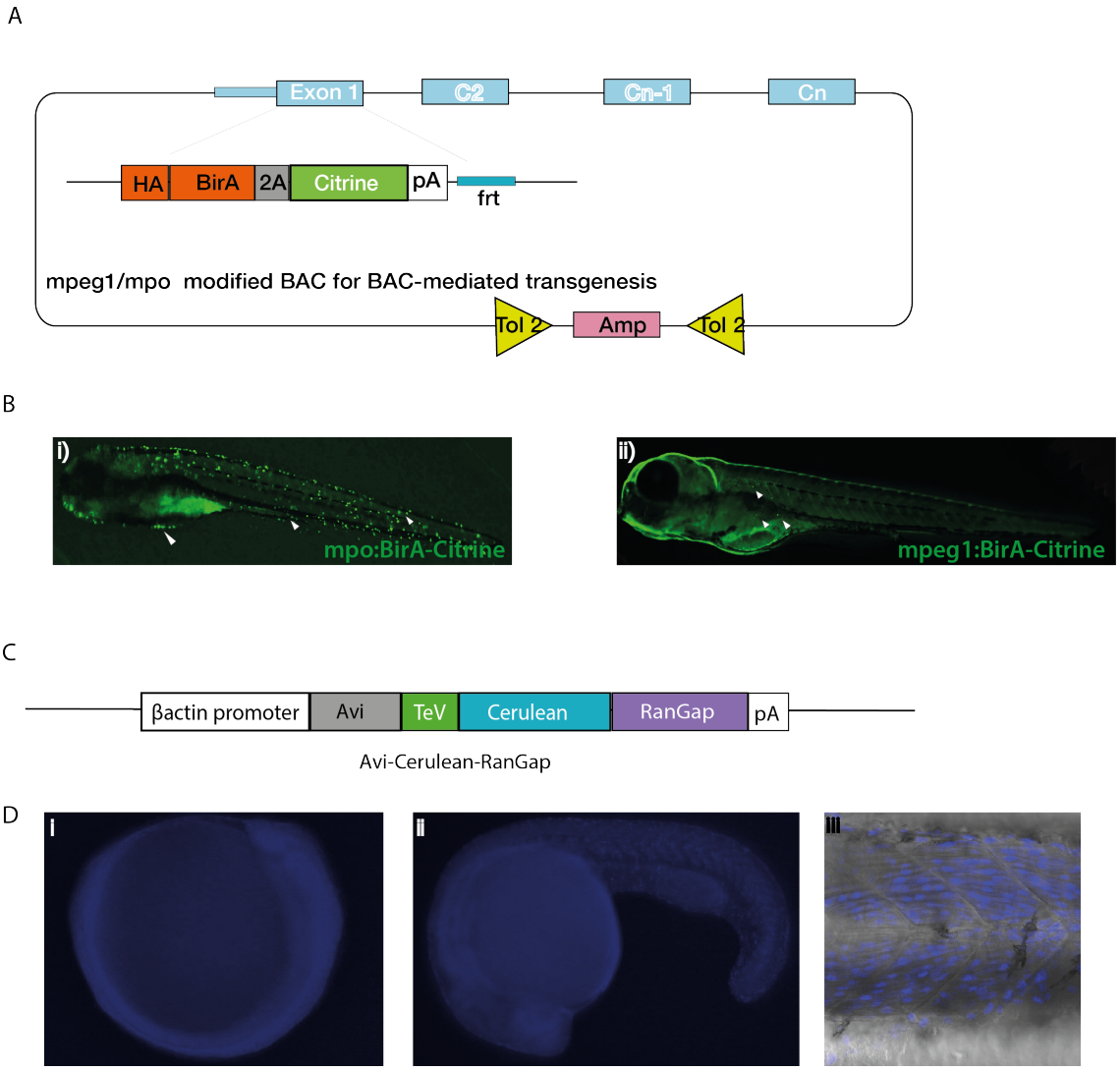
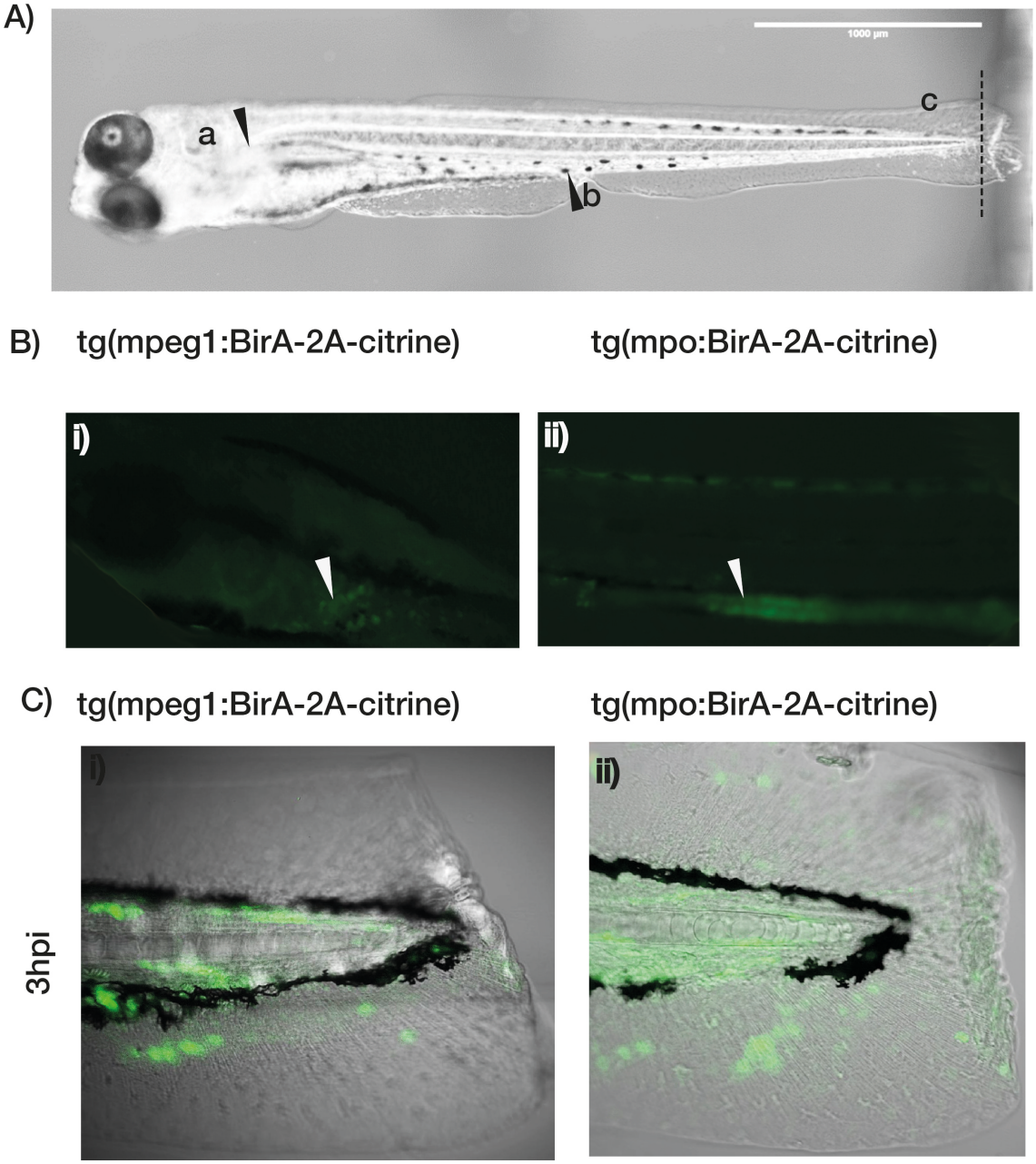


Figure 3.3 Generation of driver and effector transgenic lines. A) Schematic of mpeg1/mpo modified BAC DNA containing hemagglutinin (HA)-tagged BirA (orange), a viral self-cleaving peptide (2A, grey) and a fluorescent reporter (citrine, green), followed by a polyA tail (pA, white) and remaining Frt site, recombined into the first exon, with an ampicillin selection cassette (Amp, pink) and tol2 arms (yellow) on the BAC plasmid backbone for BAC mediated transgenesis. B) Transgene expression of neutrophils (a) and macrophages after staining with αGFP-Alexa488 (b) in founder generation as indicated by white arrows. C) Schematic of Avi-Cerulean-RanGap effector construct which contains ubiquitous βactin promoter (white), Avi-tag (grey), TeV protease site (green) fused to Cerulean (blue fluorescent reporter) and the Cterminus of RanGap (purple) (i). Wide-field image of Biotagging Avi-Cerulean-RanGap effector transgene at 14 somite stage (i) and 24 hrs post fertilization (ii). Confocal image of Avi-Cerulean-Rangap expression showing nuclear localization of Cerulean-RanGap (iii).



3.4 Macrophage ontology and behaviour in tg(mpeg1:BirA-2A-citrine) and tg(mpo:BirA-2A-citrine) stable transgenic lines. A) Schematic diagram showing anterior yolk sac (a), intermediate cell mass (b) and typical tailfin transection (c) B) Zebrafish larvae were anesthetised with 4% tricaine in E3. GFP was visualised by excitation at 492 nm on the Olympus MVX10 stereomicroscope. Images in larvae at 5dpf show citrine expression in distinct cells at 5dpf over the anterior yolk sac in *TgBAC(mpeg:BirA-2a-Citrine)*^{ox122} (i) and the intermediate cell mass in *TgBAC(mpo:BirA-2a-Citrine)*^{ox121} (ii) transgenic fish lines. C) Tailfins of anaesthetised transgenic fish were transected at 3dpf. Fish were fixed with PFA at 3hpi and stained with αGFP-Alexa488. For each image Z-stacks of fluorescent images were projected into a single image using the maximum intensity at each point. At 3hpi macrophages in *TgBAC(mpeg:BirA-2a-Citrine)*^{ox122} (i) and neutrophils *(TgBAC(mpo:BirA-2a-xt gentextext Citrine)*^{ox121}*)* (ii) can be seen responding to tail injury.

3.2.2 Mpo/mpeg1 BirA driver lines recapitulate larval inflammatory cell phenotype

The fluorescent cells in the *TgBAC(mpo:BirA-2a-Citrine)*^{ox121} and *TgBAC(mpeg:BirA-2a-Citrine)*^{ox122} transgenic lines had several defining ontological and functional characteristics, typical of macrophages and neutrophils respectively. By 5 dpf, macrophages can mainly be seen developing over the anterior yolk sac (Figure 3.4 Bi), whereas neutrophil development can be seen mainly in the posterior intermediate cell mass (Figure 3.4 Bii), as indicated by the white arrows. To show that the mpo/mpeg1 driver lines generated in this study faithfully recapitulated a larval inflammatory phenotype, we injured mpo and mpeg1 BirA driver transgenic larvae at 3 days post injury (dpi) by tailfin transection[34]. Macrophages in TgBAC(mpeg:BirA-2a-Citrine)^{ox122} (Figure 3.4 Ci) and neutrophils in TgBAC(mpo:BirA-2a-Citrine)^{ox121} (Figure 3.4 Cii) migrate in response to wounding.

3.3 Discussion

Although the current mpeg1 and mpo transgenic fish lines allow for functional studies of the myeloid component at a cellular level, deciphering the intricacies of regulatory programmes that govern inflammation is difficult. The binary system established in this study for the biotagging of nuclei of either macrophages or neutrophils provides a powerful platform to study gene expression of these individual cell types under different conditions of inflammation. Unlike traditional fluorophore-based cell sorting techniques, biotagging allows for the isolation of relatively rare cell types from complex tissues. Furthermore, given the high specificity and avidity of the biotin-streptavidin interaction, this system requires less starting material. Since both BirA driver lines were generated by BAC-

mediated transgenesis, unlike the previous mpeg1 line[37], highly reproducible expression of BirA at endogenous levels and tightly controlled spatiotemporal pattern was obtained in these lines. Transgenic BirA driver lines using a minimal promoter and proximal enhancers to drive expression, often don't recapitulate the expression patterns seen by *in situ* hybridisation[122].

Making use of the ease with which zebrafish genetics can be manipulated, the binary systems described here can be coupled to genome editing technology to further dissect the complexities of the inflammatory response. One of the biggest advantages of the zebrafish animal model is the translucency of the fish and the ease with which the dynamic interactions of different cell types can be visualised *in vivo.* Since both the mpeg1 and mpo driver lines, also have a Citrine reporter, gene regulatory studies can be coupled to functional studies using genome editing of previously identified targets. In particular, models of the early larval stages which are optimally suited for imaging and genetic and chemical screens, can give much insight into the host response to pathogens and other inflammatory stimuli[124]. Furthermore, use of this system is not only limited to studies of immunity and inflammation. Distinct aspects of haematopoiesis, cell fate mapping and understanding the development of these two distinct haematopoietic lineages could be further explored[121].

In vivo modelling of inflammation is essential for understanding the complexities of disease and translating them into therapies. In conclusion, this model will provide an exciting new platform to study inflammation, *in vivo,* in response to a range of both sterile and non-sterile stimuli.

Chapter 4

Early transcriptional signature of neutrophils in *M. marinum*-induced mycobacterial infection

4.1 Introduction

4.1.1 Neutrophils and *M. tuberculosis*

Neutrophils are rapidly recruited to sites of infection where they destroy invading microorganisms either by phagocytosis of the pathogen or extracellular release of microbicidal granules[125]. Neutrophils are the less well-studied cell type in mycobacterium infection, and much controversy surrounds their role in the pathogenesis of the disease. In pulmonary tuberculosis, neutrophils are present both in newly forming and established granulomas[125]. *Ex vivo* studies of neutrophils are not robust because they are terminally differentiated cells with a short half-life, cannot be cryopreserved and are easily activated during methods of cell isolation from blood[62]. The studies on neutrophils in mammalian models of *M. tuberculosis* present conflicting results with some studies suggesting that neutrophils play a protective role in early infection, whilst others report no such effect[62]. Some studies have focused on pathological roles of neutrophils, where excessive neutrophil accumulation is seen in advanced infection and a higher neutrophil count is associated with poorer prognosis[126].

Dogma suggests that neutrophils are recruited early to sites of infection where they recognise mycobacteria either directly or by opsonisation, mediated by activation of the complement cascade[127]. Activated neutrophils phagocytose bacteria, secrete numerous cytokines, chemokines and growth factors and controversially, may or may not kill internalised bacteria[62]. It is likely that both outcomes exist and contribute to the progression of the disease. If neutrophils

do kill bacteria, they would stop the infection at a very early stage. In some patients when neutrophils fail to kill bacteria, neutrophils may traffic bacteria to distal sights, resulting in the subsequent onset of latent disease[62]. Both oxidative and non-oxidative mechanisms have been reported for neutrophil killing of bacteria[62]. Oxidative burst may induce apoptosis in bacteria-infected neutrophils via a NADPH oxidase-dependent pathway[128]. MPO, present in neutrophil granules, was shown to kill mycobacterium tuberculosis in the presence of hydrogen peroxide[72]. Human neutrophil peptides (HNPs), members of the α-defensin family, are cationic proteins that are also stored in neutrophil granules. By a non-oxidative mechanism, HNPs bind anionic molecules, including the plasma membrane of pathogens and cause leakage of bacterial contents by disrupting the integrity of the membrane wall[62, 129].

4.1.2 Zebrafish as a model to study neutrophils in mycobacterium infection

Zebrafish are naturally susceptible to *M. marinum,* the closest genetic relative to the *M. Tuberculosis* organism complex[102]. *M. marinum* infection in zebrafish has become an accepted model to study *M. Tuberculosis* because *M. marinum* infection in zebrafish shares the main pathological and histological features, as *M. Tuberculosis*[130]. Zebrafish infection results in the necrotic, caseating granulomas, seen in human tuberculosis[131]. Furthermore, infection of larvae, recapitulates the steps leading to human tuberculosis[125]. A recent study of *M. marinum* in zebrafish revealed how neutrophils are rarely recruited to the initial sites of *M. marinum* infection, but rather neutrophil interactions with mycobacteria, occur only once granulomas have formed. Neutrophils

were shown to be recruited to nascent granulomas in response to signals from dying infected macrophages, which they phagocytosed[125]. Furthermore, a subpopulation of neutrophils killed the mycobacterium in phagocytosed macrophages by NADPH oxidase-dependent mechanisms as was shown by morpholino knockdowns of two key subunits of NADPH, $gp91^{phox}$ and $p22^{phox}$[125].

This chapter highlights how the zebrafish model previously developed to study myeloid-mediated inflammation by biotagging of nuclei can be used to study neutrophils in non-sterile inflammation using *M. marinum* as a model. This model circumvents previous problems with the variability of these short-lived cells following isolation from blood by rapidly sequestering the nuclei. We show that not only is this an ideal system to study the role of neutrophils in M. *marinum* infection, but that the results obtained are relevant to human *M. tuberculosis.*

4.2 Results

4.2.1 Optimization of granuloma infection model using *M. marinum*

To ensure optimal infection experiments, *M.* marinum was transformed with a pmsp12 vector carrying a tdTomato fluorescent reporter. The pmsp12 vector is a constitutively expressed micobacterial vector and the codon optimised tdTomato provides optimal brightness with low larval autofluorescence[102]. *M. marinum* cultures were processed to make a single cell suspension for consistent microinjection and infection dosing[102].

A previous study showed that injection into the yolk sac of developing zebrafish embryos up until 1024-cell stage, recapitulates the well-established caudal vein injection method for granuloma development in zebrafish larvae[132]. This method relies on the fact that the bacteria stream through the yolk syncytial layer (YSL), to reach the circulation of the embryo. This approach results in variation in the amount of bacteria in the circulation between different embryos. Therefore, embryos were injected with 20 CFU *M. marinum,* directly into the cell mass (32-512 cell stage) (Figure 4.1 Ai). Bacterial growth was seen in the yolk, head and trunk of the embryo by 3 days post infection (dpi) (Figure 4.1 Aii). When *M. marinum* was injected into *TgBAC(mpo1:BirA-2a-Citrine)*^{ox121} embryos, co-localisation of *M. marinum* with neutrophils was visible at 5 days post infection (dpi). Other groups have shown that neutrophils interactions with *M. marinum* are initiated only once granuloma formation has occurred[125].

