In Vivo Accuracy of a Macrophage-Based Drug Delivery System Demonstrated in Zebrafish

by

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Declaration

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Abstract

Targeted drug delivery systems are widely regarded as being the ‘magic bullet’ in the drug delivery research niche. Coined by Paul Ehrlich in 1900, the 'magic bullet’ is an immunological concept for a treatment intervention that targets only damaged or diseased cells in the body, leaving healthy cells intact. Targeted drug delivery systems achieve this by combining a drug carrier capable of precisely and accurately targeting specific tissues, cells, or mechanisms with a therapeutic agent. Many drug carriers have been developed and tested within the last decade with some being more effective than others. Based on prior *in vitro* work completed by our group, showing that human macrophages could be modified to ingest matter, appropriately translocate and expel the matter, all without degrading said cargo, we selected these cells as potential drug carriers.

Based on the high degree of physiological conservation between humans and zebrafish, high fecundity, cheap maintenance, optical transparency - allowing tracking of injected cells - and the fact that primary human macrophages have been found to survive for extended periods in zebrafish larvae, we set out to experimentally determine whether these would be suitable model organisms in which to study and develop a human macrophage-based drug delivery system.

First, cell microinjection and fluorescent staining parameters were optimised for long-term tracking of cells in zebrafish larval circulation. The latter parameter was optimised using immortalised, undifferentiated human monocytic leukemia cells (THP-1) which were shown to exhibit endothelial adherence to the caudal hematopoietic region of the larval blood vessels. This behaviour is similar to that of endogenous zebrafish macrophages/monocytes and illustrates a significant degree of conservation between human and zebrafish immune cells. The undifferentiated human THP-1 monocytes also underwent rapid proliferation in response to zebrafish inflammatory stimuli as a result of tail fin transections, suggesting some degree of cross-species reactivity to inflammatory cues.

Next, the THP-1 cells were differentiated and polarised to M1 macrophage-like cells to determine if these cells were suitable for drug delivery in zebrafish larvae. These cells were also shown to exhibit adhesion to the zebrafish caudal hematopoietic tissue (CHT) and associated blood vessels, but all became unviable and lost fluorescent signal within 24 hours post injection, without undergoing migration to the transected inflammatory site. The experiment was repeated with unpolarised THP-1 macrophages and yielded similar results, suggesting that THP-1-derived macrophages may be unsuitable for drug delivery research in zebrafish larvae.

Finally, the experiments were repeated employing primary human M1 polarised macrophages. These cells proved to exhibit a more suitable survival capacity, maintaining cellular viability to the experimental endpoint, however migrational capacity ultimately remained insufficient for drug delivery. This series of experiments lays a solid foundation for future studies which could definitively answer whether zebrafish larvae are suitable models in which to investigate a macrophage-based drug delivery system.

Research Outputs

1. Conference contributions:
   - International poster presentation (PSSA Conference 13/09/2021) *Assessing the in vivo feasibility of a human macrophage-based drug delivery system in zebrafish larvae.*
   Johnny van der Walt 1st place prize winner.

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List of Abbreviations

ActA	Actin assembly-inducing protein
AGM	Aorta-gonad-mesonephros
CCL1	Chemokine ligand 1
CCR8	Chemokine receptor 8
CD	Cluster of differentiation
CHT	Caudal hematopoietic tissue
CMP	Common myeloid progenitors
DAMP	Damage associated molecular patterns
DC	Dendritic cells
dpf	Days post fertilization
DPW	Days post wounding
EHT	Endothelial-to-hematopoietic transition
EMPs	Erythro-myeloid progenitors
FET	Fish Environmental Toxicity
G-CSF	Macrophage colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HMGB1	High motility group box protein 1
hpi	Hours post injection
hpf	Hours post fertilization
HPW	Hours post wounding
HSC	Hematopoietic stem cell
ICAM	Intracellular cell adhesion molecule

ICM	Intermediate cell mass
IFN-gamma	Interferon-gamma
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
LFA-1	Lymphocyte function-associated antigen-1
LFA-1	Lymphocyte function-associated antigen-1
LLO	Listeriolysin O
LPS	lipopolysaccharides
Mac-1	Macrophage-1-antigen
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloprotein
MSC	Mesenchymal stem cell
NK	Natural killer
PDGF-BB	Platelet derived growth factor BB
PSGL-1	P-selectin glycoprotein ligand-1
qRT-PCR	Quantitative realtime polymerase chain reaction
ROS	Reactive oxygen species
SFK	Src family kinase
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-a	Tumour necrosis factor alpha
TNFR1	TNF-a receptor 1
VCAM	Vascular cell adhesion molecule
VDA	Ventral wall of the dorsal aorta

VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
ZF	Zebrafish

Units of Measure

%	Percent
g	Grams
g	Gravitational force
l	Liters
mg	Milligrams
mg/l	Milligrams per liter
ml	Milliliters
mM	Millimolar
n	Number of larvae
nl	Nanoliters
U	Units
µm	Micrometers
µl	Microliters
uS/cm^3	Microsiemens per cubic centimeter

Chapter 1: Introduction

In the drug delivery research niche, intensive focus is currently being placed on the development of effective targeted drug delivery systems comprising both drug and drug carriers. Such systems should be able to overcome biological barriers *in vivo* and accurately guide therapeutic cargo to a therapeutic target site or target mechanism. This would significantly improve drug efficacy, as focal delivery of the drug would be assured, and improve drug safety by mitigating deleterious off-target drug effects. To this end, in the last decade, a plethora of targeted drug delivery systems, utilising a variety of novel targeting strategies and showing striking success *in vitro* have been designed and developed (Singh, Biswas, Shukla and Maiti, 2019). Often, however, this success is poorly translated *in vivo* (Yun, Lee and Park, 2015). We posit that, in many cases, one of the largest factors holding these systems back is the inability to accurately model the biological complexity of *in vivo* conditions, *in vitro*. A pre-clinical *in vivo* model, which would allow for real-time visualization of the fate of drug carriers (e.g., carrier cells) and their cargo, would provide valuable information to facilitate optimization of targeted drug delivery systems.

To avoid the aforementioned pitfall in the development of drug delivery systems, we have selected zebrafish larvae as early models in which to test the feasibility of a targeted drug delivery system. This organism has been selected due to appropriately complex, highly conserved vertebrate biology with humans, high fecundity, and optical transparency, allowing for long-term tracking of labeled drug carriers *in vivo.* Given the rapid rate of development of the larvae, they hatch already displaying a fully functional innate immune system and sufficiently developed organ and circulatory systems for our purposes. The former is of especial importance, as we have selected human macrophages as our potential targeted drug carriers. To date, very few studies have reported on injection of human macrophages into zebrafish larval circulation, but the early data does indicate immunocompatibility and long-term survival of human macrophages in zebrafish larvae. These studies did not assess whether human macrophages resumed normal immune functioning – a crucial factor for our uses - in the foreign organisms, however.

Our group has developed a macrophage-based drug delivery system in previous years. This system is predicated on the innate ability of classically activated (M1) macrophages to phagocytose matter and extravasate from circulation to migrate towards sites of inflammation (Visser and Smith, 2017). Briefly, these macrophages were also modified with the addition of purified *L. monocytogenes* effector proteins to facilitate the vomocytosis of cargo loaded through phagocytosis. Autophagic degradation of the cargo in macrophage transit was also successfully inhibited so as not to reduce the cargo load at the site of inflammation. This system offers tremendous potential as a broad application drug delivery system, able to shuttle any packaged drug to a site of inflammation. Unlike with conventional drug application, the therapeutic site need not be adequately supplied with blood, as macrophages are found in high numbers in many difficult-to-target and hypoxic areas, including the center of chronic diabetic ulcers and solid cancerous tumors (Loots et al., 1998) (Lee et al., 2013). However, while the proof-of-concept experiments have yielded promising results, *in vivo* data is still lacking. The work presented in

this thesis was therefore aimed at investigating whether zebrafish larval models of acute inflammation may be used for assessment of the *in vivo* functionality and ultimate fate of macrophage-based drug delivery systems.