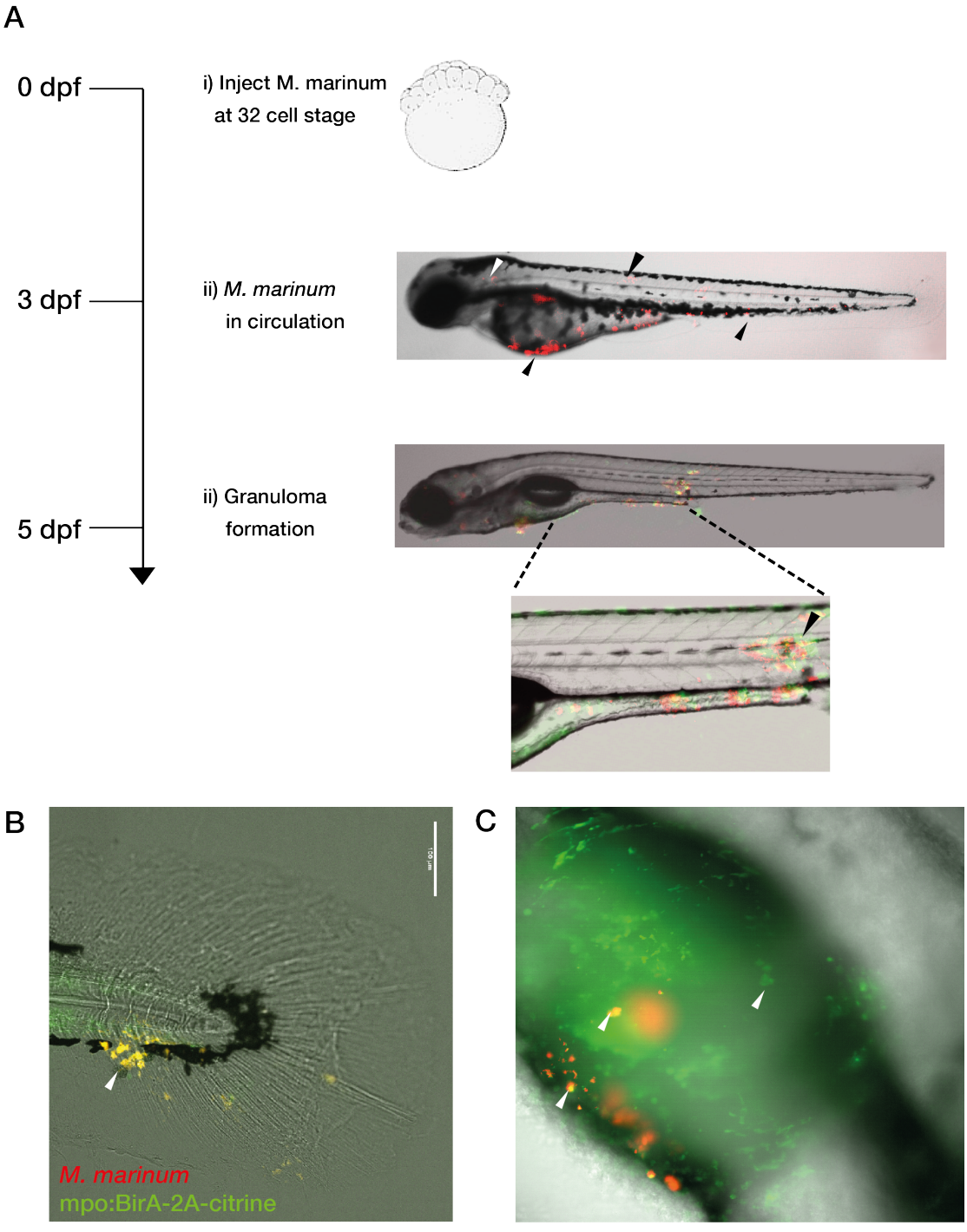
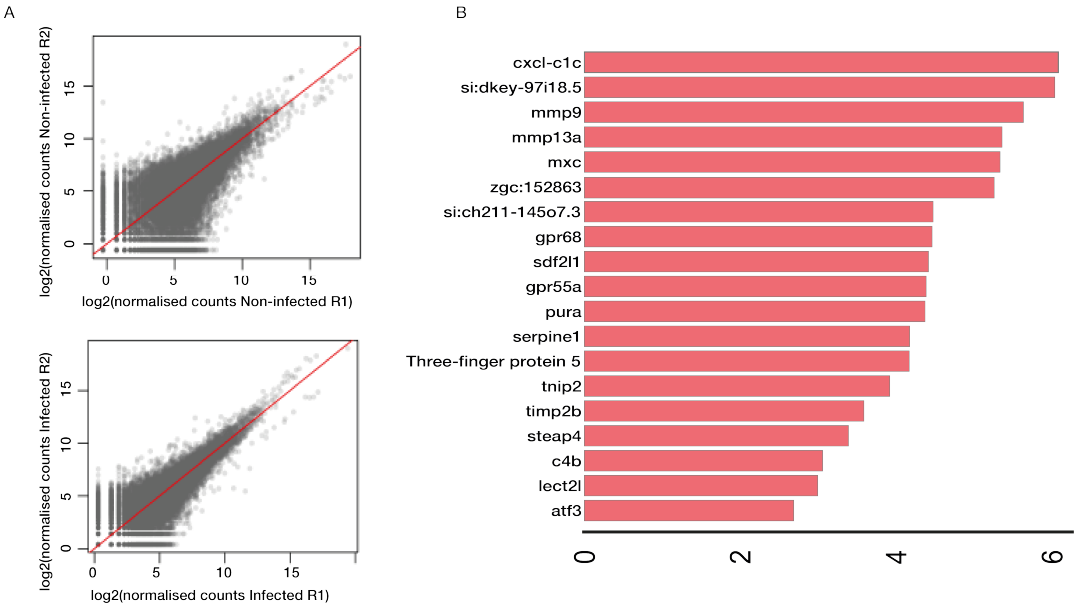


Figure 4.1 Bacterial localisation and neutrophil recruitment following 32-cell stage microinjection of *M. marinum*. A) Schematic representation of *M. marinum* infection process. 20 CFU of bacteria, transformed with a tdTomato expression plasmid were microinjected into the 32-cell stage of a *TgBAC(mpo1:BirA-2a-Citrine)*^{ox121} embryo (i). At 3 dpi *M. marinum* (red) is present in the yolk, head, trunk and tail (ii). By 5dpi neutrophils have infiltrated *M. marinum* and putative granuloma formation occurs (iii). B) Confocal stack of the tail of 5dpf Tg(mpo:BirA-2A-citrine) embryo shows co-localization (yellow) between *M. marinum* (red) and neutrophils (green). C) Localisation of *M. Marinum* in the yolk sac shows co-localisation (yellow) of neutrophils (green) and *M. Marinum* (red) and uninfected neutrophils (green) as indicated by the white arrows. Images were captured on a Zeiss Z1 lightsheet microscope.

4.2.2 Transcriptional analysis of neutrophils in *M. marinum* model of infection by *in vivo* biotagging

To obtain the active transcriptome of neutrophils following *M. marinum* infection we utilised the transgenic model previously generated for 'biotagging' of nuclei of neutrophils. Double transgenic *Tg(mpo:BirA-2a-Citrine;bactin:Avi-Cerulean-RanGap)*^{ox128} fish were in-crossed and embryos were injected with *M. marinum* directly into the cell mass (32 – 512 cell stage) and allowed to develop until putative granuloma formation had occurred. 'Biotagged' neutrophil nuclei were isolated from 5 dpf embryos by nuclei pulldown[122]. Nuclear RNA pools were sequenced using Illumina HiSeq technology. Scatterplots of the replicates show technical reproducibility between the samples (Figure 4.2 A), consistent with a large effect on the host transcriptome as a result of the infection[133,134]. Differential expression analysis of infected vs uninfected embryos showed 19 genes to be upregulated with a high stringency cut-off ($p<0.05$). A number of genes involved in proteolysis (serpine1, mmp9, mmp13a, timp2b, and steap4), cell motility (cab701040055.1, cxcl-c1c, lect2l, gpr55a and gpr68), gene regulation (atf3, pura and si:dkey91i18.5), host defence (mxc, sdf2l1, c4b) and some uncharacterised proteins (zgc:152863 and si:ch211-145o75) were significantly upregulated (Figure 4.2 B-C).



C

Ensembl Gene ID	Associated Gene Name	Description	log2FoldChange	pvalue
ENSDARG00000075045	cxcl-c1c	chemokine (C-X-C motif) ligand c1c	6.1	2.48E-10
ENSDARG00000090352	si:dkey-97i18.5	non-coding RNA	6.0	2.21E-14
ENSDARG00000042816	mmp9	Matrix Metalloproeinase-9	5.6	1.17E-10
ENSDARG00000012395	mmp13a	Matrix Metalloproeinase-13	5.4	1.01E-09
ENSDARG00000024789	mxc	Interferon-induced GTP-binding protein (MXA-related)	5.3	1.10E-08
ENSDARG00000069338	zgc:152863	Subfamily not named	5.3	5.48E-07
ENSDARG00000061548	si:ch211-145o7.3	Subfamily not named	4.5	4.39E-05
ENSDARG00000053711	gpr68	Ovarian Cancer G-protein coupled receptor 1	4.5	4.08E-05
ENSDARG00000035631	sdf2l1	Stromal cell-derived factor 2-like protein 1	4.4	2.34E-05
ENSDARG00000043447	gpr55a	G-protein coupled receptor 55	4.4	1.13E-05
ENSDARG00000024890	pura	Subfamily not named	4.4	6.04E-06
ENSDARG00000056795	serpine1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	4.2	1.59E-09
ENSDARG00000086869	cabz01040055.1	Three-finger 5 protein	4.2	5.27E-07
ENSDARG00000074501	tnip2	TNFAIP3 interacting protein 2	3.9	1.86E-05
ENSDARG00000075261	timp2b	Metalloproteinase Inhibitor 2	3.6	3.98E-07
ENSDARG00000055901	steap4	Metalloreductase STEAP family member 4	3.4	4.29E-07
ENSDARG00000038424	c4b	Complement C4b	3.1	6.63E-05
ENSDARG00000033227	lect2l	Leukocyte cell-derived chemotaxin 2 like	3.0	7.98E-05
ENSDARG00000007823	atf3	Cyclic AMP-dependent transcription factor ATF-3	2.7	8.48E-05

Figure 4.2 In vivo biotinylation of nuclei and RNA-seq analysis reveal transcriptomic signature of neutrophils in response to M. marinum infection. *Tg(mpo:BirA-2a-Citrine;bactin:Avi-Cerulean-RanGap)*^{ox128} fish were incrossed and embryos injected with *M. marinum* at the 32-cell stage. At 5 dpf, embryos were lysed in a hypotonic buffer and nuclei isolated with streptavidin dynabeads and processed for RNA extraction, library preparation and sequencing. 19 genes showed significant up-regulation during M. marinum infection with a high stringency cut-off (adjusted P-value <0.05).

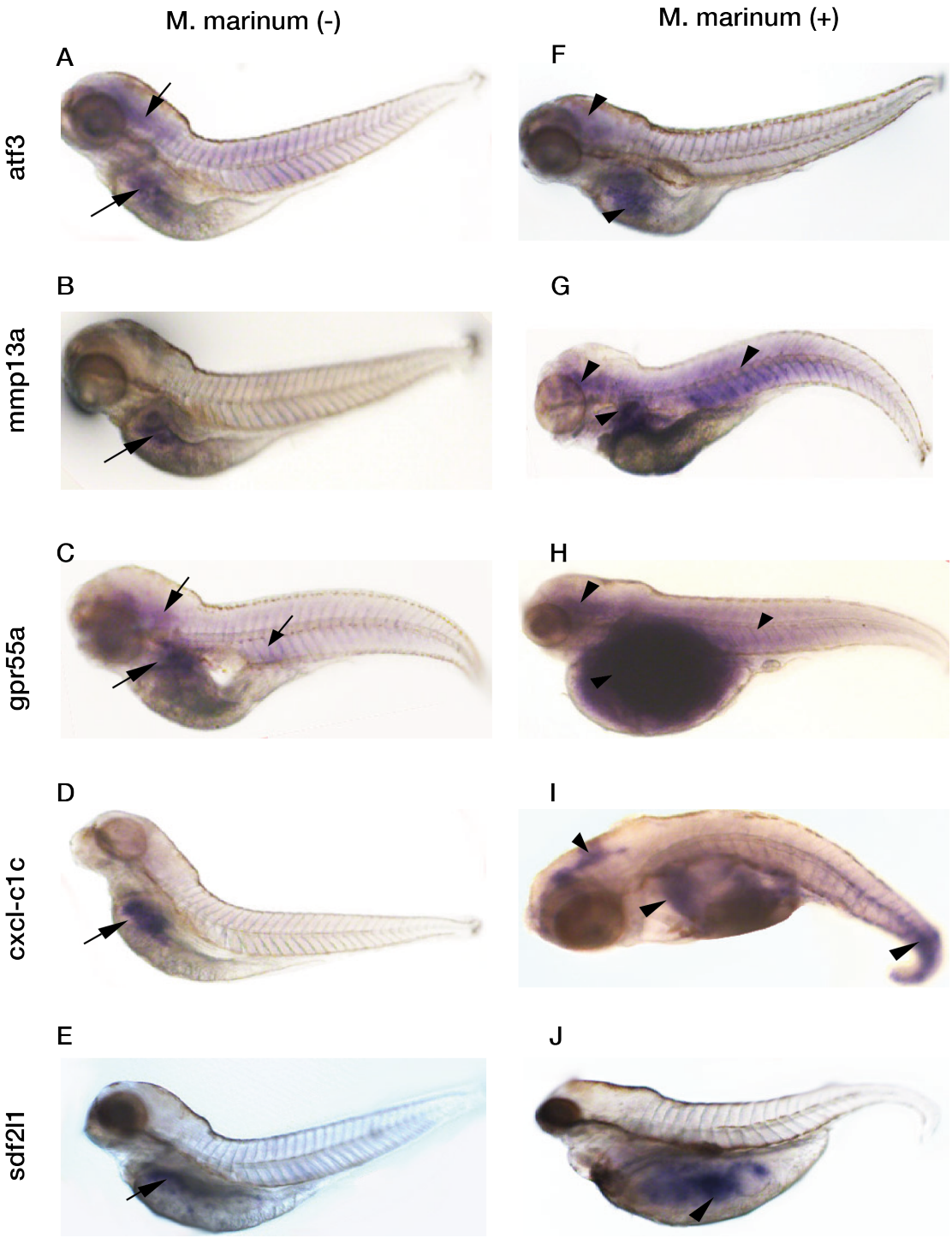


Figure 4.3 Spatial analysis of *M. marinum*-induced upregulated genes. Embryos were injected at the 32-cell stage with *M. marinum.* Expression patterns and gene transcript levels were analysed by i*n situ* hybridisation using a digoxigenin-labelled RNA probes for atf3, mmp13a, gpr55a, cxcl-c1c and sdf2l1 at 4dpi, in uninjected controls (A-E) and *M. marinum* injected embryos (F-J). Control expression patterns are indicated by the black arrows with black arrowheads used to indicate ectopic expression patterns.

4.2.3 *In situ* hybridisation of selected upregulated genes confirms RNA-seq data

To begin to validate the RNA-seq data and to give us an idea of the expression patterns of some of the upregulated genes, whole mount *in situ* hybridisation was carried out. The images shown in Figure 4.3 reflect representative images. From the RNA-seq data, we selected five different candidates to validate: a transcription factor (atf3), a matrix metalloproteinase (mmp13a), a GPCR (gpr55a), a chemokine (cxcl-c1c) and gene involved in host defence (sdf2l1). The expression of the transcription factor, atf3, is seen predominantly in the anterior yolk sac, with lower levels of expression detected throughout the developing embryo in both the uninjected control (Figure 4.3 A) and *M. marinum*-injected embryos (Figure 4.3 F). In contrast, mmp13a is predominantly expressed over the anterior yolk sac in the uninjected control (Figure 4.3 B), with a general increase in expression seen throughout the embryo (Figure 4.3 G). Gpr55a, transcripts are detected in the anterior yolk sac and at low levels in the entire embryo (Figure 4.3 C), with a general increase in expression seen following *M. marinum* infection (Figure 4.3 H). Cxcl-c1c expression is seen in the anterior yolk sac in the uninjected control (Figure 4.3 D). However, in a representative embryo injected with *M. marinum,* cxcl-c1c expression levels are increased in the anterior yolk sac but also, in distinct areas in the embryo, presumably where the *M. marinum* had localised (Figure 4.3 I). Sdf2l1 is expression in the anterior yolk sac in the uninjected control (Figure 4.3 E). As previously described, *M. marinum is* frequently localised to the yolk sac following injection. *In situ* hybridisation for

sdf21l shows punctate expression in the centre of the yolk, presumably a site of *M. marinum* infection, where an increase in sdf21l expression is seen (Figure 4.3 J).

4.2.4 Neutrophil migration in zebrafish larvae is mediated by cxcl-c1c

One of the advantages of the zebrafish is the ease of directed mutagenesis for the functional analysis of specific genes. We use Crispr/Cas9 genome editing approaches to target genes of interest and induce double stranded breaks, followed by non-homologous end joining (NHEJ) pathways, hence causing gene perturbation[135]. Cxcl-c1c, had the highest log2fold change as revealed by RNA-seq and the *in situ* hybridisation data showed Cxcl-c1c localised to distinct sites in the embryo, presumably sites of granuloma formation. Therefore, two single guide RNAs (sgRNAs) directing Cas9 nuclease (nls-Cas9-nls) to specific genomic target sites in the cxcl-c1c coding region were designed and high resolution melt analysis (HRMA) carried out to determine the efficiency of the sgRNA targeting. HRMA of sgRNA: cxcl-c1c_1 (Figure 4.4 Ai) and of sgRNA: cxcl-c1c_2 (Figure 4.4 Aii) revealed a melting difference over the sgRNA target area when compared to wildtype controls although one of the chosen guide RNAs (cxcl-c1c_1) appeared to be more efficient. To maximise the possibility for disrupting gene function, both of sgRNA: cxcl-c1c_1 and sgRNA: cxcl-c1c_2 were injected into the 1-cell stage of the zebrafish embryo together with Cas9 mRNA. Subsequently, when they reached 32-cell stage, these embryos were injected with *M. marinum* to investigate whether interfering with neutrophil migration, would have an affect on granuloma size and formation. At 4 dpi, embryos were fixed and neutrophils detected by immunohistochemistry for mpo marker. Since the *M.*

marinum had previously been transformed with a td-tomato expression plasmid, the td-tomato signal was amplified with an RFP-booster. However, the RFP-booster did not sufficiently amplify the *M. marinum* signal and this experiment will be repeated in the future. As it stands it still serves to give an indication of the role of cxcl-c1c function in the zebrafish. Disrupting cxcl-c1c function by sgRNA lead to a significant decrease in the number of neutrophils in the tail, although neutrophil numbers in the head and in the vicinity of the anterior yolk sac remained consistent with control embryos not injected with sgRNA, but cas9 mRNA only (Figure 4.4 B, Ci, Cii). Additional sgRNAs for mmp9, sdf2l1 and lect2l have been designed and validated, indicating they efficiently target and lead to editing of the targeted genomic regions. These sgRNAs will be used in the future to elucidate the function of their target genes in *M. marinum* pathogenesis.

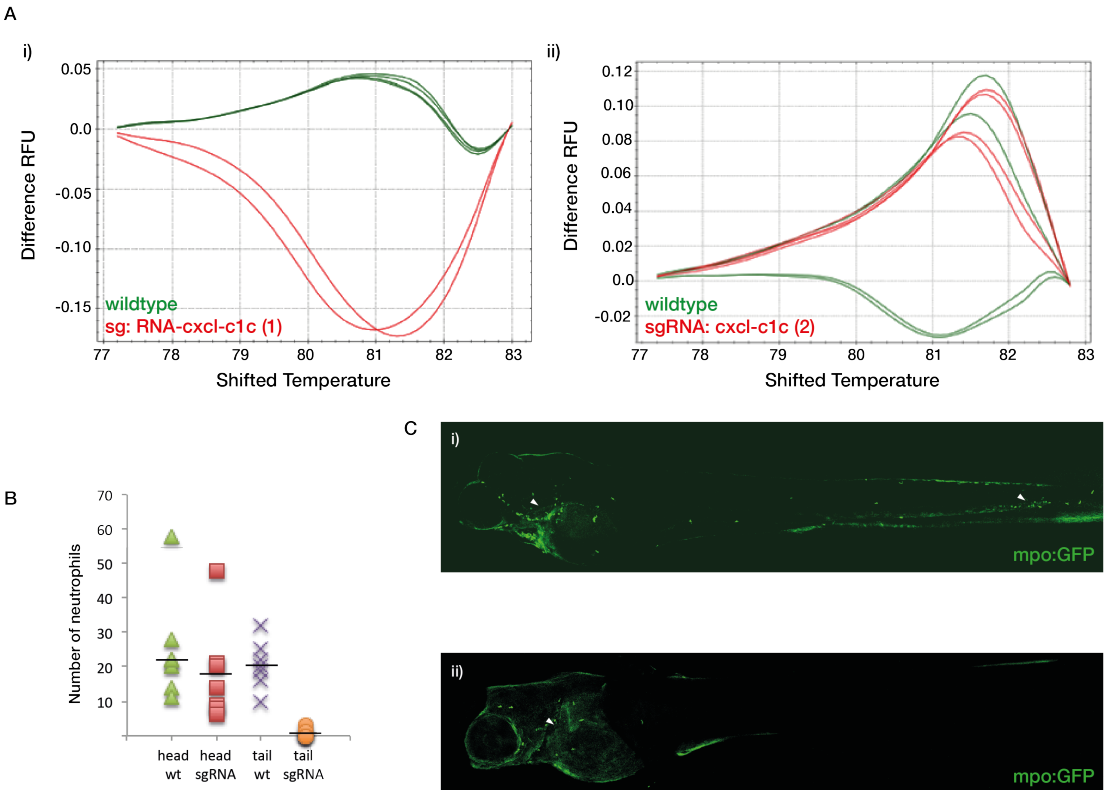


Figure 4.4 Cxcl-c1c controls migration of neutrophils in zebrafish larvae. A) sgRNAs designed to target cxcl-c1c were injected into the 1-cell stage of the developing zebrafish embryo, along with mRNA for Cas9. A subset of sgRNA: cxcl-c1c injected embryos (red lines) were analysed by high resolution melt analysis and normalised to uninjected wildtype controls (green lines) revealing the melting difference over the sg-RNA target area for of sgRNA: cxcl-c1c_1 (i) and of sgRNA: cxcl-c1c_2 (ii). Embryos were injected with Cas9 mRNA only or Cas9 mRNA and sgRNA: cxcl-c1c, followed by injection of *M. marinum* into the 32-cell stage. Embryos were fixed at 4 dpi and stained with for mpo (green). B) Graph showing number of neutrophils in the head and tail of Cas9 mRNA only or Cas9 mRNA and sgRNA: cxcl-c1c embryos. C) Representative confocal images of Cas9 mRNA only (i) or Cas9 mRNA and sgRNA: cxcl-c1c (ii) injected embryos where neutrophils (green) are indicated by the white arrows.