In this thesis, I review the evidence in literature for or against the cross-species compatibility of human macrophages and the zebrafish internal environment. This extends to an assessment of whether the human cells would theoretically be able to migrate within the zebrafish, as well as the capacity for bi-directional crosstalk between the zebrafish inflammatory environment and human cell secretory products. The literature review is followed by a combined methods and results section to chronologically describe the series of pilot studies and experiments undertaken. Discussion and conclusion chapters contextualize newly generated data with the available relevant literature and provide recommendations for future study.

Chapter 2: Literature review

2.1 Introduction

The defining goal of the current generation of drug delivery research is the overcoming of biological and chemical barriers *in vivo* for unprecedented accuracy and precision (Yun, Lee and Park, 2015). Practically, a large focal point in current drug delivery research is the development of targeted drugs, capable of site- or mechanism-specific targeting (Alvarez-Lorenzo and Concheiro, 2014). Such a system would entirely mitigate the deleterious off-target effects and drug accumulation which are often dose-limiting factors in treatment of disease. This generation has heralded several advancements in the field with breakthroughs in stimuli-responsive systems, including both endogenous (e.g. Reactive oxygen species (ROS) and specific enzymes) and exogenous (e.g. sonic and thermal bombardment) stimuli, and nanoparticle-based and ligand-targeting systems among others (Alvarez-Lorenzo and Concheiro, 2014). Despite these breakthroughs, few current-generation drug delivery systems returning promising results *in vitro*, have showed the same promise *in vivo* (Yun, Lee and Park, 2015).

We posit that the principal reason for the poor *in vivo* translatability of many successful *in vitro* drug delivery systems is the inability to accurately model the biological complexity of living organisms *ex vivo*. For this reason, the resulting delivery systems have not necessarily been developed to overcome biological barriers. We propose to avoid these pitfalls in two independent manners: firstly, the drug delivery vehicle we have selected is an innate immune cell capable of overcoming biological barriers *in vivo* as a facet of its normal function. Secondly, we will avoid the use of an *ex vivo* model completely. This is possible through the selection of an appropriate first line *in vivo* model. In this review, I will present the rationale for our selection of zebrafish larvae as an *in vivo* model in which to assess the feasibility of a novel macrophage-based drug delivery system. This will include assessment of the physiological similarities and differences between humans and zebrafish, particularly in terms of immunology. I will also briefly provide a background on cell-based drug delivery and the rationale for the use of macrophages as drug carriers.

2.2 Overview of current cell-based drug delivery systems

Cell-based drug delivery systems have captured the attention of many researchers as a promising avenue for safe and efficacious drug delivery (Gutiérrez Millán, Colino Gandarillas, Sayalero Marinero and Lanao, 2012). Drug carriers are central to targeted drug delivery systems and improve the efficacy of drugs by extending their half-lives and increasing their specificity (Tewabe et al., 2021). In the context of drug delivery, modified cells can represent a specialized subcategory of drug carriers with benefits extending beyond those conferred by traditional carriers (Li, Dong, Zhang and Mo, 2018). Typically, circulating cells are employed as drug carriers due to their high motility and fluidity in circulation, hence transportability of cargo. Native cell-based drug carriers also exhibit low immunogenicity and high biocompatibility, thereby

improving the biological half-life of a drug (Timin et al., 2017). Finally, once the therapeutic cargo is released, these biological drug carriers will eventually undergo biodegradation, usually facilitated by the reticuloendothelial system, thereby circumventing the toxic effects often associated with traditional drug carrier accumulation (Timin et al., 2017).

Cell-based drug carriers confer additional, cell-specific benefits based on the cell-type employed. Researchers have investigated employing a wide range of circulating cells for this purpose. Table 2.1 provides a brief overview of the range of cells and cell fractions currently being investigated for drug delivery, as well as the benefits and drawbacks associated with them.

Cell Type	Attachment	Benefits	Drawbacks
Monocytes/ Macrophages	Encapsulation (via phagocytosis (Visser and Smith, 2017); electroporation (Evangelopoulos et al., 2020)) or surface attachment (Doshi et al., 2011)	Inflammation-homing (Wynn, Chawla and Pollard, 2013); can be activated to release intracellular contents (Visser, J. PhD thesis, 2020); efficient, easy drug loading (Liang et al., 2021); ability to cross the blood brain barrier and deliver payload to CNS in mice, and difficult-to-target hypoxic areas (Tong et al., 2016) (Loots et al., 1998) (Lee et al., 2013).	Autolysosomal maturation may degrade encapsulated drugs (Yousefpour and Chilkoti, 2014)
Red Blood Cells	Encapsulation and membrane resealing (via endocytosis (Ginn, Hochstein and Trump, 1969), electroporation (Lizano, Sanz, Luque and Pinilla, 1998), osmosis-based methods (Koleva, Bovt, Ataullakhanov and Sinauridze, 2020)) or surface	Most abundant circulating cells (Nemkov et al., 2018); small; deformable; high surface area; can travel through capillaries (Koleva, Bovt, Ataullakhanov and Sinauridze, 2020)	Non-homing; modified RBCs can be targeted by the reticuloendothelial system which also increases risk of off-target drug release; encapsulation methods may disrupt membrane integrity (Pitt et al., 1983) (Yousefpour and Chilkoti, 2014)

	binding (via electrostatic and hydrostatic forces (Sun et al., 2019), biological bridges including biotin (Muzykantov et al., 1996), antibodies, receptor ligands, etc.) (Muzykantov, 2010)		
Lymphocytes	Surface binding (e.g., via maleimide-thiol conjugation (Stephan et al., 2010)) Encapsulation (via electroporation; endocytosis (Steinfeld et al., 2006).	Abundant in circulation; *in vitro* isolation and expansion; long lifespan in circulation (B-cells: 4 days to 5 weeks; T-cells: months to years); inflammation and tumor homing (Yu et al., 2020)	Under researched in recent years
Platelets	Encapsulation (via phagocytosis (Male, Vannier and Baldeschwieler, 1992); loading through the open canalicular system (Xu et al., 2017), electroporation (Banning et al., 1997)) Surface binding (via chemical ligation to primary amine groups (Anselmo et al., 2014))	Abundant in circulation; natural carriers of biologically active substances which are released upon platelet activation; inflammation and tumor homing and adherence to damage and tumor sites; high drug loading and encapsulation efficiency (Yu et al., 2020) (Yousefpour and Chilkoti, 2014)	Limited application potential beyond anti-tumor therapy; erroneous platelet activation could lead to off-target drug effects; increased deleterious thrombotic risk in disease state (Lu, Hu, Jiang and Gu, 2019).
Mesenchymal Stem Cells	Encapsulation (via endocytosis, diffusion, or with transporters (Babajani et al., 2020))	Self-renewable, allowing for expansion after harvesting (Compte et al., 2009); inflammation and tumor homing	Can be recruited and trapped within lung and cerebral tissue where they can cause microembolisms (Zhang et al., 2014) (Wu, Zhou, Tabata

		(D'souza et al., 2012); natural ability to traverse endothelium (Schmidt et al., 2006)	and Gao, 2019); risk of spontaneous tumor formation after intravenous injection (Su, Zhang, Huang and Gao, 2021); risk of promoting tumor growth at high concentrations (Karnoub et al., 2007)
Neutrophils	Nanoparticle uptake (Xue et al., 2017)	Most abundant circulating immune cell (Xue et al., 2017); most rapid circulating cell inflammation homing (Rosales, 2018); natural ability to traverse the endothelium (Cooper et al., 1995).	Short half-lives (8 hours) (Summers et al., 2010); uncontrolled release of nanoparticles (Chu et al., 2018).

Table 2.1: Benefits and Drawbacks associated with different cell-based drug delivery strategies

We have selected macrophages as our preferred cellular drug carriers because of the innate inflammation sensing and homing capacity of these cells and excellent migrational ability. Unlike neutrophils and mesenchymal stem cells (MSCs) which also exhibit these benefits, the lifespan of macrophages far exceeds that of neutrophils, allowing for maximal tissue targeting and exposure to therapeutic agents, and without the risk of tumorigenicity associated with MSCs.