4.3 Discussion

Previous transcriptomic analyses using *M. marinum* in zebrafish have focused on a whole organism response, rather than the contribution of a single responding cell type. This is the first study of its kind to look at the role of neutrophils in *M. marinum* infection. Genes that overlap between human and murine responses to *M. tuberculosis* were found, confirming that this is a good *in vivo* model for studying mycobacterium infection. A number of genes that had previously been recognised to contribute to the immune response to *M.*

marinum and *M. tuberculosis* were identified, which can now, at least in part, be attributed to neutrophils. Here we report a number of novel neutrophil-derived factors that may either contribute to disease progression or elicit an early defensive function against *M. marinum* infection, providing new insight into the complex mechanisms by which neutrophils affect mycobacterium infection.

4.3.1 Factors involved in proteolysis

MMPs are zinc-containing proteolytic enzymes that degrade the extracellular matrix (ECM)[136]. MMPs have been linked to *M. tuberculosis* immunopathology and play a clear role in ECM and tissue destruction[136]. MMP9 is highly expressed in human tuberculosis as well as in mouse models of tuberculosis. However, in mice, MMP9 activity was shown to correlate with an increase in macrophage migration and granuloma formation and reported to be a host restriction factor[136]. In humans, however, MMP9 correlates with worse prognosis[137]. In a zebrafish model of *M. marinum* infection, mycobacteria were shown to actually drive granuloma formation in an mmp9-dependent pathway[138]. The bacterial secreted protein, ESAT-6, was shown to induce mmp9 overexpression in epithelial cells surrounding infected macrophages. This resulted in the recruitment of uninfected macrophages to the site of infection, granuloma formation and bacterial expansion. The same study showed that although there is a minor contribution from neutrophils within the granuloma, it is actually distil neutrophils that express mmp9 in response to *M. marinum*. Therefore, although the mmp9 expression in neutrophils is not necessarily relevant for granuloma formation, it may still contribute to the

pathogenesis of the disease[138]. Although the role of mmp9 in granuloma formation is well characterised, an increase in mmp9 expression seen in this study, serves to validate the approach. MMP13, a collagenase, is associated with tissue damage and destruction in tuberculosis pathogenesis[139]. Mmp13a has similarly been reported to be upregulated in response to *M. marinum*[134, 140]. Interestingly, a more virulent strain of *M. marinum* was shown to upregulate mmp9 and mmp13 more potently than an attenuated strain, even though in the same study, the majority of genes were equally upregulated[134]. We provide the first evidence that neutrophils are a source of mmp13. The *in situ* expression data obtained in this study for mmp13a shows a general upregulation throughout the embryo. Although the *in situ* analysis is not neutrophil-specific, an increase in mmp13a expression appears to not be localized to granulomas. Further analysis would need to be conducted to determine whether the neutrophils overexpressing mmp13a were present only in granulomas and whether knockdown of mmp13a would reduce *M. marinum* pathogenesis. In the ECM, tissue inhibitors of metalloproteinases (TIMPs) contribute to regulating MMP activities. As was shown for mmp9, ESAT-6 similarly induced timp2b overexpression[141]. However, whereas an upregulation in mmp9 expression was seen by 1dpi, timp2b overexpression was evident at 5dpi. Therefore, it is likely that the increase in timp2b is a direct result of the increase in MMP expression, as seen by us and others[138].

Serpine1 is a member of the serine protease inhibitor family (also known as plasminogen activator inhibitor type I, Pail) and is the main inhibitor of the fibrinolytic system[142]. Tuberculosis patients have elevated plasma levels of

Serpine1. Serpine1, inactivates tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPa), which ultimately leads to fibrin degradation because plasmin production is inhibited[142]. The murine model of tuberculosis in the Serpine1$^{-/-}$ background has a higher bacterial load compared to wildtype (WT) mice at 5 weeks but not 29 weeks post infection, suggesting a role for Serpine1 in host defence at the early phases of infection[142]. Of particular interest is the role of Serpine1 in regulating leukocyte cell migration by modulating extracellular matrix degradation via the regulation of plasmin levels, regulating cell adhesion and by mediating chemo-attractant gradients[143]. Furthermore, serpine1 has been shown to support IL-8 mediated neutrophil transendothelial migration[144]. A recent study showed Serpine1 upregulated in response to *M. marinum* in a Traf6-dependent mechanism[48]. In a study of *Klebsiella pnemoniae,* Serpine1 deficiency reduced neutrophil recruitment to the lungs, whereas Serpine1 overexpression in healthy lungs resulted in neutrophil recruitment[145]. Therefore, this study provides the first evidence that serpine1 is a neutrophil-derived factor. Further investigation into neutrophil-derived Serpine1 as a host restrictive factor early in infection is necessary. This coupled with its potential to control neutrophil migration into sites of *M. marinum* infection, make serpine1 an interesting factor to harness in fighting the infection.

4.3.2 Host defence mechanisms

Identification of mannose residues on the surface of *M. tuberculosis* has been shown to result in the innate immune response-mediated activation of the complement system[146]. C4 is cleaved to give C4a and C4b, which forms part

of the C3 convertase, a serine protease, which is further cleaved to form the C5 convertase resulting in subsequent cytolysis of bacteria but in excess may mediate tissue damage. Increased serum levels of C4b of *M. tuberculosis* patients with advanced disease has been reported, suggesting that C4b is a marker for higher bacterial infection and tissue damage[146]. The increase in c4b in this study provides evidence of an innate immune response-specific mechanism that may result in tissue destruction and disease pathogenesis[146].

Our study also shows the upregulation of two tumour necrosis factor (TNF)-induced genes, TNFAIP3-interacting protein 2 (tnip2) and six-transmembrane epithelial antigen of prostate 4, steap4. Steap4, otherwise known as, TNF alpha-induced protein 9, TNFAIP9, is a plasma membrane metalloreductase. In a study using non-human primates early *M. tuberculosis* lesions showed increased levels of TNF-related genes including TNIP2[147]. However an increase in expression of tnip2 has not previously been reported in response to *M. marinum.* Previous groups have similarly seen an increase in expression levels of Steap4 in response to *M. marinum* in both adult[133] and larval[48, 140] zebrafish. Although the role of TNFα in stimulating neutrophils to kill mycobacterium is well established, this study highlights new downstream mediators of TNFα-induced function in neutrophils[148].

An increase in expression of Myxovirus (influenza virus) resistance C gene (MXC) following *M. marinum* infection, induced with a more virulent strain of the bacteria, has previously been reported. Mx proteins are usually associated with the type I IFN system, a powerful antiviral defence mechanism in

vertebrates. The IFN response-induced Mx proteins inhibit viral replication[149]. Similarly, virulent strains of *M. tuberculosis* induce higher levels of type I IFNs[150]. Previous studies have shown that patients with active TB have a whole blood transcriptional signature which was dominated by a neutrophil-driven IFN response and correlated with diminished outcome[61]. Therefore, taken together, this data suggests a role for type I IFN signalling in neutrophils not only in active disease but also at the earliest stages of mycobacterium infection.

Stromal cell-derived factor 2-like 1 (sdf2l1) encodes an endoplasmic reticulum (ER) stress-inducible gene and is a factor that is involved in the processing of defensins, cationic antimicrobial peptides that play a central role in host innate immunity[151, 152]. Myeloid α-defensins constitute about 5% of the protein mass of human neutrophils and are discharged into the phagosome during phagocytic ingestion of microbial particles[152]. Therefore, we provide the first evidence that sdf2l1 may play an important role in neutrophil mycobacterium killing by processing of defensins released from primary granules either in the extracellular space or phagosomes.

4.3.3 Chemokines and G-protein-coupled receptors (GPCRs)

Chemokine (C-X-C motif) ligand – c1c (cxcl-c1c) or CXCL18B, is a chemokine that is annotated in the zebrafish genome but has no known human ortholog. Increasing evidence suggests its role in the immune response, and in particular, in bacterial infection. Cxcl-c1c expression was upregulated in response to *S. typhimurium* infection in zebrafish embryos with *in situ*

hybridisation data showing its localization to discrete cells at the site of infection, presumably innate immune cells[153]. Increased expression levels of cxcl-c1c have previously been seen in response to *M. marinum* in whole organism transcriptomic anaylses[31, 140]. Similarly, the *in situ* hybridisation data in this study, shows cxcl-c1c localised to discrete locations, within the developing embryo, presumably sites of granuloma formation. This suggests that cxcl-c1c expressed by neutrophils and potentially other immune cells at the site of infection, plays a key role in trafficking of leukocytes. The sgRNAs for cxcl-c1c in this study will provide a novel tool with which to dissect neutrophil function at sites of infection, by preventing neutrophil trafficking into the infection site.

Gpr68 is also known as ovarian cancer GPCR1 and is a proton sensing GPCR that has previously been shown to play a role in extracellular acidic pH-induced attenuation of neutrophil function[154]. Although Gpr68 has not previously been known to play a role in mycobacterium pathogenesis, one might speculate that the in the acidic environment of the granuloma necrotic core gpr68 may negatively regulate neutrophil function. This is a novel finding and further investigation is necessary to determine, just how Gpr68 signalling affects neutrophil function within the granuloma. Gpr55a belongs to the GPCR family and has been described as a cannabinoid receptor[155]. At sites of infection, a gradient of bacterial products and members of the complement system, C5a, IL-8 and endocannabinoids released by macrophages and damaged tissue, are responsible for the migration of neutrophils to sites of inflammation[155]. GPR55, which is expressed on neutrophils, was shown to

modulate the directed migration of neutrophils to sites of inflammation. GPR55 receptor signalling also served to mediate inhibiting effects on neutrophil functions, including degranulation and respiratory burst. If the same is true for neutrophils in response to mycobacteria, this will limit tissue damage, but also result in less effective killing of the bacteria. Therefore GPR55 represents an exciting new target to explore further and investigate the role of neutrophils in granuloma formation and mycobacterium infection. The *in situ* expression data confirms the increase in gpr55a expression, although the analysis is not restricted to neutrophils and does not appear to be localized to distinct sites as one would expect if it was simply overexpressed by neutrophils in the granulomas. Therefore, gpr55a expression may be upregulated in other cell types as well as neutrophils at distal sites.

Other groups similarly found leukocyte cell-derived chemotaxin 2-like (lect2l) levels increased after *M. marinum* infection[140]. It was not previously known that this molecule was neutrophil-derived, and hence the increase in expression revealed in this study and others, is probably as a result of the directed migration of neutrophils to the granuloma. Interestingly, a proteome mapping study on zebrafish marrow neutrophils listed the most abundant neutrophil proteins, including lect2l[156].

Although largely uncharacterised in zebrafish, cabz01040o55.1, is a three-finger protein, which was previously identified as a novel member of the ly-6 protein family and was similarly seen by other groups as upregulated in

response to *M. marinum*[140]. Ly6G is used as neutrophil-specific marker in murine biology and is thought to play a role in neutrophil migration. It may be that cabz01040o55.1 is the zebrafish ortholog of Ly6G. Although further confirmation is required, this may serve to further highlight the similarities between zebrafish and murine neutrophils, hence having additional implications for the field as a whole.

4.3.4 Gene regulatory factors

Increased expression levels of activating transcription factor 3 (atf3) have previously been seen in response to *M. marinum* in studies using adult and embryo models[134, 157]. In zebrafish larvae, atf3 expression was upregulated when embryos were challenged with *S. typhimurium* suggesting a conserved bacterial response mechanism, rather than a response specific to mycobacteria. Atf3 was described as an ER stress marker and shown to increase in macrophage-rich areas of granulomas in a murine model of tuberculosis[158]. In murine studies, atf3 was shown to be a negative regulator of TLR pathways, thus altering the response to immunological challenges. *In situ* hybridisation analysis does not reveal an obvious increase in expression. Since, Atf3 is a transcription factor and expressed throughout the embryo, and not only at sites of bacterial infection or only in neutrophils, this analysis is not sufficient to confirm the increase in expression revealed by the RNA-seq data. Investigation of the downstream targets of atf3, are needed to understand the role of this transcription factor in *M. marinum* infection.

Purine-rich element-binding protein A (PURA) is a regulatory protein involved in DNA replication, gene transcription, RNA transport and mRNA translocation[159]. It does not have a previously described function in the immune response to mycobacteria and hence is a novel candidate to explore further.

Si-dkey-97i18.5, is a non-coding RNA, the function of which has not been characterised. However an increase in expression in response to *M. marinum* has been seen by us and others[140]. Further analysis is required to determine whether this non-coding RNA is transcribed in other cell types and whether it has a gene regulatory role in attenuating the role of neutrophils in *M. marinum* infection.

Taken together, these results show that the binary transgenic system for studying the myeloid response to non-sterile inflammation generated in this study is a valuable resource. The initial transcriptome analysis generated in this study highlights the potential for the data obtained using this model to explore the contributions of specific cell populations to the pathogenesis of human tuberculosis. The role of neutrophils in mycobacterium pathogenesis is not well studied and this analysis reveals some exciting new findings, elucidating how neutrophils may both be a source of host restrictive factors and eliminate infection but may also contribute to the pathogenesis of the disease by tissue damage. The genetic tractability of the zebrafish provides potential for the data generated in study to be explored using guide RNA's and Cas9 to target specific genes and elucidate their function further. In the

future, profiling on the macrophage response to *M. marinum* will be carried out. Since we have identified a number of novel neutrophil-derived targets, we would like to validate this dataset in patient samples.

Chapter 5

Generation of a binary inducible zebrafish model for melanocyte oncogenic transformation

5.1 Introduction

Infiltration by innate immune cells is not only limited to pathogens, but also includes immune cell infiltration characteristic of tumours[35]. Immune cells and their associated molecules are responsible for detecting and eliminating cancer cells as they establish themselves as the precursors of a tumour[35]. As with infection, cancer cells can evade the immune system. By the time a patient has a detectable solid tumour, cancer cells have escaped the initial immune response mechanisms. Further studies are needed to understand the different inflammatory signalling pathways in tumour initiation and progression and how they may be modulated for immunotherapy. Because we cannot predict when and where transformed cells may arise in the human host, very little is known about the sterile inflammatory signalling pathways that ensue somatic cell transformation[160]. The zebrafish is now an accepted model in cancer research, with many aspects of carcinogenesis being conserved between fish and humans[161]. Unlike mammalian models, the zebrafish is easily amenable to techniques that will allow us to study both cancer initiation and progression[36]. Zebrafish are particularly useful in studying melanoma because zebrafish melanocytes are externally visible and large single cells can be analysed in living fish[79].

5.1.1 Establishing a zebrafish melanoma model

5.1.1.1 Zebrafish melanoma models

Classically, formation of melanoma has been described as a stepwise process in which melanocytes acquire mutations in oncogenes leading to their

uncontrolled proliferation and ultimately giving rise to benign nevi. From here transformed melanocytes undergo a radial growth in the epidermis followed by a vertical growth phase into the underlying dermis[79, 116]. However, it should be noted that 50% of melanoma arises from normal skin rather than dysplastic nevi[116].

Taking this into account, the current zebrafish models for benign nevus and cutaneous melanoma all rely on the targeted expression of a human oncogene in melanocytes. Transgenic zebrafish melanoma models with oncogenic BRAFV600[162], NRasQ61K[163], HRasG12V[164, 165] under the melanocyte specific microphthalmia-associated transcription factor a (mitfa) promoter have previously been generated. Mitfa is the zebrafish ortholog of microphthalmia-associated transcription factor (MITF), and has been described as the master regulator of melanocyte development in vertebrates. Mitfa is expressed in a tissue-specific fashion during zebrafish development[162]. In zebrafish, null alleles of mitfa result in the absence of melanocytes as well as the earlier markers of melanoblast specification including dopachrome tautomerase (dct) and kita[79]. However, in order to develop melanoma both the BRAFV600- and NRasQ61K-expressing models require a further mutation in the tumour suppressor gene, p53[162, 163]. In the mitfa-driven HRasG12V transgenic line, melanoma development is rare but contributing mutations in the PI3K signalling pathway contribute to melanoma formation[162, 163, 165]. Santorelli *et al.,* subsequently developed a conditional transgenic zebrafish line using the melanocyte-specific kita promoter to drive HRasG12V. The murine ortholog of KIT is a type III receptor tyrosine kinase

that is required for melanocyte development, migration and survival in mammals and fish[79]. Kita:HRasG12V induced early onset melanoma without additional mutations in tumour suppressor genes. Therefore, it might be that the ability of oncogenes to induce melanoma is correlated with the driving promoter, rather than the oncogene itself. Kita, however, is also expressed in goblet cells[36].

5.1.1.2 Hormone-inducible binary transgenic zebrafish model

Previous melanoma transgenic models mostly relied on driving an oncogene under the control of a melanocyte-specific promoter. However, constitutive expression of the oncogene makes maintenance of stable transgenic lines difficult because these models may develop severe tumours before the fish reach reproductive age[166]. Conversely, if the transgenic zebrafish can withstand the expression of the oncogene, it is unlikely that the oncogene is expressed at high enough levels to drive tumour formation. In the Et*(kita:GalTA4,UAS:mCherry);Tg(UAS:eGFP-HRAS_GV12)* melanoma model generated by Santoriello and collegues, they made use of the combinatorial genetics for conditional transgene activation with the Gal4-upstream activating sequence (UAS) system. An optimized Gal4-activator for efficient activation in zebrafish[167], KalTA4, was used to drive the expression of effector transgene, in this case HRasV12, under the control of Gal4-binding region (5XUAS)[167], thus driving high levels of the oncogene. However some reports suggest that high levels of Gal4 expression can be toxic and may result in developmental defects[96]. Moreover, UAS sequences have been shown to be susceptible to DNA methylation leading to transcriptional

silencing of the transgene, which is minimal in the first generation, but exacerbated upon propagation through later generations[168, 169].

Emelyanov and Parinov designed a novel, hormone-inducible binary system for targeted gene expression in zebrafish[96]. This approach is based on use of two independent transgenic lines, the effector line carrying a transcriptionally silent gene of interest, and a driver line expressing a transcriptional activator in the cell type of interest. In animals carrying both alleles, cell type-specific expression of the transcriptional activator results in activation and expression of the target gene of interest in the same cells[96]. The transcriptional activator expressed by the transgenic driver is a ligand-dependent chimeric transcription factor, termed LexPR transactivator. LexPR transactivator is a fusion protein containing DNA-binding domain of the bacterial LexA repressor, a truncated ligand-binding domain of the human progesterone receptor and the activation domain of NF-κB/p65 protein. The effector transgenic fish line harbours the operator-promoter sequence, consisting of the synthetic LexA operator fused to a minimal 35S promoter sequence from the Cauliflower mosaic virus, upstream of the target reporter gene. In the presence of the progesterone antagonist, mifepristone, that binds to its ligand-binding domain, the LexPR transactivator activates the transcription of the target gene of interest by binding to the LexA binding sites present in the LexA operon (LexOP) driving the reporter gene. When this system was used to drive the expression of EGFP-KRasG12V under the control of the liver specific Pfabp10 promoter rapid liver tumour progression, coupled with 100% incidence was observed[166].

We therefore sought to generate an inducible melanoma model in zebrafish using the LexPR/LexOP system[96, 166]. Two melanocyte-specific driver lines (mitfa and kita) and three Ras-effector lines were generated (NRasK61Q, HRasG12V and KRasG12V). In our system we placed the LexPR transactivator, followed by Cerulean reporter, under the control of the melanocyte-specific promoters, kita and mitfa. The effector lines were generated such that different Ras-oncogenes (NRasK61Q, HRasG12V and KRasG12V), fused to a mCherry fluorescent reporter were placed downstream of LexOP operator. When a driver line is crossed to an effector line, in the presence of the synthetic progesterone ligand, mifepristone, the mCherry-Ras oncogene is transcribed only in the melanocytes. The Ras-effector line also contains the selection marker, ECFP, under the control of lens-specific zebrafish crybB promoter (crystallin beta β), which results in blue fluorescence in the lens, used to screen for transgene integration, without driving the oncogene (Figure 5.1)[170].