The primary focus of current cell-based drug delivery research is the development of efficient and safe anti-cancer treatments. To this end, many researchers have shown success both *in vivo* and *in vitro*. For our purposes, however, we aim to assess the *in vivo* efficacy of a novel macrophage-based drug delivery system with the potential for broader therapeutic applications. This proposed drug delivery system, first described by Visser and Smith in 2017, mitigates the largest drawback in the use of macrophages as vectors for drug delivery (Table 2.1). This novel system involves the incorporation of two proteins purified from the *L. monocytogenes* bacterium to enable the release of the therapeutic cargo from within the macrophages without disrupting the membrane integrity of the cells (Visser and Smith, 2017).

The two proteins used in this system are listeriolysin O (LLO), which enables pore formation in the membrane of the macrophages upon acidification of the autophagolysosome as the macrophages attempt to degrade the cargo, and actin assembly-inducing protein (ActA)

(Osborne and Brumell, 2017). ActA is chiefly responsible for the motility of L. *monocytogenes* both intracellularly and extracellularly and facilitates cell-to-cell transfer. This is accomplished immediately post-lysosomal escape, through the phosphorylation of ActA, manipulation of the host cell cytoskeleton and subsequent actin spike formation (Darji et al., 1998). Together, these purified proteins combined with cargo - in this case 6µm polystyrene beads - and facilitated their escape from the macrophages *in vitro* while leaving these cells intact, after lysosomal maturation was allowed to recommence after being transiently inhibited (Visser and Smith, 2017).

Given the promising *in vitro* results, we aim to assess the potential of this system *in vivo*, and we therefore must select an appropriate *in vivo* model for this task.

2.3 The Case for Zebrafish as *in vivo* Models for Drug Delivery

Zebrafish embryos younger than five days post fertilization (<5dpf) are attractive pharmacological and physiological models, particularly in the fields of drug discovery and drug delivery. This is in large part due to the fact that zebrafish are relatively inexpensive to raise and house, offer high fecundity and therefore high throughput and statistical power; and larvae develop rapidly, predictably, and transparently, the latter allowing for ease of *in vivo* live imaging. This model confers the additional advantage of a high degree of genetic homology and physiological conservation between humans and zebrafish. In fact, approximately 82% of genes implicated in human disease have at least one obvious zebrafish orthologue and the same can be said for 71.4% of all human protein-coding genes in zebrafish (Howe et al., 2013). Despite obvious anatomical and environmental differences between humans and zebrafish, and the fact that humans are homeothermic while zebrafish are poikilothermic, in many cases zebrafish embryos can be a helpful, faster, cheaper, and higher-throughput bridge between *in vitro* and higher-order *in vivo* testing compared to other animal models (Sieber et al., 2019) (Teame et al., 2019).

In many cases, zebrafish embryos in research are not a compromise, but the best-suited models for the application. One example of such a case is research on the innate immune system and inflammation in zebrafish. Cellular components of the innate immune system are detectable and functional from the first day of fertilization and are already fully developed when they hatch at 2-3 days post fertilization. The zebrafish adaptive immune system only develops between 4-6 weeks post fertilization, allowing researchers a temporal window in which to observe and investigate the innate immune system in isolation (Novoa and Figueras, 2012). In terms of composition, both the zebrafish larval and adult innate immune system is virtually identical to the human counterpart, sharing similar cell types and proportions, with neutrophils being the most abundant innate immune cell type, followed by macrophages and their precursors (Novoa and Figueras, 2011). Robust host-pathogen interaction and wound healing research has demonstrated that the larval and adult zebrafish immune system functions in a similar manner to the human counterpart, although with a few notable exceptions (Renshaw and Trede, 2012). This will be further elaborated upon in a later chapter.

A key advantage of zebrafish in this line of research as opposed to other models include the optical transparency of the zebrafish in early stages of life. This allows for long-term, real-time tracking of immune cells in response to damage or infection. Furthermore, zebrafish develop and heal rapidly as evidenced by well characterized wound models, specifically caudal fin transection models which heal within days in a predictable manner (Miskolci et al., 2019).

Other research avenues that can take good advantage of zebrafish embryo models are drug delivery, discovery, and toxicity. Robust pharmacovigilance tests such as the Fish Environmental Toxicity (FET) assay take advantage of the high-throughput nature of zebrafish larvae research for a high degree of accuracy and statistical power (Rothenbücher et al., 2019). Despite the relatively simple anatomy of zebrafish larvae compared to higher-order animal models, they offer far more complexity than *in vitro* models by supplying physiologically relevant biological, physical, and chemical barriers for researchers to overcome in the development of drug delivery systems. However, importantly for drug discovery and drug delivery, although most organ systems are fully developed from 2dpf, some others, such as the blood-brain barrier, pancreas and liver, lag one day behind, while the gastro-intestinal system is only fully mature by 4-5dpf (van Wijk et al., 2016). Researchers must therefore be aware of the temporally distinct development patterns of the different organs and stage the larvae correctly for their research.

In terms of ethical considerations, the '3R's' were proposed in 1959, as guideline for research using animal models. These principals of 'reduction' of numbers and 'replacement' of experimental animals used in research, and 'refinement' of animal welfare and use (The 3Rs | NC3Rs, 2021) are still employed today, in order to limit the numbers of *sentient* animals subjected to potential pain or discomfort. The use of embryonic zebrafish models significantly advances this ideal, as zebrafish embryos before the age of 5dpf are not recognized as sentient animals as they are not free-swimming and cannot self-feed at this stage. These are considered early putative indicators of a yet incomplete neural circuit (European Directive 2010/63/EU). Internationally, these ethical standards err on the side of caution compared to, for example Switzerland and the UK, where embryos up to 7dpf are unprotected by animal ethics guidelines.

In terms of refinement, the handling and maintenance of zebrafish embryos and early larvae (<5dpf) without causing stress or exposure to pathogens is relatively straightforward and cost-effective. This is accomplished through optimization of lighting and water conditions, minimization of sound and vibrations, and keeping the laboratory environment and equipment clean.

Next, we will discuss, in more detail, the physiological basis for the use of zebrafish larvae in the development of a human macrophage-based drug delivery system. This will be accomplished by reviewing the components of the innate immune system, particularly macrophages and their precursors, and the similarities and differences between these components in humans and zebrafish larvae.

2.4 The Innate Immune System

The innate immune system is the most evolutionarily conserved defense strategy against pathogens in living organisms. It is the dominant anti-microbial defense system in plants, fungi, and most lower order multicellular organisms, and is crucial to sustain all multicellular life in a non-sterile environment (Suckale, Sim and Dodds, 2005). Through generalized, non-specific responses, the innate immune system offers a rapid response to injury and infection, and as such represents the first line of defense against pathogens (Warrington, Watson, Kim and Antonetti, 2011).

The innate immune system comprises anatomical barriers including skin and mucosal linings, chemical and humoral elements, such as clotting factors and compliment proteins, and a cellular component. For the purposes of this review, only the cellular components of innate immunity will be discussed in detail, with particular emphasis being placed on the roles of macrophages in immunity.

2.4.1 Monocyte and Macrophage Ontogeny: A Species Comparison

Macrophages represent a heterogeneous population of phagocytic cells with diverse ontological backgrounds. The current theory is that in both humans and zebrafish, macrophages originate from three distinct but temporally overlapping hematopoietic waves in early development (Theret, Mounier and Rossi, 2019). In both species, primitive macrophages are the first leukocytes that are observed in development, and these result from the first hematopoietic wave, dubbed the primitive hematopoietic wave (Palis, 2001).

In mammals, primitive hematopoiesis takes place in the blood islands of the extra-embryonic yolk sac. These anatomical features are clusters of cells comprising a central concentration of heterogenous, primitive hematopoietic precursors surrounded by sparse epithelial cells. In these islands, both unipotent myeloid progenitors of the macrophage lineage and bipotent progenitors of the primitive erythrocyte and megakaryocyte lineage are generated. This process takes place around the second to third week of human gestation (Tavian and Peault, 2005) (Palis et al., 1999). Once fetal circulation is established, around 21 days post fertilization, these cells are released from the blood islands into the blood and are thereafter introduced into the embryo proper (Tavian and Peault, 2005). The primitive macrophages proliferate within the embryo and eventually colonize the developing embryonic tissues, including the brain. Macrophages generated from subsequent hematopoietic waves are theorized to later dilute and replace these primitive cells, with the only notable exception being those that colonized the developing brain, which ultimately give rise to microglia (McGrath, Frame and Palis, 2015). These yolk sac-derived macrophages are the only known macrophage lineage to arise in the absence of monocyte precursors (Takahashi, Yamamura and Naito, 1989) (Wittamer and Bertrand, 2020).

Primitive hematopoiesis in zebrafish is facilitated by two spatially distinct sites, the combined functions of which are orthologous to the mammalian extra-embryonic yolk sac. These sites are

the intermediate cell mass (ICM), located intra-embryonically between the notochord and trunk endoderm above the yolk sac, which facilitates the generation of primitive erythrocytes, neutrophils, and angioblasts, and the rostral blood island, formed from the anterior lateral plate mesoderm, which generates early myeloid progenitors (Bennett et al., 2001). These progenitors can differentiate into primitive neutrophils and macrophages, the latter of which begin to infiltrate the embryonic cephalic mesenchyme in response to neuronal cell death between 22-23 hpf, as shown *in vivo* (Herbomel, Thisse and Thisse, 2001) (Xu et al., 2016). Unlike in mammals, this process is independent of circulation which is established around 24 hpf (Herbomel, Thisse and Thisse, 1999). By 60 hpf, the primitive macrophages, mostly residing in the optic tectum, mature into primitive microglia and rapidly populate the midbrain (Herbomel, Thisse and Thisse, 2001).

The second hematopoietic wave facilitates the generation of macrophages via erythro-myeloid progenitors (EMPs): cells generated by endothelial-to-hematopoietic transition (EHT) of hemogenic vascular endothelium, first observed in zebrafish (Bertrand et al., 2007) (Ferrero et al., 2018). Here, these cells arise in, and seed the posterior blood islands, also referred to as the caudal hematopoietic tissue (CHT), between 26 and 36 hpf. Anatomically, the CHT is located ventrocaudally to the yolk tube extension and is physiologically homologous to the mammalian fetal liver as the site of maturation for definitive hematogenic progenitors (Murayama et al., 2006). The significance of macrophages differentiated from EMPs in zebrafish embryos is speculative, as recent lineage tracing studies have demonstrated that adult tissue-resident macrophages in the gut, heart, liver, and brain are hematopoietic stem cell- (HSC-), not EMP-derived (He et al., 2018). Although this wave and resultant cells are poorly categorized in humans, the fetal liver is seeded by increasing numbers of CD34+/CD45+ between the third and fourth week of gestation and represent a putative analogue of zebrafish EMPs (Ivanovs et al., 2017). Here, the EMPs give rise to the first enucleated erythrocytes, mast cells, and bipotent progenitors of macrophage and granulocyte lineages. Sparse data is available on the generation of these cells in humans, but early studies indicate that these cells may emerge in the human yolk sac from 28-35 days post conception (Migliaccio et al., 1986). These cells have also been found in hemogenic tissue of the placenta and umbilical cord during this wave (Dzierzak and Speck, 2008).

Almost all tissue-resident macrophages in zebrafish are descendants of EMPs and are maintained throughout adulthood by means of self-renewal. Exceptions include the brain, as mentioned before, and certain other organs such as the skin and gut, which do undergo monocyte-dependent renewal (He et al., 2018). As the cells that differentiate from the EMPs resemble the mature cells (enucleated erythrocytes and mature tissue resident macrophages) seen in adult organisms, this wave is termed the first ‘definitive’ hematopoietic wave (Wu and Hirschi, 2021). This is in contrast to the aforementioned primitive wave, which generates cells that are mostly absent in mature organisms. The EMP wave is one of two definitive waves and, because EMPs are short-term progenitors, their resultant wave is termed the ‘transient definitive wave.’

The final developmental hematopoietic wave is termed the definitive hematopoietic wave. In mammals, macrophage progenitors originating from the third hematopoietic wave are the first that are partially generated within the embryo proper. As with EMPs, progenitor cells of this wave originate from EHT of hemogenic endothelium but differ from EMP in both site of origin and transcriptional regulation. Hemogenic endothelium giving rise to these cells is found in the dorsal aorta of the aorta-gonad-mesonephros (AGM) region of the developing embryo, but also in vitelline and umbilical arteries (Medvinsky and Dzierzak, 1996) (Zovein et al., 2010).

In mammals, these cells bud from the hemogenic endothelium, towards the lumen of the arteries, where they aggregate in clusters before migrating via circulation to the fetal liver. In zebrafish, budding of these cells occurs in the opposite direction, that is, into the subaortic mesenchyme, and subsequently into circulation via intravasation of the axial vein (Bertrand et al., 2010). In humans, the cells mature in, and colonize the fetal liver, undergoing massive cell expansion. This is the primary site of human fetal hematopoiesis. After some time, the cells expand to the developing fetal bone marrow, where they persist into adulthood (Hoeffel et al., 2015. Bone marrow-derived hematopoietic progenitors only become fully functional several days post birth however, meaning the liver remains the primary contributor of these cells into circulation until this point. Secondary to the liver, the fetal spleen also serves as a reservoir of these cells (Hoeffel et al., 2015). Due to the multipotent potential of these cells, able to differentiate into any blood cell type, they are deemed hematopoietic stem cells.

In adult humans, bone marrow-derived HSC are the main contributors of circulating bipotent progenitors of dendritic cells and macrophages, called monocytes (Sawai et al., 2016). These monocytes are highly relevant to the current topic of cell-based drug delivery, as these cells would be the ones available for harvesting from the circulation of mature human patients, before being differentiated and modified *in vitro* for drug delivery. For this reason, these cells, and their zebrafish counterparts, will be the focus in subsequent chapters of this literature review.

In line with haematopoiesis in all vertebrates, the definitive hematopoietic wave in zebrafish is also facilitated by EHT of hemogenic arterial endothelium in tissue closely resembling the mammalian AGM. In zebrafish, this region is situated between the cardinal vein and dorsal aorta, an area defined as the ventral wall of the dorsal aorta (VDA) (Orkin and Zon, 2008). Zebrafish HSCs are first observed between 28 and 48 hpf (Bertrand et al., 2010). Once circulation of the HSCs is achieved (48-72 hpf), they are captured by CHT endothelium and extravasate to the abluminal side of the vessel where they trigger endothelial remodeling (Murayama et al., 2006) (Tamplin et al., 2015). Typically, 5-6 endothelial cells wrap around an individual HSC in the abluminal space in a process termed endothelial cuddling (Tamplin et al., 2015). Chemical signals within the HSC CHT niche promote expansion of the HSCs and their differentiation, maturation, and subsequent exit to circulation (Mahony, Pasche and Bertrand, 2018). Between approximately 48-120 hpf, the CHT is the primary site of hematopoiesis, after which the HSCs seed the adult hematopoietic organs, the kidney and thymus, via circulation; the latter being the zebrafish equivalent of mammalian bone marrow (Wattrus and Zon, 2018).