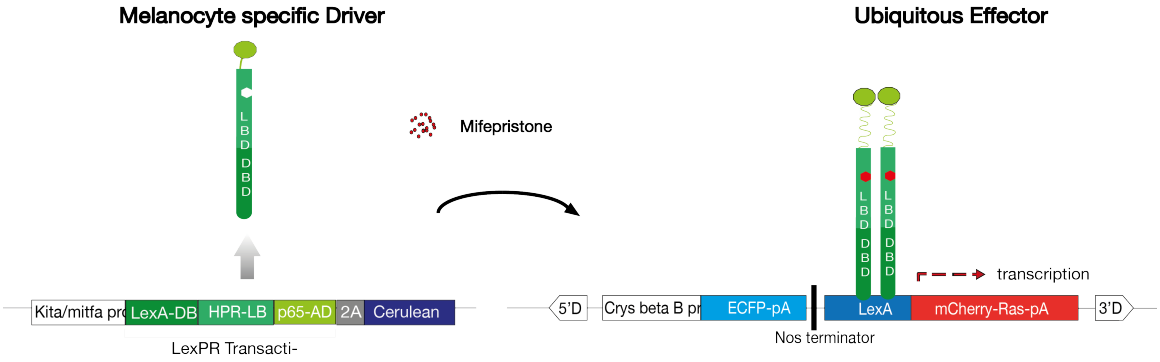


Figure 5.1. Schematic diagram of mifepristone-inducible LexPR system. The LexPR driver cassette contains the LexPR transactivator and a cerulean reporter under the control of melanocyte-specific promoters, kita and mitfa. The effector cassette contains the Lex operator sequence fused to the cherry-Ras oncogene. In double transgenic fish the cherry-oncogene fusion is activated in trans when the LexPR transactivator binds to the LexOP sequence to activate the transcription of the cherry-Ras oncogene.

5.2 Results

5.2.1 Generation of LexPR driver lines by BAC-mediated transgenesis

5.2.1.1 BAC recombination

To generate a binary inducible melanoma model in zebrafish, transgenic driver lines using both the mitfa and kita promoters were made. BAC-mediated transgenesis was used to generate melanocyte-specific LexPR driver lines as described previously (section 3.2.1.1). As before, BAC clones for the melanocyte-specific mitfa and kita genes were identified using Ensembl. A modified LexPR driver construct, with a cerulean fluorescent reporter, a polyA (pA) tail and a Frt-kan-Frt selection cassette was introduced into the respective BACs to replace the first exon of either the kita or mitfa gene (Figure 5.2 Ai-iii). Similarly, activation of Flp recombinase was used to remove the kanamycin selection cassette from the modified BAC (Figure 5.2 iv)[97, 98]. An iTol2-Ampicillin cassette (kindly provided by Prof Kawakami) was also inserted into the BAC backbone[100].

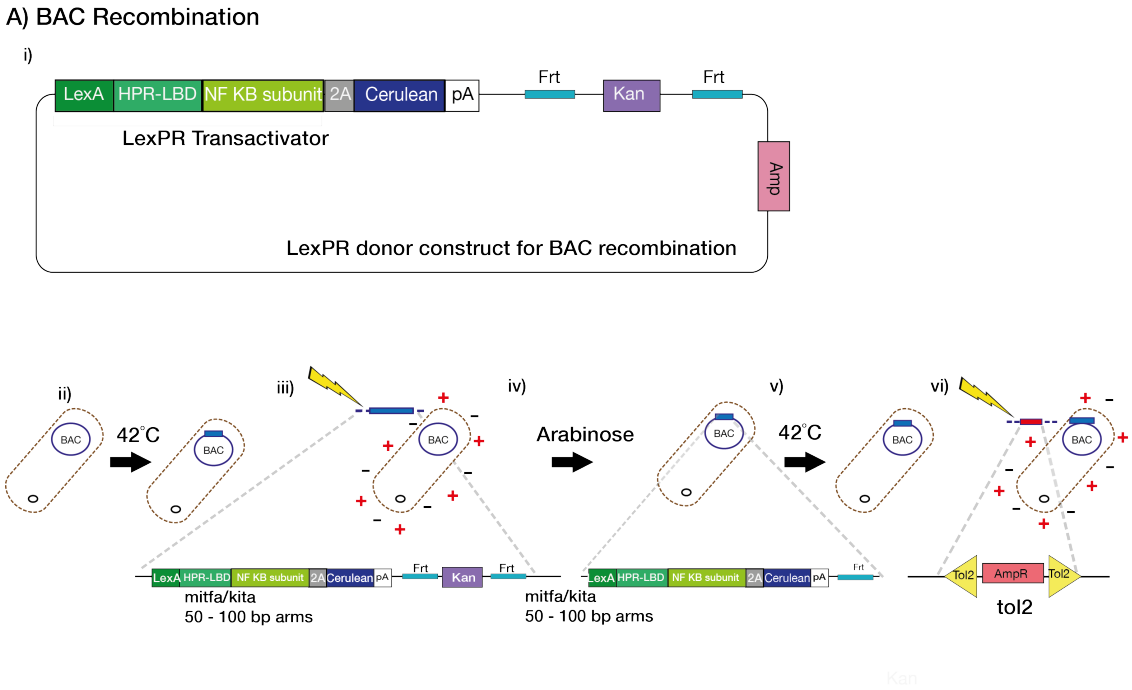


Figure 5.2 BAC recombination for melanocyte-specific LexPR driver lines. A) Schematic of LexPR BAC recombination. LexPR driver construct for BAC recombination containing the LexPR transactivator (green), a viral self-cleaving peptide (2A, grey) and a fluorescent reporter (cerulean, blue), followed by a polyA tail (pA, white) and a kanamycin selection cassette (kan, purple) embedded between Frt sites (turquoise) (i). Heat activation (42 °C) of the λ prophage homologous recombination system in SW105 competent cells containing kita or mitfa BAC DNA (ii). Electroporation of the LexPR-2A-Cerulean-pA-Frt-kanamycin-Frt cassette with 100 – 200 bp arms homologous to upstream and downstream of the first exon of either mitfa or kita into SW105 cells (iii). Activation of flippase removes the kanamycin selection marker embedded between the Frt sites of the modified BAC (iv) Heat activation (42 °C) of the λ prophage homologous recombination system (v). Electroporation of iTol-2-Ampicillin cassette into the BAC backbone (vi).

5.2.1.2 BAC-mediated transgenesis

For generation of transgenic lines, recombineered BAC DNA (Figure 5.3 A) was co-injected with tol2 mRNA into a fertilized zebrafish embryo at the single cell stage to facilitate germline integration[100]. In F_0 injected embryos, transgene expression (Cerulean) under the control of mitfa (Figure 5.3 Bi) and kita (Figure 5.3 Bii) was observed in the melanocytes. Mitfa and kita founders are usually identified by outcrossing to wildtype fish and screening for fluorescence. However, although in transient analysis of injected F_0 embryos Cerulean reporter expression was detected in melanocytes, in the LexPR

transgenic driver lines, the levels of Cerulean expression were too low for screening by fluorescence. This is presumably because following germline integration of the BAC DNA, the transgene is expressed at endogenous levels, whereas transient injections result in overexpression of the fluorescent reporter, that also possibly continues to be transcribed from the non-integrated plasmid as well. Furthermore, when endogenous levels of the transgene are expressed, melanin may mask the visibility of the fluorescent reporter. To identify founders among our injected fish, we performed genomic DNA isolation and PCR amplification of the transgene from pools of F_1 embryos. The offspring of the F_0 founders was raised and positive individuals identified by fin clipping. The penetrance of integration of the BAC construct into the germline varied between 20-30%. Two founder fish were identified for each LexPR driver line, (*TgBAC(kita:LexPR-2a-Cerulean)*^{ox123a,b} *and TgBAC(mitfa:LexPR-2a-Cerulean)*^{ox124a,b}). The F_1 colonies were subsequently expanded for future experiments.

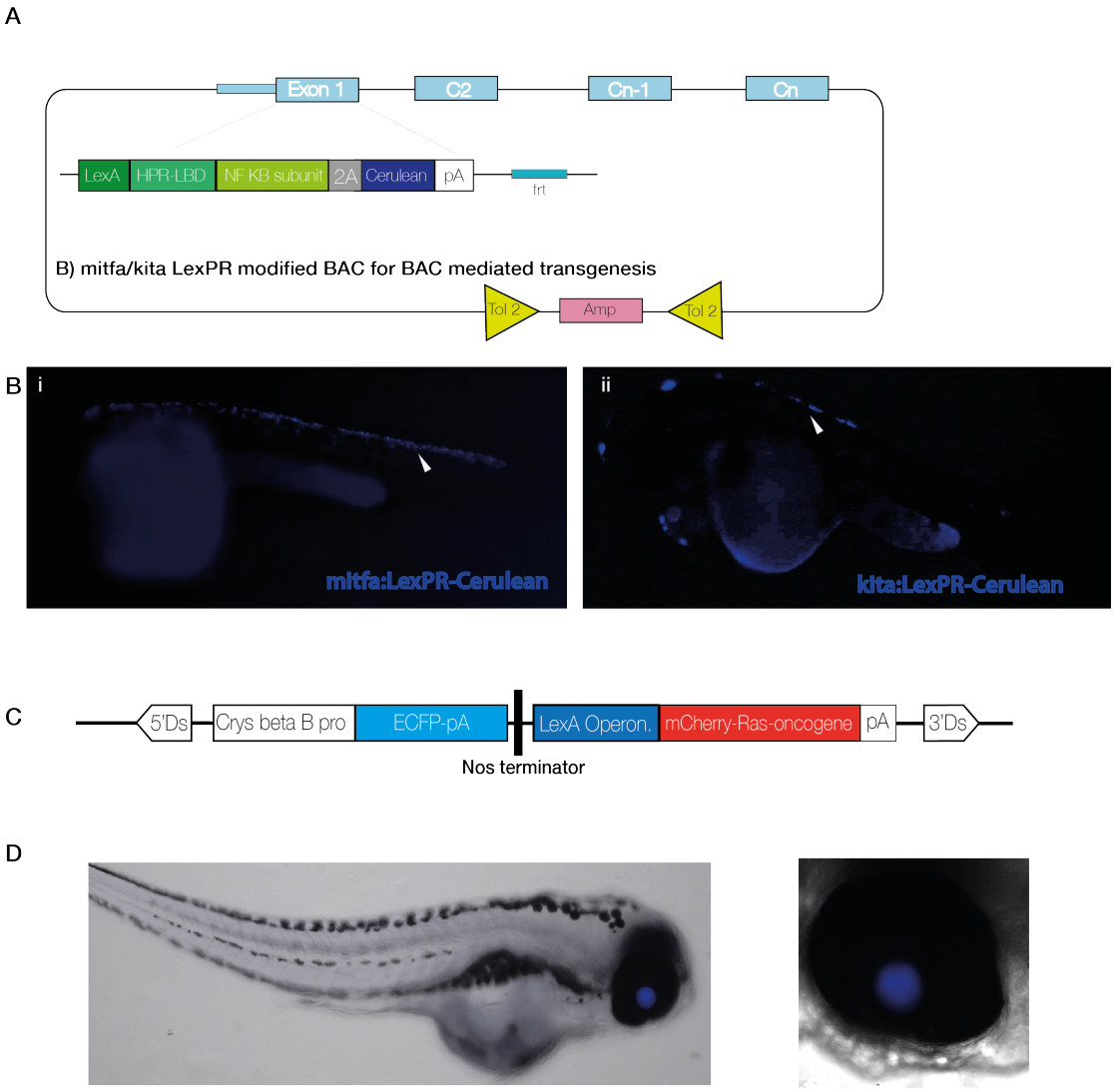


Figure 5.3 Generation of driver and effector transgenic lines for melanocyte-specific oncogene expression. A) Schematic of kita/mitfa modified BAC DNA containing the LexPR transactivator (green), a viral self-cleaving peptide (2A, grey) and a fluorescent reporter (cerulean, blue), followed by a polyA tail (pA, white) and remaining Frt site, recombined into the first exon, with an ampicillin selection cassette (Amp, pink) and tol2 arms (yellow) on the BAC plasmid backbone for BAC mediated transgenesis. B) Transgene expression in melanocytes with kita (i) and mitfa (ii) in Fo larvae as indicated by white arrows. C) Schematic PCrysB:ECFP-LexOP-mCherry-RasOncogene construct, embedded within a non-autonomous Ds element to produce Ds insertions in the zebrafish genome with the aid of a modified Ac transposase. D) The PCrysB promoter driving ECFP reporter showed fluorescence specific to the lens from 2 dpf.

5.2.2 Generation of Ras-oncogene effector lines

The Ras-effector construct was adapted from previously published LexOP effector construct[96] by inserting amplified mCherry-Ras oncogenes (NRasK61Q, HRasG12V and KRasG12V) in place of ECFP, downstream of LexA operon (Figure 5.3 C). The construct also contains the selection marker, ECFP, expressed in the eye of the injected fish under the control of lens-specific zebrafish crybB promoter (crystallin beta B) and used to screen for transgene integration (Figure 5.3 D)[170]. Instead of tol-2 mediated transgenesis this system makes use of the transposase *Activator (Ac)/ Dissociation (Dc)* element[170]. The effector constructs are placed within a non-autonomous maize dissociation (Ds) element, which allows for effective activator (Ac)-mediated transposition. This system is highly efficient and results in a very high number of independent integrations into the zebrafish genome, thus allowing for high level expression of the Ras effectors in the context of oncogenic transformation, when activated by LexPR. Three founder fish were identified for each effector line, *Tg(CrysB:ECFP,LexOP:mCherry-NRasK61Q)^{ox126a,b,c}, Tg(CrysB:ECFP'LexOP-mCherry-HrasG12V)^{ox127a,b,c} and Tg(CrysB:ECFP'LexOP-mCherry-KrasG12V)^{ox128a,b,c}*.

Mitfa and kita melanocyte-specific driver lines were crossed with the effector lines harbouring the mCherry-Ras oncogene (NRasK61Q, HRasG12V and KRasG12V) under the control of LexOP to generate transgenic zebrafish lines harbouring both alelles. Thus far, *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)^{ox129,} Tg(mitfa:LexPR-*

2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)^{ox130}, *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-HRasG12V)*^{ox131} and *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-KRasG12V)*^{ox132} have been generated. Crossing the mitfa- and kita-driver lines with the Ras-effector lines resulted in double transgenic offspring that expressed mCherry-Ras exclusively in the melanocytes following addition of mifepristone (Figure 5.4). Figure 5.4 A and B, shows representative images for *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129} and *Tg(mitfa:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox130}. Consistent spatial expression patterns of the mCherry reporter were observed when different founders of the mitfa and kita driver lines were crossed to the Ras-effector lines, suggesting that the BAC transgene recapitulates the endogenous expression patterns. It should be noted that when the kita driver was used, the mCherry-Ras oncogene expression was seen mainly in the tail (Figure 5.4 A) whereas when the mitfa driver line was mCherry-Ras oncogene expression was mainly visible over the head (Figure 5.4 B). Interestingly, in melanocytes expressing mCherry either low levels of melanin were sometimes observed or they did not appear to have melanin at all. Transformed melanocytes are seen visible in pairs or clumps of transformed red cells as indicated by the white arrows, indicative of their proliferative potential (Figure 5.4 A).

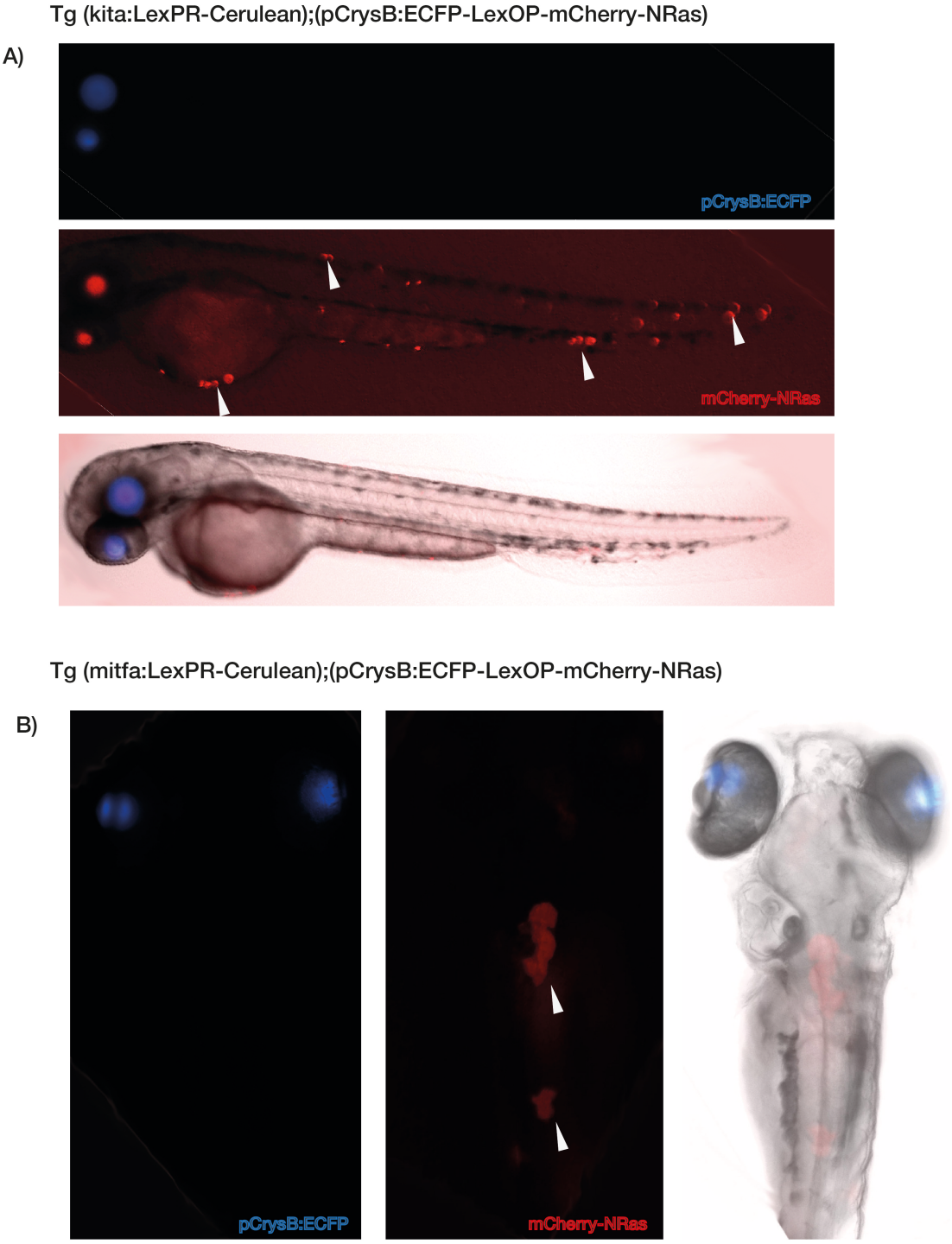


Figure 5.4 Mifepristone-inducible melanocyte-specific Ras-oncogene expression in double transgenic larvae. The melanocyte-specific expression of mCherry-NRasK61Q in transgenic fish harbouring both driver and effector alleles is activated by addition of 1 μM mifepristone (RU486) at 1 dpf in both tg(kita:LexPR-Cerulean);(pCrysB:ECFP-lexOP-mCherryNRasK61Q) (A) and tg(mitfa:LexPR-Cerulean);(pCrysB:ECFP-lexOP-mCherryNRasK61Q) (B) as indicated by the white arrows.