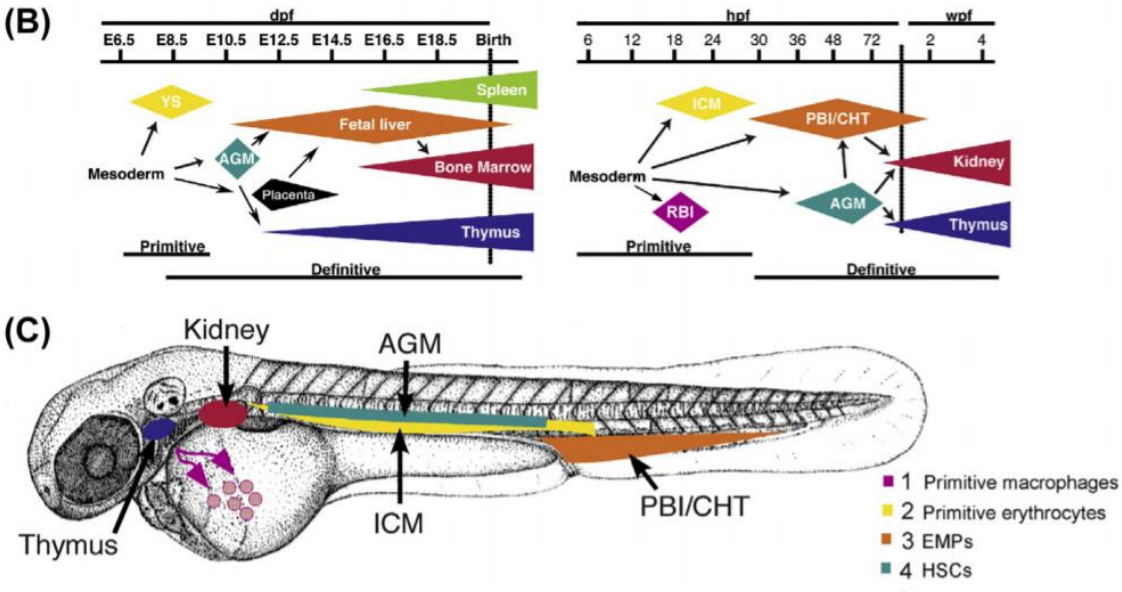


Figure 2.1: Ontological model of hematopoiesis over time. B shows timeline of main organs of hematopoiesis in humans (left) and zebrafish (right). C is an anatomical representation of the zebrafish organs mentioned. (Stachura, D. and Traver, D., 2011. Cellular Dissection of Zebrafish Hematopoiesis. *Methods in Cell Biology*, pp.75-110.)

2.4.2 Monocytes

Monocytes are the most relevant human macrophage precursors for our context. As with macrophages, monocytes can elicit a variety of immune responses depending on their activation state (analogous to monocyte phenotype) (Guilliams, Mildner and Yona, 2018). Differentiation of HSC to monocytes follows a sequence of intermediary cells with progressively reducing lineage potential. There are presently thought to be two distinct, independent HSC differentiation pathways in steady-state monopoiesis resulting in monocytes. Both pathways share intermediaries until common myeloid progenitors (CMP) and deviate from there onwards. Both pathways have the potential to produce broad classifications of classically activated (CD14++, CD16−), intermediate (CD14+, CD16+), and non-classically activated monocytes (CD14+, CD16++) (Guilliams, Mildner and Yona, 2018). Mature, circulating monocytes account for approximately 5-12% of peripheral blood leukocytes in humans under homeostatic conditions (Rosales, 2018). The lifespan of mature monocytes in circulation depends heavily on the activation state, with classical monocytes circulating for approximately 1 day before undergoing apoptosis, leaving circulation, or transitioning to intermediate monocytes. Intermediate monocytes exhibit a longer lifespan in circulation of about 4 days before transitioning to non-classical monocytes. Non-classical monocytes have the longest circulation time of approximately 7 days before undergoing apoptosis or extravasation (Patel et al., 2017).

Due to the diverse activation states of circulating monocytes that can be harvested for *in vitro* differentiation and polarization, it stands to reason that the function of the resultant

macrophages may differ depending on the activation states of the monocytic precursor. Although functional studies are sparse in literature, one study has shown that human monocytes, in any of the three activation states, can differentiate into both M1 type macrophages, upon the addition of granulocyte-macrophage colony stimulating factor (GM-CSF), and M2 type macrophages, upon the addition of macrophage colony stimulating factor (M-CSF) (Boyette et al., 2017). This was confirmed through morphological analyses, cytokine expression profiles, and strongly increased phagocytic capacity across all macrophages relative to the undifferentiated monocytes. Despite the fact that each monocyte activation state was able to differentiate into both M1 and M2 macrophage subtypes, macrophages differentiated from classically activated monocytes showed the greatest response to differentiation. This was shown by a stronger upregulation of interleukin-10 (IL-10), IL-6, and platelet derived growth factor BB (PDGF-BB) in response to M-CSF and a greater degree of phagocytic capacity in response to GM-CSF and M-CSF relative to other monocyte subtypes (Boyette et al., 2017). The motility of the resulting macrophages dependent on the precursor subtype was not assessed, though all macrophages became similarly adherent in response to differentiation. Based on these data, it is evident that macrophage phenotype is far more dependent on the differentiation stimuli that act on monocytes than the precursory monocytic activation state. Hence, for our purposes, monocytes derived from human circulation need not be typed and separated by activation status prior to differentiation.

2.4.3 Macrophage phenotypes

Macrophages are highly plastic cells, able to shift dramatically between forms and functions. It is therefore important for us to consider which macrophage phenotype would be the optimal candidate for drug delivery to an inflamed target site. Of similar importance, the lingering phenotypic effects elicited by our cellular drug carriers at the target site independent of cargo delivery should be considered.

Macrophages can be separated into naïve (M0), classically activated (M1) and alternatively activated (M2) phenotypes, the latter of which can be further subdivided into four subtypes, M2a-M2d (Orekhov et al., 2019). M1 macrophages are the most abundant phenotype that infiltrate the wound during early stages of inflammation (Martin and García, 2021). In the context of tissue damage, these macrophages are responsible for neutralizing potential pathogens and debridement of cellular debris by means of phagocytosis at the wound site (Martin and García, 2021). For these reasons, M1 macrophages are highly motile, potently pro-inflammatory cells. In the specific context of drug delivery, the generally accepted consensus is that M1 polarized macrophages are the best macrophage candidates due to their superior capacity for inflamed tissue recruitment compared to other phenotypes (Liang et al., 2021) (Pang et al., 2018). Research published in our group confirmed this choice of macrophage phenotype by demonstrating that transendothelial migration was not impaired in M1 macrophages laden with cargo (Visser and Smith, 2017).

M1 polarisation stimuli include pro-inflammatory molecules tumour necrosis factor alpha (TNF-a), interferon-gamma (IFN-gamma), and bacterial lipopolysaccharides (LPS). When considering adaptive immunity, M1 macrophages play a potent role in coordinating a positive M1 polarisation

feedback/amplification loop with T cells through 'instructing' T cells to secrete IFN-gamma. This is not necessary for M1 polarisation, however, and given the zebrafish larval model in which the drug delivery system will be employed lacks a functional adaptive immune system at the stage we will be intervening, this will not be further discussed. M1 macrophages secrete many factors that guide the early immune response, including pro-inflammatory cytokines such as IL-1b, IL-6, TNF-a and chemokines such as monocyte chemoattractant protein 1 (MCP-1) attracting and alerting further immune cells (Atri, Guerfali and Laouini, 2018). Once the initial phase of inflammation is complete, M1 macrophages should, under normal conditions, transition into an M2 phenotype to prevent collateral inflammatory damage to the host.

Beginning with M2a, "wound healing" macrophages, which rely on polarisation stimuli IL-4 and IL-13, these macrophages contribute to the resolution of inflammation by secreting anti-inflammatory cytokines transforming growth factor beta (TGF-β), IL-10, and IL-1 receptor antagonist (IL-1RA). M2a macrophages also contribute to wound healing by releasing factors that aid in remodeling of the extracellular matrix. Despite the pro-healing effects of M2a macrophages, this phenotype is also potently profibrotic through the secretion of insulin-like growth factor, TGF-β and fibronectin. The timely phenotypic shift from M2a to M2c is an important factor in limiting this fibrosis in the healing wound (Tang et al., 2017). Hence, for appropriate wound healing and refunctionalisation of damaged tissue, it is important that our macrophage drug carriers not only switch from classically- to alternatively activated polarization states, but from M2a to M2c in a timely manner.

M2b macrophages are distinct from other macrophage subtypes in that they release high levels of chemokine ligand 1 (CCL1), a chemoattractant that acts on chemokine receptor 8 (CCR8) membrane proteins of monocytes, dendritic cells (DC), immature B cells, and natural killer (NK) cells (Tiffany et al., 1997) (Sironi, 2006). Interestingly, M2b macrophages also produce cytotypical M1 hallmarks including pro-inflammatory cytokines IL-1b, IL-6 and TNF-a (Gerber and Mosser, 2001)(Mosser, 2003). Secreting high levels of anti-inflammatory IL-10 and low levels of IL-14, the functional role of M2b macrophages is not as clearly defined as either M1 or M2a macrophages (Mantovani et al., 2004). It has been suggested that a pathological abundance of M2b macrophages arise in conditions of chronic inflammation and represent an incomplete conversion of proinflammatory M1 macrophages to anti-inflammatory M2c phenotypes (Ollewagen, Myburgh, van de Vyver and Smith, 2021). For example, polarization of macrophages is shifted towards an M2b phenotype in human patients suffering from alcohol abuse, acute radiation and severe burn wounds, the latter being a result of increased plasma catecholamines in the acute phase of inflammation (Wang et al., 2018). This results in macrophages stuck in an 'indecisive' phenotype capable of ineffectively performing both pro- and anti-inflammatory functions depending on signaling factors (Ollewagen, Myburgh, van de Vyver and Smith, 2021). To further emphasize this point, M2b macrophages can be converted to all macrophage phenotypes, including naïve macrophages but with the notable exception of the M1 phenotype (Wang et al., 2018). In humans with a pathological abundance of M2b macrophages, M1 macrophages are not as easily generated, creating an immunosuppressed phenotype. A likely

explanation put forward for this is that M2b macrophages also inhibit the polarization of naïve macrophages to the M1 phenotype (Wang et al., 2018). It is especially important to confirm, when utilizing M0 or M1 macrophages for drug delivery in the context of chronic inflammation, whether the drug carriers are successfully fully converting to anti-inflammatory M2 phenotypes. If conversion is unsuccessful, and the delivered macrophages do become stuck in a M2b phenotype, for example, there is a risk of our intervention exacerbating the condition.

Researchers have long known about the inhibitory, "deactivating" effect of IL-10 on macrophages *in vitro* (Vieth et al., 1994). Macrophages stimulated with IL-10, termed M2c macrophages, produce significantly fewer proinflammatory cytokines but exhibit an increased capacity for phagocytosis. M2c macrophages also suppress the pro-inflammatory response by secreting high levels of IL-10, which also stimulates the conversion of more macrophages into an M2c phenotype. Additional stimuli capable of inducing this phenotype in macrophages have over time been documented. These stimuli are TGF-β, IL-21, and glucocorticoids (Martinez, 2008).

Other defining features of this phenotype include an upregulation of angiogenic genes and secretion of proteins involved with tissue remodeling such as matrix metalloprotein (MMP) 7, MMP8, and MMP9 (Jetten et al., 2013) (Lurier et al., 2017). It is postulated that these MMPs are primarily responsible for the anti-fibrotic effects of M2c macrophages. A rapid and complete transition from the proinflammatory M1 phenotype of the human macrophages to anti-inflammatory M2 phenotypes, particularly M2c, would be the optimal fate of our drug carriers. This is predicated on the extracellular cytokine profile acting on the drug carriers, which, although they might be shifted to yield M2b macrophages in sites of chronic inflammation, should be rectified by the delivered therapeutic agent (Ollewagen, Myburgh, van de Vyver and Smith, 2021).

The most recently accepted classification of a macrophage subtype is the M2d phenotype. Although only named M2d macrophages by Ferrante and colleagues in the early 2010's, researchers had long since observed a phenotypic alteration when M1 macrophages were co-stimulated with agonists for adenosine receptors and toll-like receptors (TLRs) (Pinhal-Enfield et al., 2003)(Grinberg, Hasko, Wu and Leibovich, 2009)(Ferrante and Leibovich, 2012). This phenotype is characterized by an upregulation of vascular endothelial growth factor (VEGF) and IL-10, as well as a downregulation of proinflammatory cytokines IL-12, TNF-a, and IL-1b (Colin, Chinetti-Gbaguidi and Staels, 2014). *in vivo*, macrophages resembling this subtype are frequently found in association with tumours. Here they promote angiogenesis and suppress the immune response, thus driving tumour progression and earning the name of tumour associated macrophages (TAMs) (Wang et al., 2010).

Although it has been suggested by a number of researchers that macrophage-based drug delivery systems could be effective against solid tumours, convincing evidence is still scarce on this topic (Liang et al., 2021). It is our belief that the grade of inflammation generated by solid tumours is too low to consistently attract sufficient macrophage drug carriers to render the system effective. However, should macrophage-based drug delivery systems be shown to be effective in this

context in the future, it is important to consider the effect the tumour may have on the drug carrier. Given M2d macrophages are derived from circulation and can be generated from M1 macrophages in response to the tumour microenvironment, it is possible that the macrophage drug carriers can be polarized to M2d macrophages in the tumour (Wang et al., 2010). A high density of M2d macrophages within cancerous tumours is associated with a poor prognosis, hence further contributing to the M2d pool would be counterproductive (Bingle, Brown and Lewis, 2002).

Most work on macrophage typing has been performed *in vitro* and either using mouse- or human-derived cells. Some early attempts to dynamically categorize macrophages *in vivo*, within zebrafish larvae have been recently established in the context of inflammation and resolution. Though along less robust measures, M1- and M2-type macrophages have been identified throughout the wound healing process in zebrafish larvae, according to the expression, or lack of expression, of *tnfa*, respectively. The phenotypes were confirmed though cell sorting and quantitative realtime polymerase chain reaction (qRT-PCR), as expression of proinflammatory genes *tnfb, il1b,* and *il6* was elevated in the *tnfa+* macrophages whereas these genes were expressed in low levels in the *tnfa-* macrophages. Conversely, the latter expressed high levels of expression of genes closely associated with M2 macrophages in mammals, such as *tgfb1*, *ccr2*, and *cxcr4b* (Nguyen-Chi et al., 2015). Of more relevance for our purposes, the early inflammatory role of tissue resident macrophages in coordinating the subsequent influx of circulating immune cells *in vivo*, will be reviewed next.

Under normal, or steady-state, conditions, tissue-resident macrophages maintain tissue homeostasis through their interactions and crosstalk with their surrounding environment. In the absence of inflammation, zebrafish generally do not display circulating monocytes or macrophages. Instead, these cells remain trapped in the CHT until mobilised by an inflammatory cue (discussed in more detail later) (Kaveh et al., 2020). Since these cells are trapped by the CHT, researchers tend to refer to these cells as CHT resident macrophages and 'conventional' tissue resident macrophages, peripheral tissue macrophages. As we are investigating the zebrafish model during acute inflammation, we will not be referring to CHT macrophages as 'resident'. Hence, all future mentions of tissue resident macrophages will refer to peripheral macrophages. This classification will also allow for easier species comparison, as peripheral tissue macrophages are orthologous to mammalian tissue resident macrophages both in origin and function as 'sentinels' of tissue damage and infection (Morales and Allende, 2019) (Wu and Hirschi, 2021). Tissue-resident macrophages exhibit highly specialized functions and gene expression profiles depending on the tissue in which they reside. In most tissues under steady state conditions, the vast majority of macrophages comprise these tissue-resident macrophages, generally displaying an "M2-like" phenotype (Davies, Jenkins, Allen and Taylor, 2013). In the event of injury or infection, tissue-resident macrophages play a significant role in the establishment, regulation, and resolution of inflammation (Liddiard et al., 2011).

By virtue of proximity, tissue-resident macrophages are the first responders to inflammatory insults within any given tissue. In steady state, these cells are important for suppressing inappropriate immune responses to minor insults. This includes non-inflammatory phagocytosis of apoptotic cells, foreign materials, and pathogens (Gordon and Plüddemann, 2018). Mouse tissue resident macrophages have also been shown to hide minor inflammatory damage from circulating immune cells. Physically, this is done through maintaining endothelial junction integrity within blood vessels, thereby preventing the extravasation of infiltrating leukocytes and through sequestering microlesions from leukocytes cells within tissue (Lapenna, De Palma and Lewis, 2018)(Uderhardt et al., 2019). The latter is accomplished by tissue resident macrophages prostrating their membranes over sites of micro-damage to physically shield the site from other immune cells. The damage is sensed by the tissue-resident macrophages through their membrane-bound RAGE: receptors that can detect damage associated molecular patterns (DAMPs) including high motility group box protein 1 (HMGB1) and S100 family proteins (Uderhardt et al., 2019). It has not yet been shown whether zebrafish tissue resident macrophages perform the same microlesion obfuscation response but given the evolutionary conservation of the innate immune system, conservation of this function is likely.