5.2.3 Expression of oncogenic NRas by kita and mitfa promoters induces continued proliferation of the melanocytes

To determine whether the observed phenotype was as a result of continuous proliferation of melanocytes, we labelled the melanocytes *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129} injected embryos with Edu by injection into the pericardium at 96 hours post fertilization (hpf). Edu will label all cells that are proliferating. Melanocytes should all be post-mitotic by 60 hpf, when the larval pigmentation pattern is largely complete. In *Tg(kita:LexPR-2A-Cerulean;PcrysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129}, where we have previously noted that many of the melanocytes expressed mCherry in the tail, we observe large clusters of proliferating cells, located immediately adjacent or in the same plane as the pigmented melanocytes (Figure 5.4 Ci) that are not present in the control where no mifepristone was added to the water (Figure 5.4 D). To confirm that these clusters were proliferating melanocytes, we boosted the mCherry signal with a RFP booster, which will boost the signal of all RFP derivatives including mCherry (Figure 5.4 Bii). Melanocytes appear in clumps with multiple dividing nuclei (Figure 5.4 Cii).

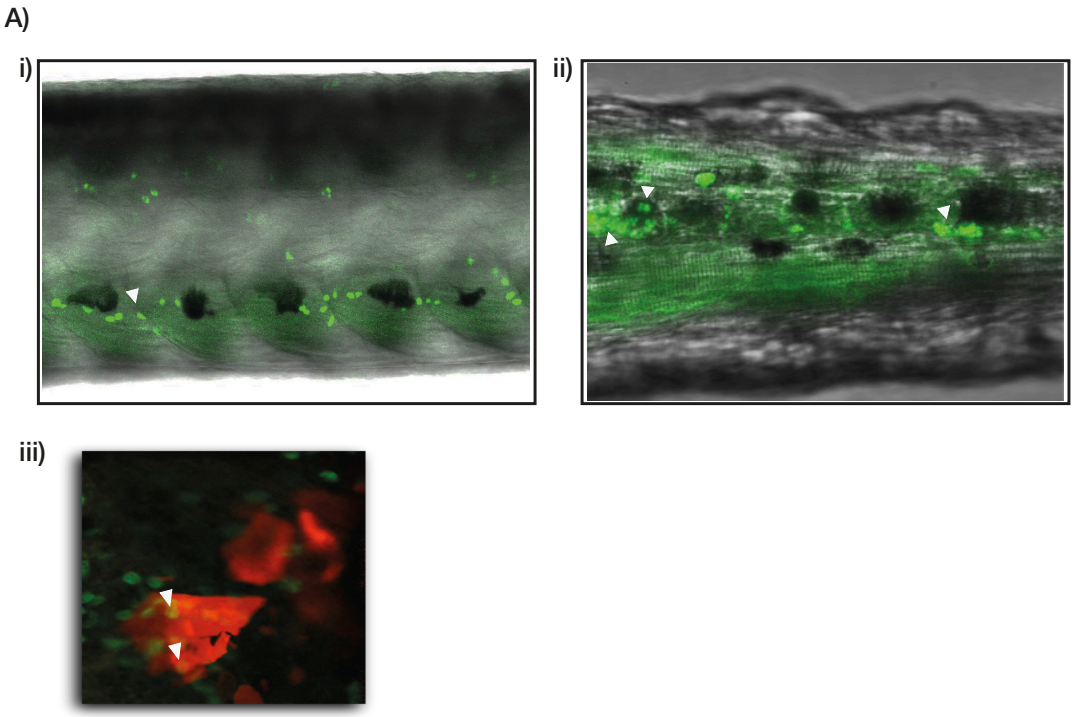


Figure 5.5 Expression of Nras-oncogene in melanocytes induces overproliferation phenotype. Embryos were injected pericardially at 96 hpf with 5 nL Edu (150 μM), placed in standard conditions overnight and labeled with the Click-iT AlexaFluor-488 Imaging Kit. A) Edu is incorporated into nuclei of cells in the same plane as melanocytes in the tail of tg(kita:LexPR-Cerulean);(pCrysB:ECFP-lexOP-mCherryNRasK61Q)(mif+) (i) and less so in controls (kita:LexPR-Cerulean);(pCrysB:ECFP-lexOP-mCherryNRasK61Q)(mif-) (ii). Immunostaining with mCherry booster shows Edu-labelled nuclei (green) in melanocytes (red) (iii).

5.3 Discussion

Although progress has been made in understanding melanoma biology, the incidence of melanoma continues to increase with the prognosis for melanoma patients still remaining poor[116]. The transgenic models generated in this study provide a powerful tool for studying different aspects of melanoma biology, including the transformation of melanocytes, melanoma progression and biology, as well as immunotherapy.

Previous melanoma models using the mitfa promoter, require the zebrafish lines to be crossed into a $p53^{-/-}$ background in order for adult fish to develop solid tumours. However, using the kita promoter, HRas expression in melanocytes resulted in overproliferation of melanocytes and ultimately a highly penetrant melanoma, without a contributing mutation in p53[74, 116]. In this study we generated two transgenic driver lines using both the mitfa and kita promoters. It may be that to form a solid tumour in adult fish, using the LexPR system, either promoter may require an inactivating mutation in p53. In the models developed in this study, transformed melanocytes mainly resided in the tail when the kita driver was used, whereas when the mitfa driver was used, transformed melanocytes were mainly present in the head. Santoriello *et. a.,l*[116] showed how melanocytic hyperplasia mainly occurs in the tail of kita-HRasG12V fish. In contrast in mitfa-NRasK61Q, tumours were not restricted to the tail but also the head and trunk of the fish. This may be because kita or mitfa-expressing progenitors may arise and concentrate in specific areas, ultimately giving rise to solid tumours in specific anatomical locations. Interestingly, in humans, the histological classification of melanomas in accordance with the site of origin, predicts the frequency of certain mutations and ultimately survival outcome[171]. Thus identifying markers linked to histological classification may be useful for diagnostic staging criteria.

In zebrafish, patterning occurs in two waves of pigmentation. For melanocytes, differentiation involves cell shape changes, the expression of pigmentation enzymes and subsequently the generation of melanin[172]. Embryonic melanocytes are directly derived from the neural crest. Adult

melanocytes are added to the embryonic pigmentation pattern by proliferation of unpigmented precursor cells and for the most part, pigmentation is associated with differentiation without evidence of cell division[173]. It should be noted, that in the developing zebrafish larvae, Taylor *et al*[173]*.,* showed that although it is a rare event, differentiated melanocytes, do have the potential to divide[173]. Therefore, in our model, the absence of melanin in transformed melanocytes, could simply be an indication that they are still proliferating and ultimately past day 5, an overproliferation phenotype as reported by others will be visible[116].

By using a binary system of transgenic fish, the same driver line, can be crossed to multiple oncogene effector lines[96]. This provides the potential to explore mechanistic detail of melanoma initiation and progression with different driver mutations. Previous studies have shown that activated components of the Ras signalling pathway drive a tumour-promoting inflammatory response[36]. However, these findings do not address the difference in the inflammatory response that different driver mutations may elicit. This model provides a novel tool to systematically address the difference in the inflammatory responses driven by different Ras driver mutations.

Unlike existing melanoma models, using the LexPR system, we have generated an inducible melanoma model that allows for temporal control of gene expression. Using this inducible system would allow us to truly study melanoma initiation. Interestingly, congenital nevi in children harbour mutations in Ras, but not BRAF, and are associated with an increase risk of melanoma development[174].

The tractability of zebrafish genetics means that using sgRNA/cas9 genome editing technology, factors that contribute to melanoma initiation and progression, both from the perspective of the immune response and the melanoma itself, can easily be studied by knockdown. Furthermore, zebrafish lines of a defined genetic background can easily be generated providing an excellent means of pathway analysis. The larval phenotype observed in this zebrafish melanoma model can provide a powerful platform for testing drug targets for pre-clinical testing of therapeutics. The mCherry positive transformed cells can provide a direct biological readout in fast, easy to score chemical screens, aimed at finding compounds or drugs that may revert the overproliferation phenotype observed in melanocytes. An alternative to more traditional melanoma treatments, which in the mammalian system is difficult to explore, is that of chemoprevention. Instead of treating patients with established melanoma, healthy subjects are given a therapy that may prevent tumour formation. Because of the inducibility of our model system and the larval phenotype we see, this model system would provide a simple and efficient tool to screen chemical compounds that may prevent clonal expansion of transformed melanocytes.

In conclusion, we propose to use this model to study the sterile inflammatory response that may occur as a result of oncogenic transformation. However the binary inducible transgenic system generated here, provides the potential to study many aspects of melanoma biology.

Chapter 6

Analysis of the regulatory networks that govern the neutrophil response to pre-neoplastic melanocytes

6.1 Introduction

A complex network of inflammation succeeds somatic cell transformation and malignant disease, and offers both targets for prevention and treatment of cancer. Myeloid cells are equipped with sensors for damage and inflammation and subsequent effector mechanisms for resolving inflammation[36]. Transformed cells are able to shape the nature of the myeloid cells and evoke an immunosuppressive response, enabling the progression of the disease. Because we cannot predict when and where transformed cells may arise in the human host, very little is known about the sterile inflammatory signals that ensue somatic cell transformation[160]. This prompts the need for models that will better our understanding of the contribution of the myeloid component as transformed cells establish themselves as the earliest progenitors of cancer.

6.1.1 Neutrophils and Melanoma

This chapter focuses on neutrophils as they respond to oncogene-transformed melanocytes and goblet cells. Although much attention has been dedicated to the role of macrophages in different cancers, the importance of neutrophils in cancer biology has only recently started to be appreciated. Neutrophils are the most abundant circulating leukocytes in humans and as previously discussed contain a range of effector mechanisms including microbicidal molecules, proteases, neutrophil extracellular traps (NETs) and oxidative mechanisms[7]. This suggests a more important role for neutrophils in tumour biology than is currently reflected in the literature. As previously

mentioned, tumour-associated neutrophils (TANs) develop a pro-tumourigenic phenotype, largely driven by the presence of TGF-β, and yet, inhibition of TGF-β, can change the cells to be tumouridical[89]. In untreated tumours, neutrophils were reported to contribute to tumourigenesis by secreting factors that promote ECM remodelling, angiogenesis, tumour growth and suppress the immune system[82]. Although studies on the role of neutrophils in melanoma are scarce, they do suggest that in general, TANs are tumour promoting[95, 175]. For example, melanomas that express granulocyte chemotactic protein-2 (GPC-2) induced a strong influx of activated neutrophils, which expressed gelatinase B/mmp9 and promoted angiogenesis[175]. IL-8 secreted by melanoma cells, promotes neutrophil recruitment into the tumour and mediates an increase in expression of the β2 integrin on their cell surface. β2 integrin signalling with intercellular adhesion molecule (ICAM)-1 on the melanoma surface facilitated lung metastasis[95]. However, it has been shown that under certain conditions, it is possible to shift the neutrophil phenotype to being tumouricidal[176, 177, 178, 179, 180]. In a study using human cell lines, it was shown that in primary melanoma cells, increasing the levels of IL-8 resulted in reduction in tumour growth[176]. In contrast, in a metastatic melanoma cell line, increasing levels of IL-8 correlated with tumour growth. Cytotoxicity assays with isolated neutrophils confirmed the preferential killing of primary melanoma cells rather than metastatic melanoma cells by neutrophils[176]. This study further proves the ability of melanoma cells to shape the microenvironment and the outcome of the disease, begging the question of the plasticity of the immune cells as the disease progresses and the microenvironment changes[176]. Melanoma cells

were shown to produce secreted protein acidic and rich in cysteine (SPARC), which plays a role in tumour evasion from immune surveillance by inhibiting neutrophil infiltration and anti-tumour activity[177]. When SPARC was knocked down, neutrophil infiltration into the tumour, as well as their activation, was triggered[177]. Melanoma cell lines, transfected to secrete IL-4, resulted in neutrophils able to diminish tumour growth, once again suggesting that the immunogenicity of the tumour polarises the neutrophils and directly affects the outcome of the disease[178]. Similarly, recombinant IL-1β, injected intratumourally was shown to inhibit the growth of B16-melanoma in mice. This correlated with an increase in number of polymorphonuclear leukocytes in the tumours[179]. One mechanism by which neutrophils, polarised to an anti-tumour phenotype, are able to mediate tumour cell killing is by reactive oxygen species (ROS)[180]. To study ROS-dependent activities of neutrophils in melanoma, melanoma-bearing mice were injected with sephadex, known to cause an aseptic inflammation resulting in activation of neutrophils. This study in no way recapitulates the role of neutrophils in melanoma biology but it suffices to say that in the presence of sephadex, which would switch the neutrophils to an N1 phenotype, neutrophils were able to kill tumour cells by oxidative mechanisms[180]. Conflicting reports over the role of neutrophils in cancers suggests that their function is context dependent and largely determined by the tumour microenvironment.

6.1.2 Zebrafish studies of the innate immune response to melanoma

The zebrafish embryo has previously been used as a model organism to study the interactions between macrophages and neutrophils and oncogene-

transformed melanocytes and goblet cells using a Kita:HRasG12V transgenic line. This study revealed a recruitment of both neutrophils and macrophages to oncogene-transformed cells. The authors reported frequent cytoplasmic tethers between the immune cells and transformed cells, as well as phagocytosis of transformed cells by macrophages[36]. HRasG12V-positive cells were shown to trigger H_2O_2 production in the vicinity of the transformed cells which lead to leukocyte recruitment[36]. This mirrors the phenotype previously reported for the migration of leukocytes in response to a H_2O_2 gradient following tailfin transection on the zebrafish embryo[181]. A subsequent study in the same model system highlighted the fact that myeloid cells contribute to neoplastic cell growth by releasing prostaglandin $(PGE)_2$[160]. In the adult zebrafish harbouring the HRasG12V mutation, with a previously reported predisposition for developing tumours in the ventral and tailfins, repeated wounding of the tailfins for three months resulted in an increase in pigment intensity in the wounded tailfins suggesting local recruitment and proliferation of pre-neoplastic melanocytes[36, 182]. The increased proliferation of pre-neoplastic cells was attributed to neutrophils rather than macrophages, and that PGE2 derived from immune cells contributes to the proliferation. Interestingly, patients with ulcerative melanomas had an increase in neutrophil number, which correlated with the extent of ulceration and tumour proliferation. However, no such correlation was seen for macrophages[182].

Using the binary zebrafish models described in chapters 3 and 5, this chapter highlights the potential of the resource created in this study in exploring the myeloid cell role in melanoma biology and provides novel insight into the

transcriptional profile of neutrophils as they respond to oncogene-transformed melanocytes.

6.2 Results

6.2.1 Generation of a double binary system to study the myeloid response to oncogene-transformed melanocytes

To generate a double binary system to study the myeloid response to oncogene-transformed melanocytes, the inducible LexPR binary system (chapter 5) was coupled to the *in vivo* biotinylation system for the transcriptional profiling of macrophages and neutrophils (chapter 3). Although two different melanocyte-specific driver lines (kita and mitfa) and three different mCherry-Ras oncogene lines were generated in this study, the kita driver and NRas oncogene were prioritised. This decision was based on the previous studying showing that when the kita promoter was used to drive HRasG12V expression in melanocytes, a highly penetrant melanoma resulted without the need for a contributing p53 mutation[116]. Since, NRasK61Q is the most relevant to human melanoma, this oncogene was prioritized[79]. As described in chapter 5, *TgBAC(kita:LexPR-2A-Cerulean)*^{ox123} (Figure 6.1, fish a) was crossed to *Tg(crysB:ECFP, LexOP:mCherry-NRaK61Q)*^{ox125} (Figure 6.1, fish b) to generate double inducible transgenic line *Tg(kita:LexPR-2A-Cerulean;crysB:ECFP,LexOP:mCherry-RasK61Q)*^{ox129} (Figure 6.1: Binary System 1, fish ab). Similarly, as described in chapter 3, *TgBAC(mpo:BirA-2A-Citrine)*^{ox122} (Figure 6.1, fish c) was crossed to crossed to *Tg(bactin:Avi-Cerulean-RanGap)*^{ct700a} (Figure 6.1, fish d) to generate *Tg(mpo:BirA-2a-citrine;bactin:Avi-Cerulean-Rangap)*^{ox128} (Figure 6.1: Binary System 2, Fish cd). Subsequent analyses were carried in embryos by crossing binary system

1 (Figure 6.1, fish ab) with binary system 2 (Figure 6.1, fish cd). However as illustrated below, multiple permutations of the double binary system are possible.

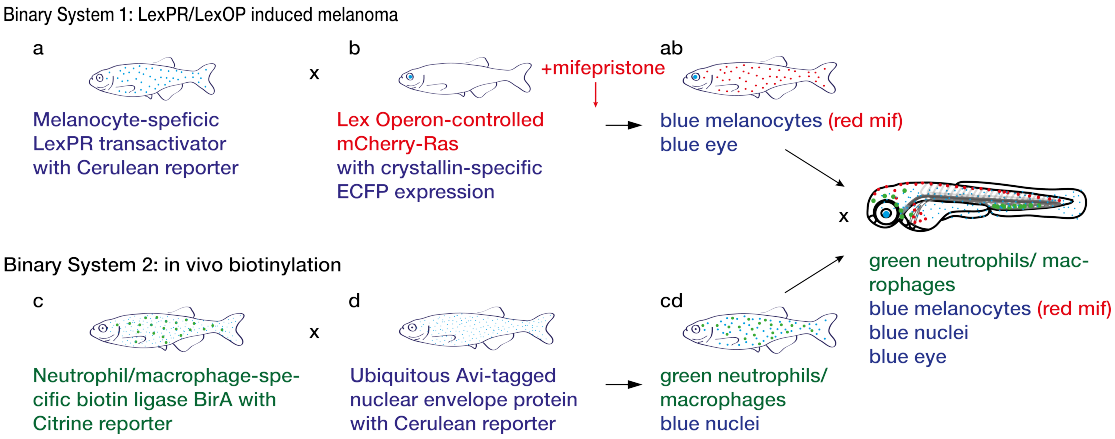


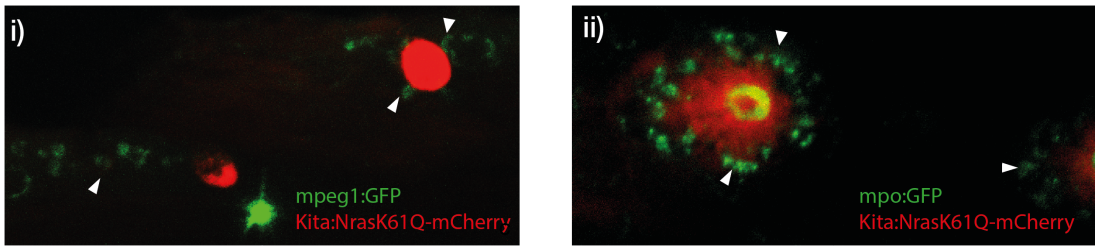
Figure 6.1 Schematic representation of double binary system to study the myeloid response to melanoma. Binary System 1: The LexPR transactivator expressed under the control of a melanocyte-specific promoter activates mCherry-Ras oncogene in the presence of a specific ligand (mifepristone). Fish a) Melanocyte-specific LexPR transactivator with Cerulean reporter. Fish b) Lex Operon controlled mCherry-Ras with crytallin-specific ECFP expression. Binary System 2: Nuclear envelope protein tagged with a biotin acceptor peptide (Avi-tag) is biotinylated in macrophages/neutrophils allowing for cell-specific isolation of nuclei and genome-wide analysis. Fish c) Macrophage/neutrophil-specific biotin ligase, BirA, with Citrine reporter. Fish d. Ubiquitous Avi-tagged nuclear envelope protein with Cerulean reporter. Fish a X Fish b ➔ Fish ab (blue eyes and blue melanocytes (red in the presence of mifepristone). Fish c X Fish d ➔ Fish cd (green macrophages/neutrophils and blue nuclei). Fish ab X Fish cd ➔ embryos for analysis (green neutrophils/macrophages, blue melanocytes (red in the presence of mifepristone), blue nuclei, blue eyes).

6.2.2 Kita-driven oncogenic NRas melanocytes are immunogenic

To show that the NRasK61Q-transformed melanocytes generated by the inducible LexPR binary system were immunogenic, *Tg(kita:LexPR-2A-Cerulean;crysB:ECFP, LexOP:mCherry-NRasK61Q)*^{ox129} were incrossed and mifepristone added to the water of 24 hpf embryos, followed by subsequent fixing of the larvae at 5 dpf and staining for mpeg1 and mpo to detect macrophages and neutrophils, respectively. Macrophages can be seen in the vicinity as well as directly interacting with the NRasK61Q-transformed cells

(Figure 6.2 Ai), in the tail of the zebrafish larvae. The neutrophils appear to form a ring-like structure surrounding the transformed melanocytes (Figure 6.2 Aii).

A



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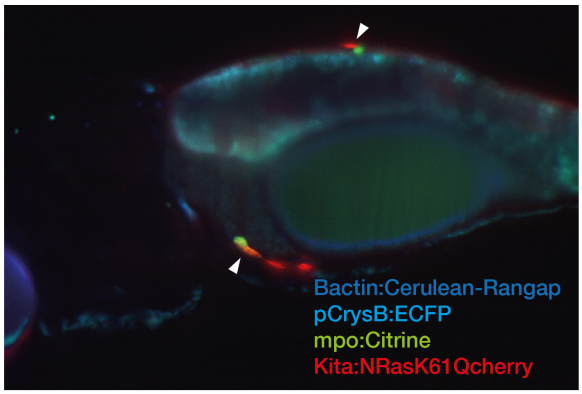

Figure 6.2 Infiltration of mCherry-NRasK61Q transformed melanocytes by macrophages and neutrophils. A) *Tg(kita:LexPR-2A-Cerulean;pCrysB:ECFP-lexOP-mCherry-NRasK61)* were incrossed and 1 µM of mifepristone added to the water at 24 hpf. At 5dpi, larvae were fixed and stained for mpo (neutrophils) and mpeg1 (macrophages). Confocal images of macrophages (green, Ai) and neutrophils (green, Aii) interacting with a mCherry-NRasK61Q-transformed melanocyte (red) as indicated by the white arrows. B) *Tg(kita:LexPR-2A-Cerulean;crysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129} was crossed to *Tg(mpo:BirA-2a-citrine;bactin:Avi-Cerulean-Rangap)*^{ox128}, 1 µM of mifepristone added to the water at 24 hpf and embryos allowed to develop until 5dpi. Image of neutrophils (green) interacting with NRasK61Q-transformed melanocytes (red) as indicated by the white arrows, in larvae harbouring all four (blue eye, blue nuclei, green neutrophils and red melanocytes), was captured on a Zeiss Z1 lightsheet microscope.

Similarly, melanoma-inducing binary system 1 transgenic line, *Tg(kita:LexPR-2A-Cerulean;crysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129} was crossed to *Tg(mpo:BirA-2a-Citrine;bactin:Avi-Cerulean-RanGap)*^{ox128}, oncogene constitutive expression was activated in the melanocytes and goblet cells by addition of mifepristone to the water at 24 hpf and embryos were fixed and imaged at 5dpf. A fish harbouring all four alleles features a blue eye, blue

nuclei, green neutrophils and NRasK61Q-transformed red melanocytes/goblet cells. The green neutrophils were localised in the vicinity of the transformed melanocytes (Figure 6.2 B).

6.2.3 Transcriptional analysis of neutrophils in response to NRasK61Q-transformed melanocytes by *in vivo* biotagging

This chapter focuses on the results obtained in genome-wide transcriptional profiling of neutrophils, whilst the equivalent analysis for macrophages is currently underway. To obtain the active transcriptome of neutrophils, *Tg(kita:LexPR-2A-Cerulean;crysB:ECFP,LexOP:mCherry-NRasK61Q)*^{ox129} and *Tg(mpo:BirA-2a-Citrine;bactin:Avi-Cerulean-RanGap)*^{ox128} transgenic lines were crossed and NRasK61Q expression driven in the melanocytes by addition of mifepristone to the water at 24 hpf. Embryos expressing mCherry-NrasK61Q in the melanocytes were selected for the profiling experiments. Roughly, 45 larvae from each replicate were dissected into trunk and tail, to reduce background levels from non-responding neutrophils (Figure 6.3 A). The dissections were done in such a fashion that analysed transformed cells and their environing neutrophils in the tail are melanocytes and exclude the goblet cells. The neutrophil population in the trunk is reflective not only of cells responding to the transformed melanocytes and goblet cells but also developing neutrophils in the intermediate cell mass and anterior yolk sac. 'Biotagged' nuclei were isolated from 5 dpf embryos by nuclei pulldown[122]. Nuclear RNA pools were extracted and libraries prepared using SMART-seq^{TM}v4 technology for cDNA synthesis and amplification for small cell numbers followed by Illumina Nextera library preparation. Libraries were

sequenced on a Next Generation Sequencing (NGS) platform. For the purposes of this study, the analysis focuses only on the tail samples. Differential Expression analysis comparing the controls (tail control 1 and tail control 2) to tail Nras^{+} 1 sample, and was carried out using Deseq2 R package which identifies a number of differentially expressed genes with statistical significance using a high stringency cut-off (adjusted P-value <0.05) (Figure 6.3 B). Replicate analysis of genome wide studies was used to determine the reproducibility of technical duplicates. Scatterplots indicate normalised expression counts of individual genes, where normalised counts of tail control 1 vs tail control 2 (Figure 6.3 Ci), show technical reproducibility whereas for tail control 1 vs tail NRas^{+} 1 and tail control 2 vs tail NRas^{+} 2, an increase in the number of differentially expressed genes is evident (Figure 6.3 Cii-iii).

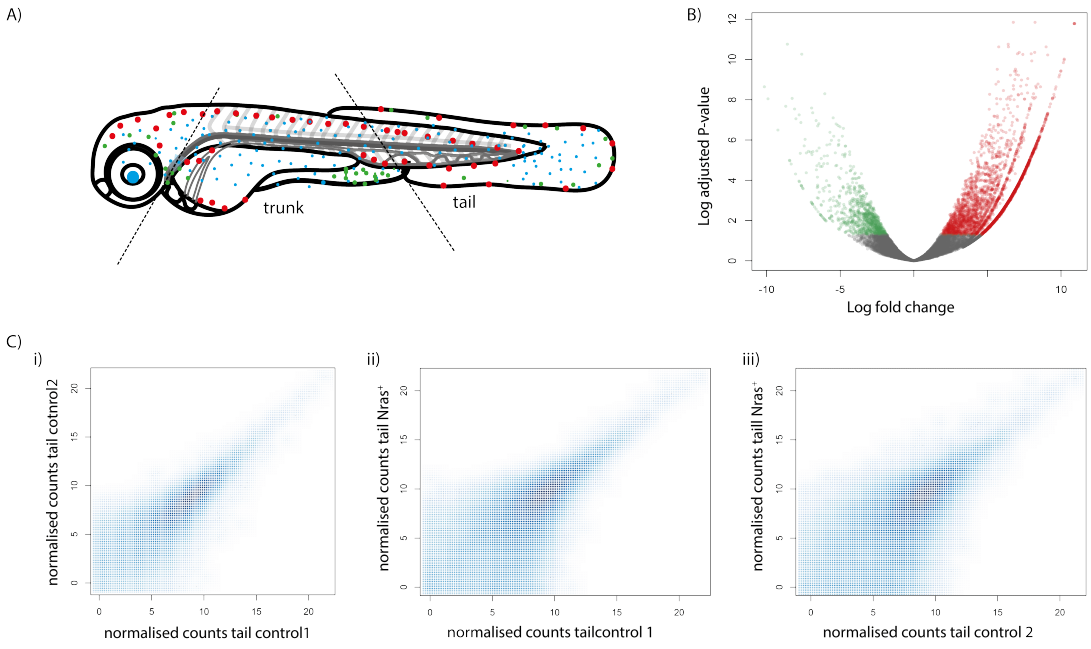

Figure 6.3 Analysis of technical replicates to obtain differentially expressed genes. A) Larvae were dissected into tail and trunk. B) Volcano plot showing differentially expressed genes with a high stringency cutoff (adjusted p-value <0.05), with upregulated genes in red and downregulated genes in green. C) Scatterplots show normalised counts of tail control 2 vs tail control 1 (i), tail NRas^{+} vs tail control 1 (ii) tail NRas^{+} vs tail control 2 (iii).

To gain an understating of the role of neutrophils in the earliest stages of melanoma, we focused on key pathways essential for neutrophil function or those previously associated with the role of neutrophils in tumours. Genes were classified into those involved in cytoskeletal rearrangement, cell adhesion, chemokines and integrins, ERK/MAPK signalling, apoptosis and p53 DNA damage, growth factors, neutrophil granule proteins, NOS, ROS and respiratory burst, metalloproteinases, metallopeptidases and associated proteins, TLRs, MHC-related molecules and T cell-activation. A global analysis of all the categories analysed shows a number of genes, both upregulated and downregulated, although a strong bias towards genes that are downregulated is evident (Figure 6.4).

To link genes to specific pathways and functional classifications, we used gene ontology tools. Interestingly we found a general downregulation in genes involved in cytoskeletal rearrangement (Figure 6.5 A) including myosin, heavy chain 11a (myh11a), cdc42 effector protein (Rho GTPase binding (cdc42ep3), as well as molecules involved in cell adhesion (Figure 6.5 B), including CD36 (thrombospondin receptor) (cd36) and a number of cadherins (cdh6, cdh15 and cdh13).

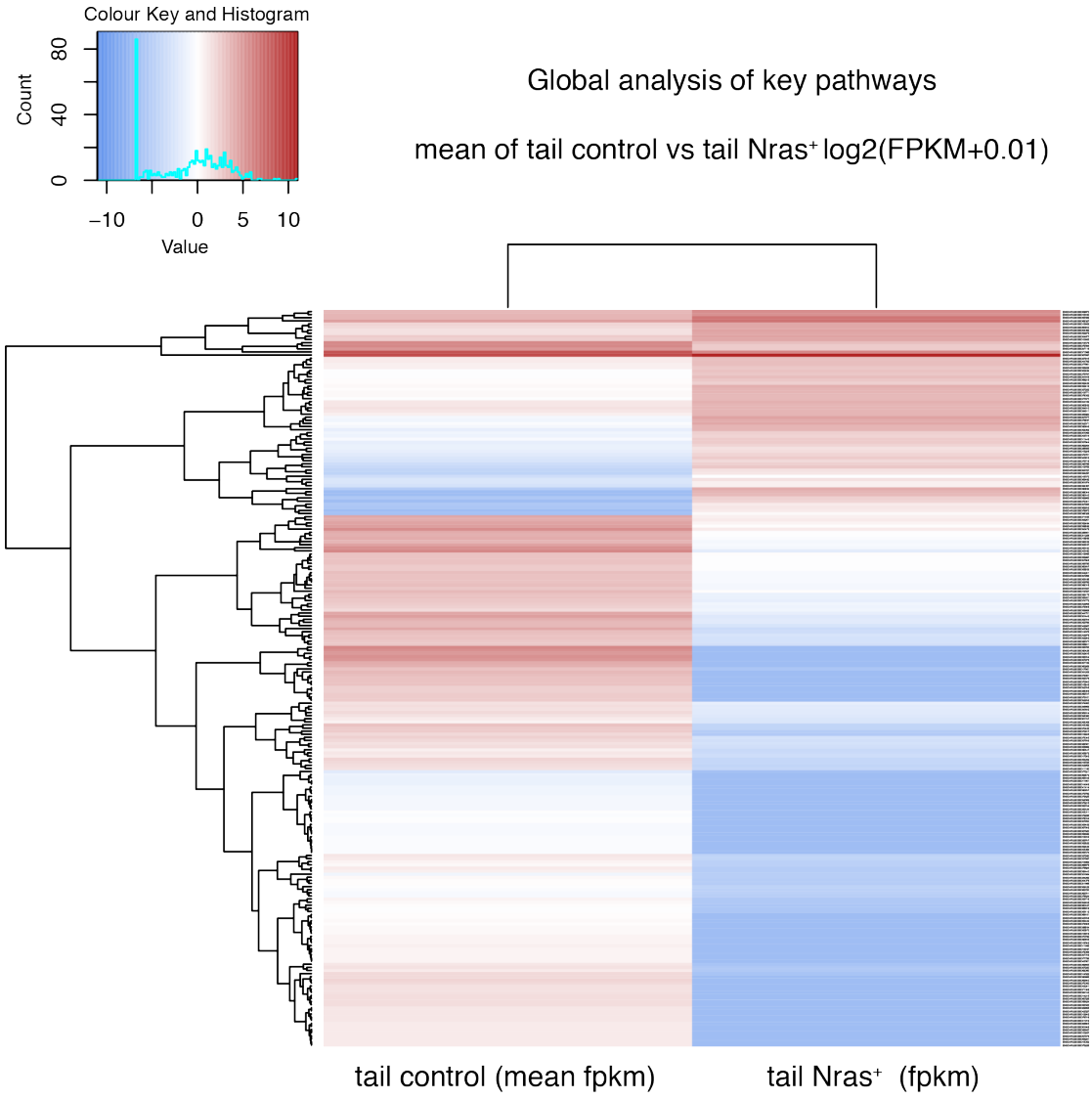

Figure 6.4 Heatmap comparing differentially expressed genes. Heatmap shows the $\log_2$ (Fragments Per Kilobase of transcript per Million mapped reads (FPKM) +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Differentially expressed genes were selected from different pathways or functional classifications (cytoskeletal rearrangement, cell adhesion, chemokines and integrins, ERK/MAPK signalling, apoptosis and p53 DNA damage, growth factors, neutrophil granule proteins, NOS, ROS and respiratory burst, metalloproteases, metallopeptidases and associated proteins, TLRs, MHC-related molecules and T cell-activation). Red - high expression. Blue - low expression. White - medium expression.

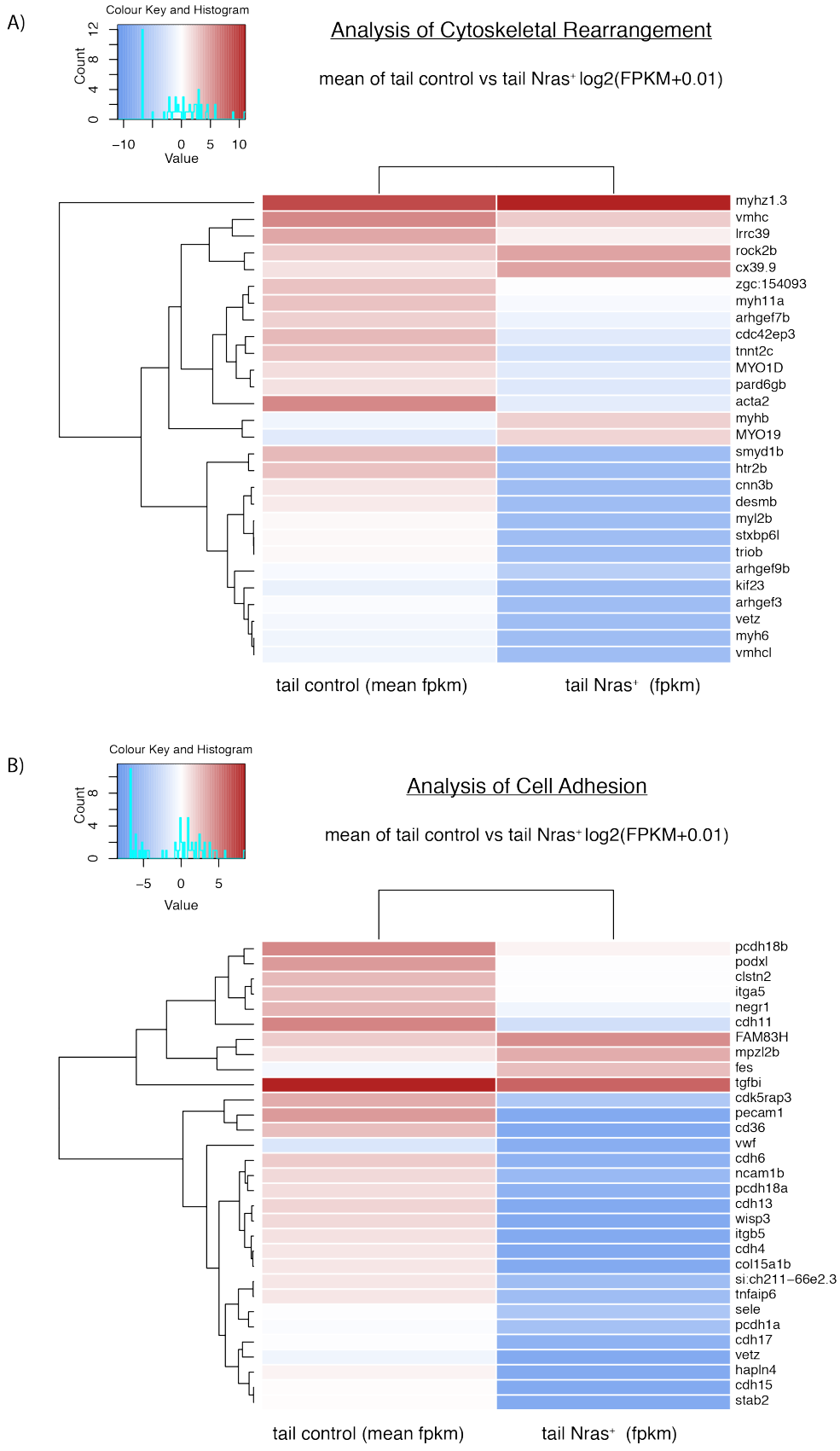

Figure 6.5 Heatmap of differentially expressed genes involved in Cytoskeletal Rearrangement (A) and Cell Adhesion (B). Heatmap shows the $\log_2$(FPKM +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Red-high expression. Blue-low expression. White-medium expression.

A number of integrins (itgb1bp1, itfg3, itga11b, itgb5, itga9) and chemokines (cxcl18b, cxcr4, cxcl12b, cxcl12a, ccl19a.1, ccl44) were also downregulated (Figure 6.6 A). Similarly there was a striking downregulation of genes in the ERK/MAPK signalling pathway (jnk1/mapk8-associated membrane protein (jkamp), late endosomal/lysosomal adaptor, MAPK and MTOR activator 4 (LAMTOR4), mapk8a) with only mapk8b found upregulated (Figure 6.6 B).

Since tumour-associated neutrophils are thought to be longer-lived cells[183], we looked at pathways involved in apoptosis (Figure 6.7 A). Strikingly, the pathway analysis revealed a strong bias towards downregulation of genes in involved in apoptosis. Particularly striking were the number of genes downreguled in the p53 DNA damage pathway including (atr, cdk2, growth arrest and DNA damage-inducible (gadd45bb)), with only a few pro-apoptotic genes found upregulated (caspase7, TNF receptor-associated factor 1 (traf1), checkpoint kinase 2 (chek 2)) (Figure 6.7 A). Among growth factors and associated proteins, there were many that were downregulated, such as insulin-like growth factor 2b (igf2b), vascular endothelial growth factor c (vegfc), neuropilin 1b (nrp1b) (Figure 6.7 B). Interestingly, a number of key growth factors, such as platelet-derived growth factor (PDGFD), placental growth factor b (pgfb) and fibroblast growth factor 24 (fgf24) were highly upregulated (Figure 6.7 B).