Injured tissues, regardless of the cause, release DAMPs. These factors act as beacons of stress and are released both by dying cells and the degrading extracellular matrix (Chen and Nuñez, 2010). In bouts of moderate to severe acute tissue damage, tissue-resident macrophages undergo a similar qualitative switch to an M1 phenotype, albeit less robust than in monocyte-derived macrophages. In mammals, this switch is followed by secretion of proinflammatory mediators and a temporary phenomenon known as the ‘macrophage disappearance reaction’, a process whereby tissue-resident macrophage numbers in the site of inflammation drop extensively (Barth, Hendrzak, Melnicoff and Morahan, 1995)(Yoshida et al., 2004). This is thought to occur due to macrophage emigration to lymph nodes, increased adhesion to structural cells, and cell death (Barth, Hendrzak, Melnicoff and Morahan, 1995). Recent research has indicated that suppression of the innate tissue-resident macrophage ability to undergo self-renewal is likely a factor in this phenomenon (Mu et al., 2021).

With the increase in proinflammatory mediators being released at the site of injury, and the disappearance of tissue-resident macrophages, diapedesis of circulating immune cells towards the injured site becomes possible (Mu et al., 2021). This is different from the zebrafish tissue resident macrophage response to injury, as will be discussed in the section on zebrafish tail transection.

2.4.4 Monocyte and macrophage migration

Neutrophils are the first systemic immune cells to infiltrate the site of injury in both humans and zebrafish. These short-lived, fast-responding cells exhibit potent microbicidal activities and weak phagocytic ability before committing to apoptosis to limit inflammatory damage (Rosales, 2018). Concurrently, the slower monocytes also begin to infiltrate the site of inflammation.

Understanding the migration and 'life cycle' of macrophages is essential in understanding the role of these cells in the innate immune system and the sequence of events that unfolds in response to tissue damage. This understanding is critical in the assessment of a macrophage drug delivery system; both in its effectiveness to perform accurate drug delivery and the bimodal interaction of the drug carrier and the host internal environment.

Human monocytes involved in the promotion of inflammation, namely classically activated and intermediate monocytes, express a high level of CCL2 (or monocyte chemoattractant protein-1 (MCP-1)) receptor, CCR2. The activation of this receptor, also achievable by CCL7, is required for the egress of proinflammatory monocytes from the bone marrow (Tsou et al., 2007). Ligands for these receptors are secreted by numerous cells including bone marrow stromal cells stimulated by circulating LPS (Whelan, Caplice and Clover, 2020). Two orthologues for the CCR2 gene exist in the zebrafish reference genome Zv11: ENSDARG00000079829, which encodes for a protein that shares 44% identity with human CCL2, and ENSDARG00000105363, encoding for a 43% similar protein. A degree of MCP-1 cross-species compatibility with zebrafish has been demonstrated, showing that human recombinant MCP-1 acts on zebrafish macrophages via a CCR2 orthologue to stimulate their recruitment (Cambier et al., 2013). This finding was corroborated in a separate study showing human orthosteric and allosteric MCP-1 receptor antagonists inhibited zebrafish macrophage recruitment (Sommer, Ortiz Zacarías, Heitman and Meijer, 2021). Though it has not yet been shown if the opposite is true, i.e., whether human macrophage CCR2 can respond to zebrafish MCP1, we strongly suspect this would be the case as the active binding sites of MCP-1 and CCR2 appear to be sufficiently conserved between species.

Murine studies have elucidated that, in the steady state, classically activated, short-lived monocytes progressively convert to longer-lived, non-classically activated monocytes in the blood and bone marrow (Yona et al., 2013). This is thought to occur with the intermediate step in humans giving rise to observed intermediate monocytes (Kapellos et al., 2019). In contrast to circulating classically activated monocytes, non-classically activated monocytes are in constant contact with the blood vessel endothelium. The integrins required for this adhesion, namely β2 integrin lymphocyte function-associated antigen-1 (LFA-1), are expressed in low concentrations and intermediate affinities in these cells (Auffray et al., 2007). This allows for constant contact with the endothelium, while maintaining motility, allowing for the observed 'patrolling' function (Thomas, Tacke, Hedrick and Hanna, 2015). These cells are therefore primed to respond to tissue injury (Auffray et al., 2007).

Where classical monocytes rely heavily on a CCL2 gradient and CCL2/CCR2 interaction for extravasation and migration to tissue injury, non-classical monocytes rely on CX3CR1/CCL3 interaction for tissue recruitment (Tacke et al., 2007) (Auffray et al., 2009). An important step in monocyte migration in inflammation is activation of endothelial cells. This can take place in two distinct ways: the quicker, protein synthesis-independent pathway (within minutes) and the slower, gene-transcription- and protein-translation-dependent pathway (Pober and Cotran, 1990). Named type I and type II endothelial activation respectively, endothelial cell activation is

crucial for the expression of adhesion molecules on the endothelial cell surface (Pober and Sessa, 2007).

Type II endothelial cell activation is usually long-lasting compared to type I and is stimulated primarily by proinflammatory cytokines TNF-a and IL-1b derived from tissue-resident macrophages after tissue injury (Gerhardt and Ley, 2015). This mode of activation is much more effective at leukocyte recruitment than the more transient type I (Pober and Sessa, 2007). Stimulation by TNF-a and IL-1b upregulates the synthesis and surface expression important adhesion molecules, including P- and E-selectin, followed, after some time, by intracellular- (ICAM) and vascular cell adhesion molecule (VCAM) on endothelial cells (Sheikh et al., 2005). Upon type II activation, endothelial cells also upregulate the synthesis and surface presentation of chemoattractant proteins, including potent classical monocyte chemoattractant, CCL2 and granulocyte monocyte colony stimulating factor (GM-CSF) (Weber, Nelson, Gröne and Weber, 1999) (Takahashi et al., 2001). This strongly attracts free-flowing classical and intermediate monocytes to the endothelium where extravasation ensues (Gerhardt and Ley, 2015). Zebrafish larval macrophages have been shown to traverse blood vessels in a similar manner, once mobilized from the CHT. Interestingly however, zebrafish neutrophils and macrophages have been shown to preferentially crawl along the abluminal side of blood and lymphatic vessels during chemotaxis (Kaveh et al., 2020). This represents a clear difference between zebrafish larval, and adult human macrophage chemotaxis and may negate the need for zebrafish macrophage transendothelial migration. It is currently unclear whether this zebrafish migrational feature persists into adulthood.

Transendothelial migration of monocytes is a complex, but well characterized process that has been extensively reviewed. In short, this process comprises a strict sequence of events, known as the adhesion cascade. Due to the reduced local blood pressure that accompanies vasodilation, inflammatory monocytes that would otherwise be found towards the center of blood vessels, can flow nearer to the vessel endothelium.

The first step in transendothelial migration is endothelial capture of monocytes via the binding of their leukocyte glycoprotein (e.g., P-selectin glycoprotein ligand-1 (PSGL-1)) (also present on macrophages) to endothelial P- and E-selectin (Gerhardt and Ley, 2015). This binding is reversible, to allow further motility, but strong enough to resist shear stress from blood flow (Gerhardt and Ley, 2015). The tethering of leukocytes to P-selectin has also been shown to upregulate the endothelial expression of CCL2, further stimulating monocyte chemotaxis (Weyrich et al., 1995). Cross-species interaction is also possible with zebrafish PSGL-1 and human P- and E- selectin, as it has been shown that zebrafish PSGL-1 supports the binding and rolling of CHO cells expressing zebrafish PSGL-1, under flow conditions, on human P- and E-selectin. Although this binding is less specific and efficient than with human PSGL-1 and confers less stability, these data provide some evidence in favour of human macrophage P- and E-selectin-dependent chemotaxis being possible in zebrafish larvae (Baïsse et al., 2019). The reduction in binding efficiency may result in the

human macrophages being outcompeted by zebrafish innate macrophages *in vivo*, potentially necessitating their ablation prior to human macrophage injection.