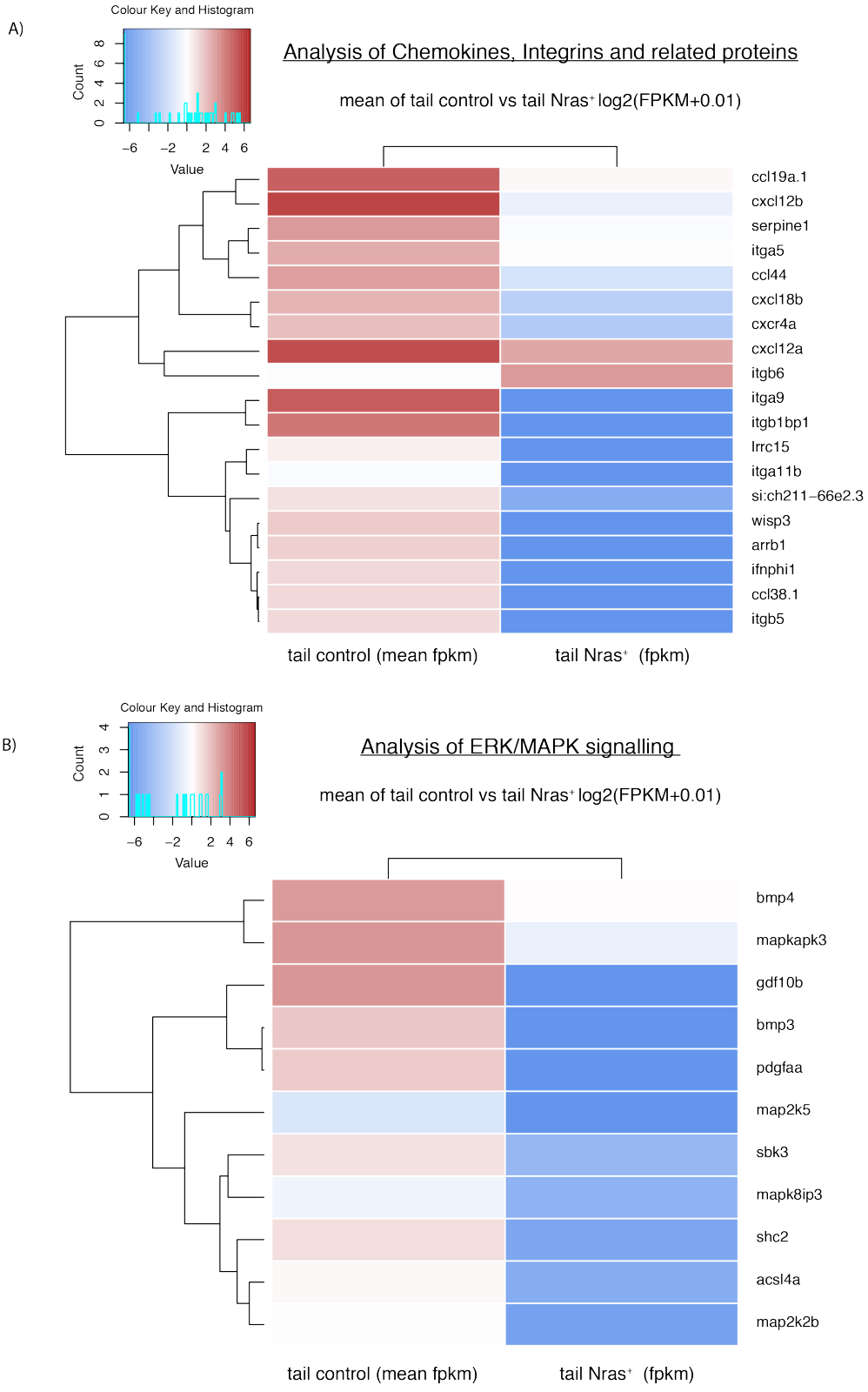

Figure 6.6 Heatmap of differentially expressed genes involved in Chemokine, Intergrin and related protein signalling (A) and ERK/MAPK signalling (B). Heatmap shows the $\log_2$ (FPKM +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Red-high expression. Blue-low expression. White-medium expression.

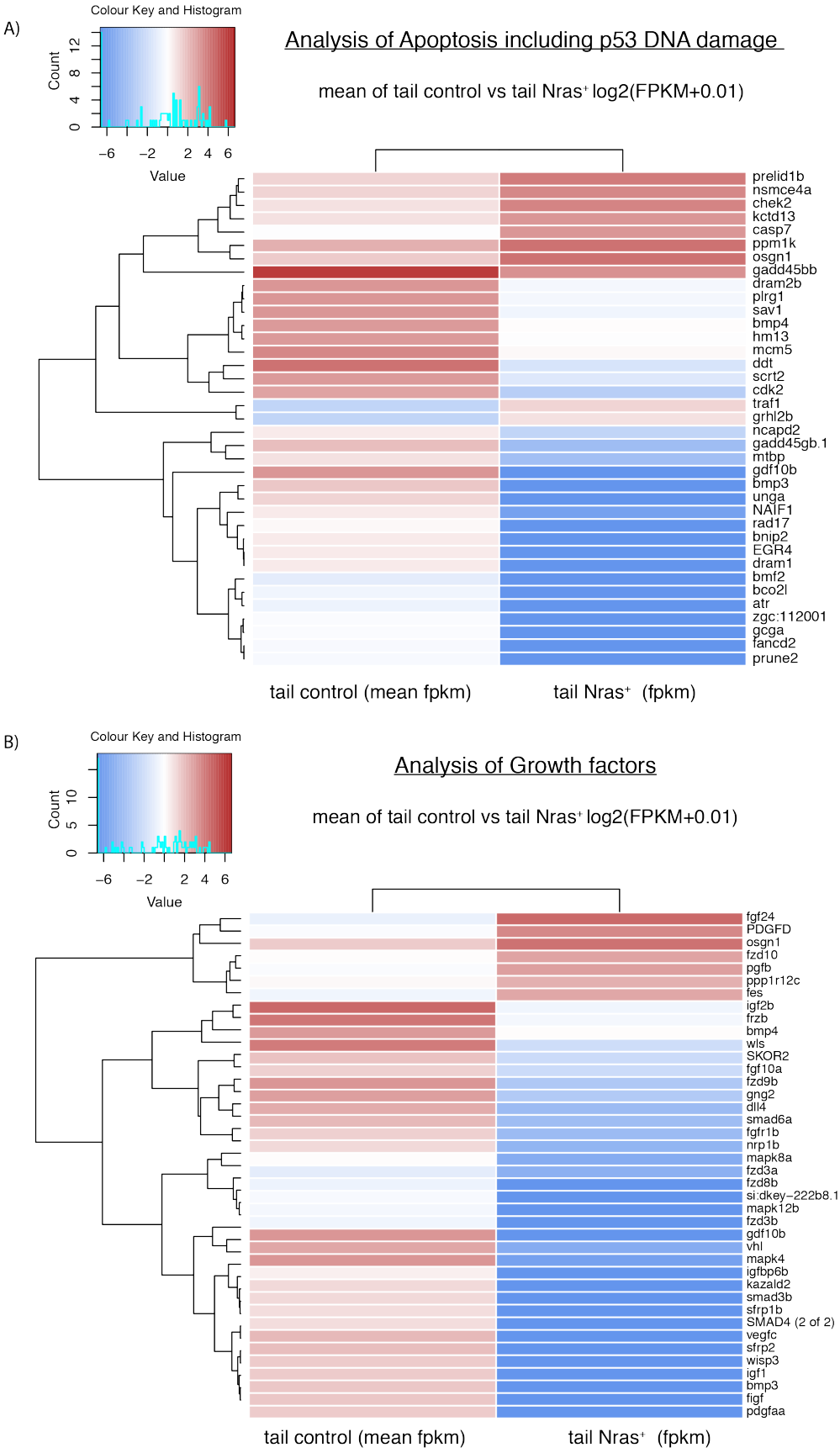

Figure 6.7 Heatmap of differentially expressed genes involved in Apoptosis including p53 DNA damage (A) and Growth Factors (B). Heatmap shows the $\log_2$(FPKM +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Red-high expression. Blue-low expression. White-medium expression.

Although neutrophil granule proteins, as an entire group were not differentially expressed, several molecules known to localise to neutrophil granules were downregulated including mpo, defensin, beta-like1 (defbl1), elastase 3-like (ela3l), elastase 2-like (ela2l), elastase 2 (ela2) (Figure 6.8 A). Through the production of cytokines and conversely cytokine receptors, neutrophils are not only responsible for influencing the inflammatory microenvironment in the vicinity of the transformed cells, but also strongly impact complex crosstalk between themselves, tumour cells and other immune cells[184]. Several cytokines, cytokine receptors and molecules associated with inflammatory signalling, such as Interleukin 7 receptor (il7r), TNF receptor-associated factor 1 (traf1), TNFAIP3 interacting protein 1 (tnip1), il-16 and il20r were upregulated, while others as c1q and tumour necrosis factor related protein 4 (c1qtnf4), interleukin 12 receptor beta 2a, like (il12rb2l), interleukin 13 receptor alpha 2a, like (il13ra2) were found downregulated (Figure 6.8 B). Interestingly, there was also a significant downregulation in the DNA binding protein HMGB1 (Figure 6.5). Increased levels of HMGB1 are associated with chronic inflammatory disorders[185].

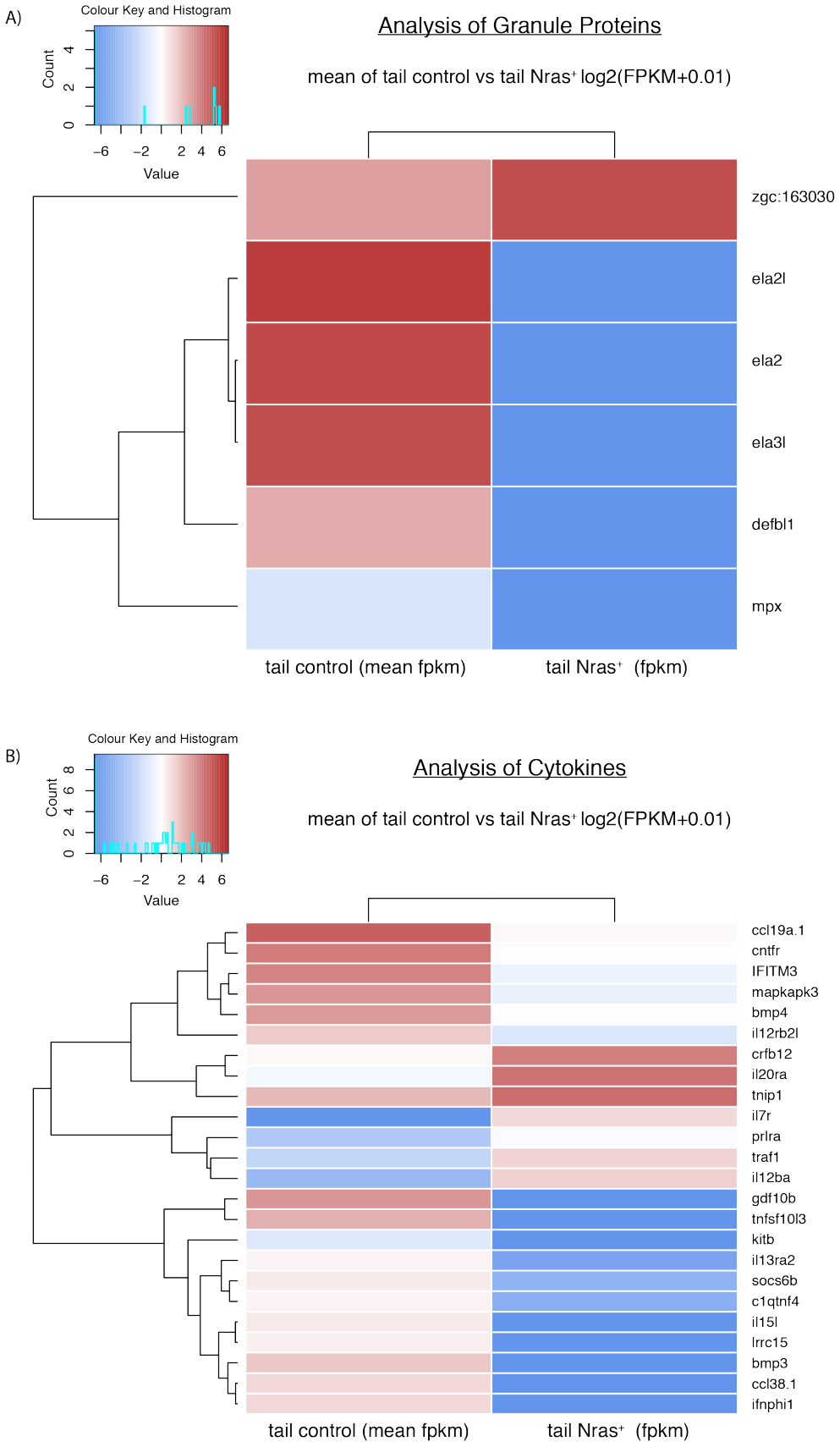

Figure 6.8 Heatmap of differentially expressed genes of Granule Proteins (A) and Cytokine signalling (B). Heatmap shows the $\log_2$(FPKM +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Red-high expression. Blue-low expression. White-medium expression.

There was a downregulation in genes associated with respiratory burst, ROS and NOS (Figure 6.9 A). Conversely, two key genes involved in H_2O_2 production, dual oxidase (duox) and neutrophil cytosolic factor 4 (ncf4, or p40phox), were upregulated (Figure 6.9 A). Similarly, nitric oxide synthase (nos)-2a otherwise known as inducible nitric oxide synthase (inos) was upregulated (Figure 6.9 A). Analysis of metalloproteinases, metallopeptidases and associated proteins showed a number of ADAM metallopeptidases were downregulated (adam19b, adamts12, adamts13, adamts16, adam12), although adam8b and TIMP metallopeptidase inhibitor 4 (timp4) were upregulated (Figure 6.9 B).

TLR signalling is an essential component of neutrophil activation and effector mechanisms. There was no clear bias towards upregulation or downregulation in TLR signalling with a few associated genes shown to be upregulated (single immunoglobulin and toll-interleukin 1 receptor (TIR) domain (sigirr) and downregulated (mitogen-activated protein kinase-activated protein kinase 3 (mapkap3)) (Figure 6.10 A). A growing body of evidence suggests that neutrophils are APCs and are able to prime T cells[17]. MHC antigen presentation and T cell activation does not show a unilateral change, but rather genes that are both upregulated (cathepsin Sb, tandem duplicate 2 (ctssb.2) and downregulated in this category (cathepsin Sb, tandem duplicate 1 (ctssb.1) (Figure 6.10 B).

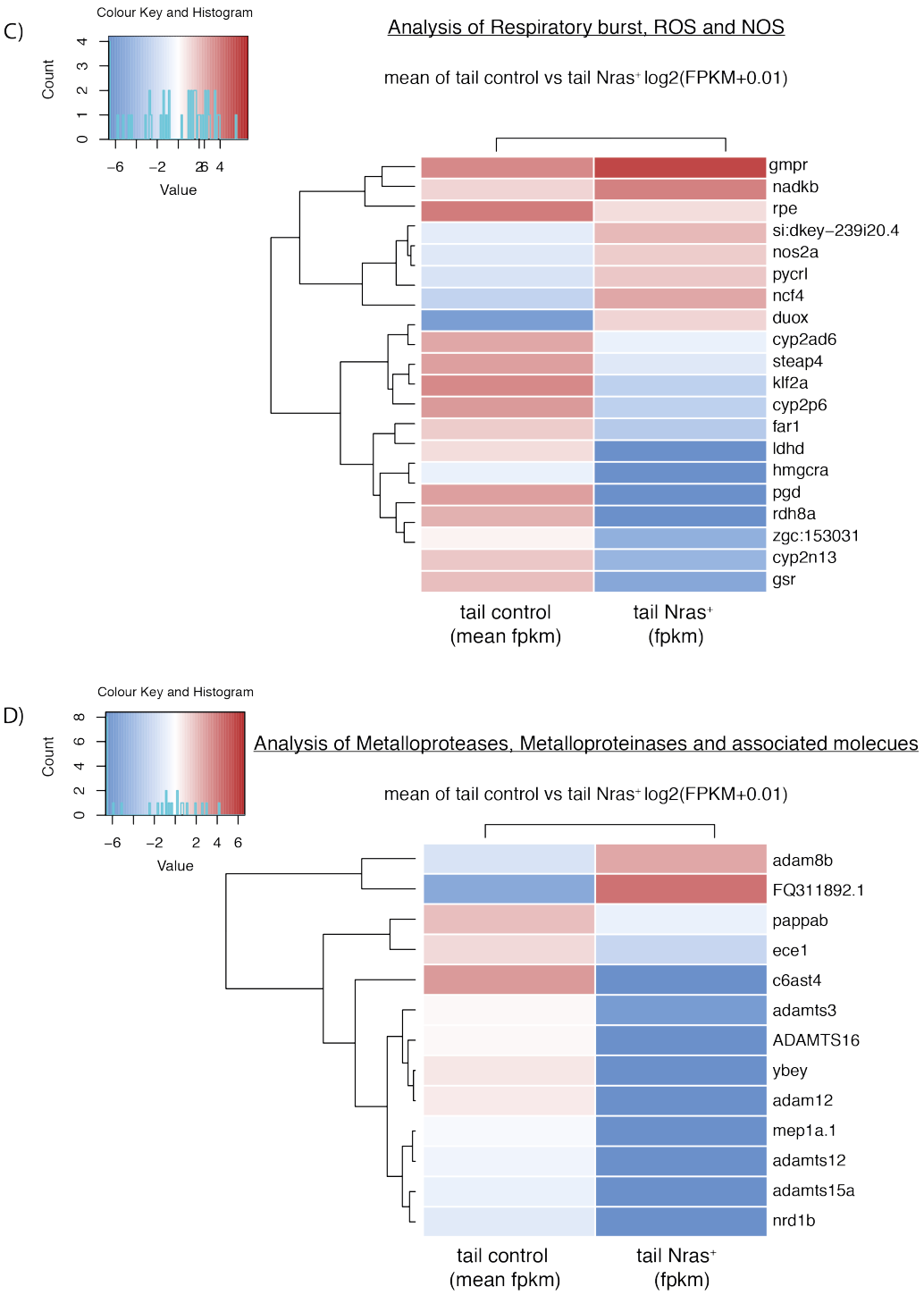

Figure 6.9 Heatmap of differentially expressed genes involved in Respiratory burst, ROS and NOS signalling (A) and Metalloproteases, Metalloproteinases and associated molecules (B). Heatmap shows the log $_2$ (FPKM +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Red-high expression. Blue-low expression. White-medium expression.

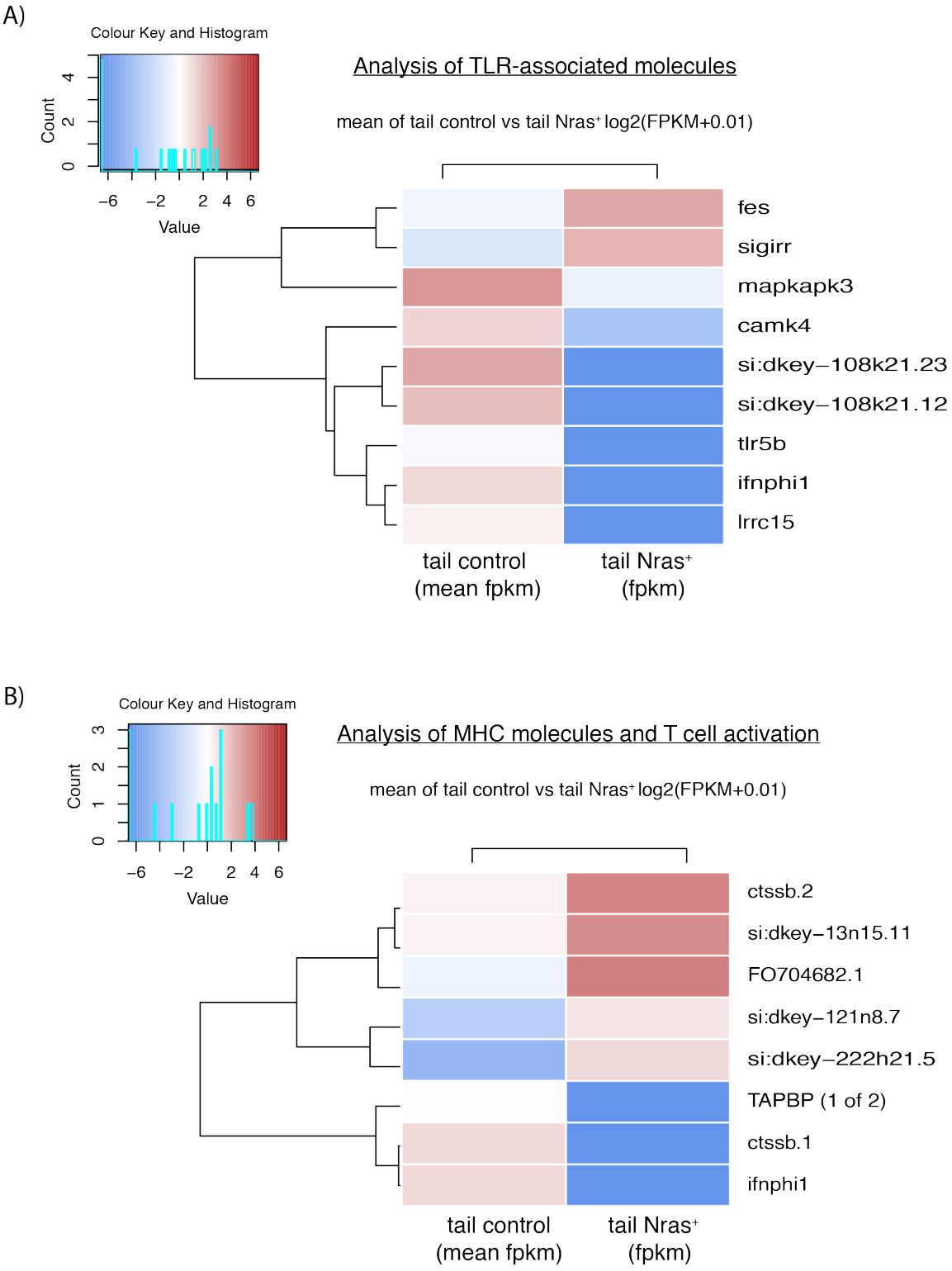

Figure 6.10 Heatmap of differentially expressed genes involved TLR signalling (A) and MHC molecules and T cell activation (B). Heatmap shows the $\log_2$(FPKM +0.01) values of the significantly differentially expressed transcripts (adjusted p-value <0.05). Red-high expression. Blue-low expression. White-medium expression.

6.3 Discussion

As shown by us and others, oncogenic transformation of melanocytes elicits an immune response, as is evident from the recruitment of both macrophages and neutrophils to transformed melanocytes[36]. At this stage of malignant transformation, the host immune system may function to detect and destroy transformed cells or it may aid tumour progression. Although, there is increasing evidence for conflicting roles of neutrophils in tumour progression, transcriptomic analyses of this cell type, in the context of cancer are few. The analysis carried out in this study, is the first of its kind where pure populations of neutrophils (and macrophages) can be profiled as transformed melanocytes and goblet cells establish themselves as the precursors of cancers.

6.3.1 A general downregulation is seen in cytoskeletal rearrangement, cell adhesion, chemokines and integrins.

There was a downregulation in genes involved in cytoskeletal reorganization, adhesion and chemokines and integrins. This may be because having reached the inciting stimulus, neutrophils downregulate a number of genes previously required for migration[186]. Similarly, one might hypothesise that neutrophils interacting with transformed cells are unlikely to be recruited to a different stimulus. Cytoskeletal rearrangement is not only required for cell migration, but also the formation of the phagocytic cup in neutrophils. Should neutrophils be phagocytosing transformed cells at this early stage of oncogenesis, one might presume to see an upregulation in this pathway. Feng *et.al.*[36], showed that although a rare event, macrophages were able to

engulf entire transformed cells whereas neutrophils appeared to extend membrane protrusions and break off pieces of transformed cells.

6.3.2 Neutrophils associating with transformed melanocytes are longer-lived cells

There is evidence to suggest that in contrast to normal neutrophils, the turnover of TANs is not as rapid. In tumour bearing mice, BrdU labeling kinetics unexpectedly revealed that not only were tumour-infiltrating macrophages replenished more rapidly than neutrophils but that in the tumours, macrophages and neutrophils lived longer than their counterparts in the spleen or blood[183]. Although relatively little is known about the transcriptional profile of tumour-associated neutrophils, a recent microarray analysis was conducted in a murine model of AB12 mesothelioma to compare the transcriptional profile of TANs and normal neutrophils[184]. Fridlander *et al.,* however reported no change in the intrinsic or extrinsic apoptotic pathways, although there was some upregulation of several anti-apoptotic members of the NF-κB family in tumour-associated neutrophils[184]. Our study, performed in the *in vivo* context using neutrophil-specific RNA-seq profiling of their active transcriptomes, provides novel evidence that neutrophils interacting with the earliest precursors of cancer do downregulate most of the components of the apoptotic pathway and are longer-lived compared to their control counterparts.

6.3.3 Neutrophils upregulate a number of growth factors

Transgenic mice expressing placental growth factor (PIGF), a member of the VEGF family[187], under the control of the keratin 14 promoter were inoculated

intradermally with B16-BL6 melanoma cells. This resulted in significantly increased tumour growth rate, compared to wildtype controls, suggesting that neutrophils may secrete PIGF and increase tumour proliferation[187]. In the described murine model, PIGF expression was shown to increase vessel size, promote tumour cell invasion and increase the number and size of metastases, making it an interesting target for therapeutic purposes[187]. The model described in this study reflects the transcriptional signature of the neutrophils as they respond to the earliest progenitors of the cancer. Hence the observed upregulation of PIGF is likely not linked to angiogenesis, but may play an important role in melanocyte proliferation and promote tumour formation. Therefore, the upregulation of PIGF seen in this study suggests that inhibitors of PIGF may represent potential therapeutic targets throughout melanoma progression.

In solid tumours, PDGF receptor signalling participates in various processes including autocrine stimulation and tumour growth, angiogenesis and the recruitment of stromal fibroblasts[188]. Different PDGF isoforms exist and PDGF-D as shown here, signals through the PDGF-β receptors. Interestingly, PDGF-D is secreted as a latent factor and must undergo proteolytic processing before it is active, presumably by proteases present in the tumour microenvironment. PDGF-D secreted from B16 melanoma cells enhanced the tumour growth rate[189]. Hence, neutrophils as shown here, may provide an additional source of PDFG-D, thus promoting melanoma cell proliferation.

Melanomas also frequently overexpress FGF2, one of 22 known human FGFs, with other family members not having been extensively explored in melanoma[190]. In melanoma cell lines, treated with recombinant FGF-2, melanoma cell migration and growth was increased. Furthermore, inhibition of FGF signalling with both dominant-negative receptor constructs and small molecule inhibitors reduced melanoma proliferation, colony formation, anchorage independent growth and interestingly apoptosis[190]. Here we provide novel evidence for neutrophils as a source of FGF-24, another member of the FGF family of growth factors, in promoting tumour growth.

Taken together, this study has revealed three new potential neutrophil-derived targets for preventing the proliferation of melanoma cells.

6.3.4 Neutrophil-derived factors that may modulate tumour growth

Adrenomedullin is a potent vasodilator and is involved in angiogenesis, cell proliferation and inflammation. The effects of TAM-derived adrenomedullin (ADM) on melanoma growth was previously investigated. This study showed that not only was ADM increased in macrophages upon co-culture with melanoma cells but that neutralising ADM resulted in a reduction in tumour growth[191]. Therefore, we provide evidence that neutrophils are an additional source of ADM, and may similarly promote early proliferation of melanocytes ADM therefore represents another exciting therapeutic target to explore in melanoma patients.

Although a number of ADAM proteins were downregulated, the upregulation of ADAM8 reveals an interesting candidate to explore further. ADAM8 is localized to the cell membrane and cytoplasmic granules of resting neutrophils. Following activation of neutrophils, ADAM8 is released into the extracellular space via a metalloprotease-dependent pathway. Both surface-bound ADAM8 and its soluble form mediate ectodomain shedding of the membrane-bound L-selectin. Neutrophils use L-selectin to roll along endothelial cells[192]. Furthermore ADAM8 has recently emerged as a mediator of inflammation in cancers. Induced expression of ADAM8 results in the cleavage of ECM components and cytokine receptors[193]. Interestingly, ADAM proteins, including ADAM8 have been shown to be able to convert growth factor receptors such as EGFR to their active form. Since ADAM proteases are known to be upregulated in certain tumours, this represents a mechanism by which they may contribute to tumour growth[194]. Although the role of neutrophil-derived ADAM8 in melanoma needs to be explored further, taken together these results suggest a putative role for ADAM8 in melanoma progression.

6.3.5 Neutrophil Effector mechanisms: a downregulation in granule proteins

One potential mechanism by which neutrophils could eliminate tumour cells, early in cancer progression is by protease inhibitors and proteases stored in primary, secondary and tertiary granules. However in this study we see no upregulation, in expression any of the neutrophil granule proteins but rather detect a downregulation in both neutrophil granule proteins and molecules associated with mechanisms of degranulation. Similarly, Fridlander et. *al.,*

found that MPO, proteinase-3 and cathepsin-G were downregulated in TANs as compared to normal neutrophils, but there was no significant change in the expression of neutrophil elastase. Similarly, secondary granule genes (MMP-8, CAMP, lactoferrin and NGAL) and tertiary granule genes such as CD11b, MMP-9 and PGLYRP were all upregulated in normal neutrophils as compared to TANs[184]. Hence, our data corroborates their findings. Previous groups have shown that under certain conditions, neutrophils may destroy cancer cells by cytotoxic mechanisms. Interestingly, our data suggests that neutrophils effector mechanisms, that are typically associated with the ability of neutrophils to eliminate pathogens, are downregulated in the presence of transformed cells and that it is an unlikely that neutrophils use such mechanism to exhibit cytotoxic effects on oncogene-transformed cells.

6.3.6 Neutrophil Effector mechanisms: Cytokines and pro-inflammatory mediators

Neutrophil-derived cytokines and conversely, their receptors, are fundamental to the role of neutrophils in the activation and suppression of the immune system. Further analysis is required to determine whether the upregulated and downregulated inflammatory mediators revealed in this study serve to drive or inhibit tumour formation in this model.

A downregulation of HMGB1 revealed in our study is an interesting candidate for further analysis. HMGB1 participates in enhancing inflammatory reactions by controlling pro-inflammatory mediators such as lipopolysaccharide (LPS) and cytokines. HMGB1 has also been shown to diminish the phagocytosis of apoptotic cells[185]. A downregulation of HMGB1 provides further evidence that

at least a portion of the neutrophils in the presence of oncogene-transformed melanocytes shuts down pro-inflammatory properties and are hence shifted to an 'N2' phenotype.

6.3.7 Respiratory burst, ROS and NOS

Enzymes capable of rapidly increasing H_2O_2 levels include the family of NADPH oxidases, of which a single isoform, Duox, has been identified in zebrafish. Tailfin transection of zebrafish larvae, induces a H_2O_2 gradient that precedes leukocyte infiltration at the site of the wound, but that decreases upon immune cell infiltration[181]. Similarly, leucocyte migration, as determined by using LysC:DsRed$^+$ cells, is dependent on H_2O_2 generation at the site of the wound, although this study did not determine the source of the H_2O_2[36]. Therefore, the upregulation of Duox and ncf4, as shown in this study, makes neutrophils an interesting source of H_2O_2, and may serves as a general mechanism whereby neutrophils are continually recruited to the transformed cells. However since the generation of H_2O_2 is typically attributed to phagocytic cells and is essential for respiratory burst, this may simply be a mechanism by which immune cells can destroy transformed clones by ROS. Jelcic and Niethammer propose that according to the 'multiple hit' principle of carcinogenesis, a single mutation will not result in melanoma[195]. Naturally, as pre-neoplastic cells divide, more mutations are likely to occur. However, it is possible that increase in ROS generated by the neutrophils amplifies the rate of DNA mutations and hence drives tumour formation[195].

Although the general trend observed in our analysis was a downregulation in genes associated with the NOS pathway, inducible NOS (iNos) was

upregulated. Since, different NOS isoforms are activated by different mechanisms, this may account for the bilateral changes in expression of different molecules within the NOS pathway. iNos expression can be transcriptionally regulated by cytokines including IFNγ, IL-1β, TNFα and LPS[196]. High levels of nitric oxide, catalysed by iNos in macrophages and neutrophils can be cytotoxic whereas low levels may drive tumour growth by genotoxic mechanisms and by promoting angiogenesis[196]. In a study using a Mutatect MN-11 tumour line that can be grown subcutaneously in syngenic C57BL/6 mice, neutrophils were the major infiltrating leukocyte and source of iNos. Neutrophil number was increased by injecting IL-8 and PGE2 into the tumours and the mutation frequency was shown to correlate with neutrophil number[197]. Although only relevant to later stages of tumourigensis, iNos may promote tumour growth through NO in a p53-dependent manner that induced VEGF and results in neovascularisation[198].

6.3.8 Neutrophil Effector mechanisms: TLR signaling

TLR signalling is an essential component of neutrophil activation and effector mechanisms and is implicated in a number of neutrophil functions which accounts for the fact that were was not a clear expression change in the group as a whole. TLRs promote phagocytosis, contribute to bacterial killing through the generation of ROS and NOS, stimulate neutrophil oxidative burst, are involved in both transcriptional and post-transcriptional (proteolytic processing and secretion) of antimicrobial factors, as well as in cytokine production and the regulation of various aspects of adaptive immunity[199, 200]. Tumour growth, in the presence and absence of tissue damage, is sensed in

some cases by TLRs, and thus triggers inflammatory mechanisms that are still poorly defined, but one might hypothesise, trigger some of the aforementioned mechanisms[199]. Furthermore, pathways that are triggered by some TLRs (TLR2, TLR4 and TLR9) can activate the PI3K pathway in neutrophils leading to signalling cascades that may result in angiogenesis, tissue remodelling and TLR-regulated tissue repair responses that can drive tumour growth and progression[199]. UV exposure of primary cutaneous melanomas in a genetically engineered mouse model increased the expansion of tumour cells and frequency of lung metastases, in a mechanism that was dependent on the activation of infiltrating neutrophils and driven by TLR4 receptor signalling[201]. Interestingly, TLRs have also been shown to regulate cell death. TLR signalling has been associated with expression of anti-apoptotic Bcl-2 family members[202]. Careful dissection and modulation of the TLR signalling pathway, may provide a useful mechanism by which to drive neutrophils to be cytotoxic.

6.3.9 Neutrophil Effector mechanisms: MHC-related molecules and T cell activation

Although in the zebrafish, the adaptive immune system is only present 2-3 weeks post fertilization, unbiased pathway analysis revealed genes that were both upregulated and downregulated related to MHC-molecules and T cell activation. Recent evidence has shown that neutrophils can participate in MHC class I and class II antigen presentation. This will require further functional studies to determine whether neutrophils can present to T cells later in tumour progression in this model. It is also possible that the genes revealed by this analysis have relevant functions in other pathways.

6.3.10 Neutrophil Polarization

Studies have shown that circulating neutrophils in both cancer patients and murine models are actually heterogeneous and consist of distinct cell populations playing conflicting functions in the context of cancer. A recent study claimed that neutrophils isolated from mice can be divided into low-density (LDNs) neutrophils that accumulate continuously as the cancer progresses and display impaired neutrophil functions, immunosuppressive properties and exhibit a reduced rate of apoptosis as well as high-density neutrophils which authors term 'normal'[203]. Interestingly, LDNs also had a reduced rate of apoptosis. Therefore, since the neutrophils isolated in this analysis represent some neutrophils in the circulation and some in direct contact with the transformed melanocytes of which some may also be exhibit different phenotpyes, the results presented here are potentially representative of a heterogenous population of neutrophils. In the context of cancer progression, it may be that the ratio of these subpopulations determines the outcome of the cancer, as determined by the microenvironment.

In conclusion, the binary transgenic system for studying the myeloid response to inflammation generated in this study is a powerful resource, with exciting potential to reveal novel inflammatory mediators that may contribute to tumour promotion and progression. The unbiased, genome wide approach, carried out at the earliest stages of somatic cell transformation is currently the only study of its kind. This gene set provides a list of interesting, novel pathways and specific candidates to explore further in human melanoma biology. The results described here contribute to the growing body of evidence that

suggests a pleiotropic role for neutrophils in tumour biology and insight into the mechanisms by which neutrophils can be harnessed for immunotherapy.

Chapter 7

Conclusion and Future Perspectives

To study the role of macrophages and neutrophils under different conditions of inflammation, a genetically encoded zebrafish transgenic model was generated. Using a binary system of transgenic fish, *in vivo* biotinylation of the nuclear envelope represents a powerful method for isolating macrophage- and neutrophil-specific nuclei from complex tissues in the *in vivo* context with unprecedented purity. This study focuses on using the model under two conditions of inflammation, in a granuloma forming infection model of M. *marinum* and investigating the myeloid response at the earliest stages of melanoma progression.

Transcriptomic analysis of the neutrophils in response to *M. marinum* infection highlighted the potential of this model to be used to study specific cell populations under different conditions of non-sterile inflammation. We do not fully understand why some granulomas function autonomously and are able to eliminate the mycobacterium, while others function as a protective niche in which the bacteria can proliferate and survive, later forming active disease. The importance of the innate immune system in protective immunity should not be underestimated because the innate immune system is able to eradicate the bacteria before the establishment of the adaptive immune response, and therefore represents truly protective immunity[61]. We reveal novel neutrophil-derived mediators that may either contribute to disease progression or elicit an early defensive function against *M. marinum.* Since so little is known about the transcriptional signature of neutrophils in tuberculosis, novel factors revealed in this study will be validated in patient samples with the hope of harnessing those that may be host protective to eliminate the

disease while targeting those that may contribute to tuberculosis pathogenesis.

One of the problems with tuberculosis is that no validated clinical correlate of risk or protection actually exists, which not only represent potential biomarkers for diagnostics, but are essential in the design of targeted therapies[204]. This is complicated since the heterogeneity of the host immune response is evident, even within the same individual. Moreover, molecular profiling in heterogeneous tissues is confounded by relative proportions of different cell types. A recent study showed that a specific transcriptional signature that was characterised by neutrophil-dependent IFN could distinguish between active tuberculosis and other inflammatory conditions[61]. Studies such as this one, which provide knowledge of the transcriptional signatures of specific cell types is therefore key to identifying biomarkers of disease progression. The model used in this study is not limited to early infection and can be used to identify macrophage and neutrophil specific biomarkers as the disease progresses.

To study the myeloid response to melanoma progression, an inducible melanoma model in the zebrafish using the LexPR/LexOP system was generated. By crossing the binary system for biotagging of the nuclei of neutrophils and macrophages to the LexPR/LexOP inducible system for the generation of oncogene-transformed melanocytes, a double binary system is created for the profiling of the myeloid cells as they respond to the precursors of melanoma. This is the first model of its kind with the potential to gain an understanding of the inflammatory processes that ensue somatic cell

transformation and the transcriptional signature of the myeloid cells as they respond to the earliest precursors of cancer. For the first time, we were able to show that neutrophils interacting with pre-neoplastic cells downregulate many of the genes usually associated with neutrophil effector functions as they respond to infection, but yet upregulate tropic factors including a number of growth factors, that may facilitate tumour growth. We also provide some evidence to show that neutrophils responding to oncogene-transformed cells are longer-lived cells. We also show that the neutrophils in this study upregulate key enzymes needed for the production of ROS and NOS. Although reflective of the earliest stages of cancer, the molecules revealed in this study go a long way to explain the pleiotropic effects of neutrophils on cancer progression, reported by other groups. Evidence suggests, that as the melanoma progresses, the tumour microenvironment changes and hence so does the phenotype of the myeloid cells. Therefore, transcriptional profiling of the neutrophils and macrophages as the disease progresses may provide novel insights into just how the tumour serves to modulate the plasticity of the myeloid cells and if the transcriptional signatures really are that different as the disease progresses. Nonetheless, the molecules highlighted here have exciting therapeutic potential, some of which will be validated in melanoma patients.

The question remains, how do we harness the potential mechanisms described here in immunotherapy? Myeloid cells provide a multi-factoral potential for tumour treatment either through the direct killing of the myeloid cells, inhibiting their recruitment to the tumours or in switching the phenotype

of the myeloid cells to be cytotoxic. For example, Trabectin, a drug generally used to treat soft-tissue sarcoma and ovarian cancers, was thought to inhibit tumour growth by selectively depleting monocytes and macrophages in caspase-8 dependent mechanism. Similarly, inhibitors of macrophage colony-stimulating factor (CSF-1) target macrophage recruitment and survival[205]. Although the mechanism has not been fully elucidated, Tasquinimoid, which has clear anti-tumour and anti-metastatic properties, is thought to bind S100-A9, a pro-inflammatory protein, and prevent macrophage and neutrophil accumulation[206]. CP-870,893 is a CD40 agonist thought to activate CD40 and thus shift macrophages to an anti-tumour phenotype because activation of this receptor is needed for APC-mediated priming and activation of tumour-specific T cells[207]. Therefore, the genes and pathways generated in this analysis, provide novel insight into potential mechanisms to harness neutrophils for immunotherapy either by eliminating neutrophils, by switching their phenotype or preventing their recruitment to transformed cells. For example, can we simply reverse the anti-apoptotic signature of the neutrophils? Would simply targeting the growth factors revealed here reduce tumour growth? If we used an agonist to upregulate neutrophil granule proteins and their drive their release, could we force their cytotoxic effects. Although this analysis provides insight into the role of neutrophils in driving tumour formation, it also brings to the fore many unanswered questions and many avenues to explore.

In conclusion, the binary model generated here, provides an exciting new platform for studying the active transcriptome of the myeloid cells, *in vivo,* in

response to a range of both sterile and non-sterile stimuli. In our hands the model has provided novel insight into the mechanisms by which neutrophils contribute to both mycobacterium infection and melanoma progression, but is in no way limited to these studies.

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