Human monocytes roll in a cyclical manner along the endothelial surface in a selectin-dependent manner, flattening out and extending microvilli and rear tethers for increased surface area and stability. Other monocyte-endothelium interactions at this point include the binding of endothelial VCAM to monocyte-expressed very late antigen-4 (VLA-4) and macrophage-1-antigen (Mac-1); stronger adhesions that facilitate slower rolling and subsequent monocyte arrest (Gerhardt and Ley, 2015) (Schenkel, Mamdouh and Muller, 2004). Primarily governed by VLA-4/VCAM and CCL2/CCR2 interactions, the monocytes stop rolling and adhere strongly to the endothelium. Intraluminal crawling of the monocytes is still made possible through leucocyte integrins lymphocyte function-associated antigen-1 (LFA-1) and endothelial ICAM as the leukocytes migrate towards potential exit sites for extravasation (Schenkel, Mamdouh and Muller, 2004). The orthologue for ICAM has been identified in the mRNA of zebrafish, showing a high degree of conservation in the functional domain with human ICAM, and confirmed in another member of the teleost family, grass carp. The teleost expression of ICAM was also shown to be inducible, increasing expression and leukocyte binding capacity after the addition of IL-1b and LPS – a feature consistent with mammalian ICAM. Furthermore, grass carp leukocytes facilitated this binding to ICAM via Lfa-1 (Wei et al., 2018). It is unknown to what extent human LFA-1 can bind to zebrafish ICAM; however, it is likely that some degree of binding will occur. The success of human macrophage extravasation from zebrafish circulation will depend, in part, on the degree of conservation of these integrins between species.

Extravasation of human and zebrafish monocytes/macrophages has been shown to occur both paracellularly and transcellularly through the endothelium with the former being preferred in mammals (>90%) (Mickael et al., 2021). It has been elucidated in mouse studies how leukocytes may extravasate paracellularly without disrupting endothelial integrity: Leukocytes extravasate paracellularly through endothelial pores that do not exceed ~4µm in width and close behind the extravasating cells to prevent vascular leakage (Heemskerk et al., 2016).

Next, myeloid cells need to navigate through the underlying basal membrane to enter the interstitium of the inflamed tissue. The basement membrane is a specialized, rigid form of ECM that subtends the endothelial layer. Mostly comprising a tight network of covalently-linked collagen type IV fibers, non-covalently associated with structural proteins called laminins, the basement membrane also contains cells including pericytes, adipocytes and nerves (Rowe and Weiss, 2008). Extravasation through the endothelium and subsequent interaction with the basal membrane are thought to be crucial for monocyte-to-macrophage differentiation (Li et al., 2020).

During extravasation, monocytes interact with a host of proinflammatory cytokines, both from the activated endothelium (crosstalk) and from surrounding monocytes. Importantly, this includes GM-CSF, a monomeric glycoprotein that upregulates the monocytic expression of CCR2 and stimulates monocyte-to-macrophage differentiation (Lotfi, et al., 2020). This has been shown extensively *in vitro* and is a frequently used stimulus to trigger monocyte differentiation in *in vitro*

research. A recent study has elucidated that, *in vivo,* differentiation of monocytes is likely a two-step process involving not only crosstalk with the endothelium, but interaction with laminin 511 in the sub-endothelial basement membrane (Li et al., 2020).

Macrophage migration is more complex than monocyte migration as they utilize a combination of two distinct migratory mechanisms for movement. The predominant migratory mode is variable depending on the rigidity and porosity of extracellular matrix through which they migrate (Rumianek and Greaves, 2020). The two modes that macrophages may employ for movement are termed 'amoeboid' and 'mesenchymal' migration. In both cases, the initial step is migrational polarization of the cells. In this case, 'polarization' is the reorganization of the macrophage cytoskeleton resulting in a breaking of symmetry and separate poles at the front and back of the cells and is distinct from macrophage polarization (Meili and Firtel, 2003).

Of particular relevance to the models employed in this thesis, a recent study in zebrafish demonstrated that although macrophages use ameboid migration, they are far more reliant on mesenchymal migration in the context of a tail transection model (Barros-Becker, et al., 2017). This mode of migration is consistent with human macrophages navigating through dense matrices with few defined fibrils (McWhorter, Davis and Liu, 2014). In mesenchymal migration, the front, or leading edge of the macrophage develops actin projections, known as lamellipodia for broad and flat projections, and filopodia for longer, thinner projections. These projections are rich in focal clusters of integrins. This allows the macrophage actin projections to form nascent weak adhesions to substratum proteins to 'tow' the macrophages forward. The human basement membrane is typically 100-300nm thick with pores only around 50nm (Rowe and Weiss, 2008). Although a laminin-rich sub-endothelial basement membrane is known to exist in zebrafish larvae, it is poorly described in literature (Gross-Thebing et al., 2020). Given human macrophages are fairly large leukocytes, with a diameter of about 21µm, mesenchymal migration of cells is protease-dependent, and leads to remodeling of the ECM. A key protease involved with human macrophage migration through the basement membrane is membrane type 1 matrix metalloproteinase (MT1-MMP) (Bahr and Weiss, 2018). The extent of proteolytic and mechanical ECM remodeling is dependent on the macrophage phenotypic polarization. *Ex vivo* studies demonstrated that human M1 macrophages degrade approximately twice as much ECM than do naïve and IL-4-stimulated (M2-type) macrophages, with twice the average pore size (Bahr and Weiss, 2018). Since mesenchymal migration relies on integrins that are only somewhat conserved between humans and zebrafish, it is possible that zebrafish innate macrophages will outcompete the human injected macrophages. Therefore, if the injected macrophages are observed to migrate in circulation to the inflammatory site but fail to undergo mesenchymal migration beyond the vascular tissue, temporary zebrafish macrophage ablation should be considered.

Also present in the human basement membrane are ~1µm in diameter preformed portals that macrophages and dendritic cells have been shown to selectively pass through in a protease-independent manner. This is likely indicative of an amoeboid-type migration and requires extensive deformation of the migrating macrophages to pass through, though they leave the ECM

intact (Renkawitz et al., 2019)(Bahr and Weiss, 2018)(Pflicke and Sixt, 2009). As a general rule, macrophages are morphologically plastic and may squeeze through pores ~≥10% nuclear diameter. Although literature is scarce on this topic, the zebrafish basement membrane also contains pores of a similar size to the human counterpart (van den Berg et al., 2019).

Finally, macrophages must navigate the tissue interstitium. This specialized layer of ECM comprises primarily type I and III collagen fibrils, elastin, glycoproteins, proteoglycans, and glycosaminoglycans (Rowe and Weiss, 2009). The key integrins involved with the interstitial mesenchymal migration of human macrophages have been identified to be members of the β2 integrin family, namely αDβ2 and αMβ2 (Cui, et al., 2018). Macrophages expressing moderate concentrations of these integrins are the most efficient macrophage migrators in tissue. Macrophages expressing very low concentrations of αDβ2 and αMβ2 are unable to adhere to (and therefore pull themselves along) a matrix, and macrophages expressing high concentrations of these integrins adhere too strongly to the matrix to efficiently migrate (Cui, et al., 2018. A number of *in vitro* studies have shown that M1 macrophages are less proficient at migrating in a 3D environment than naïve macrophages and M2 cells. This is in part due to the high expression the integrin αDβ2, promoting a more static phenotype at the site of inflammation. Resident macrophages are also static but this is due to a high expression of αMβ2. In contrast, naïve and M2-type macrophages express these integrins in moderation, allowing for efficient migration (Cui, Ardell, Podolnikova and Yakubenko, 2018). These data indicate that M1 macrophages are relatively immobile once they have reached the target inflammatory site. Here the M1 macrophages will either undergo apoptosis or a phenotypic shift towards a more mobile M2 state, allowing for wound healing and migration away from the wound site. Hence, although *in vivo* macrophage typing is beyond the scope of this thesis, we may infer that, if our injected M1 macrophages are mobile at the wound site after the inflammatory phase, it is likely that they have undergone a phenotypic shift to an M2 state.

An important physiological detail relevant to the translation of the novel human macrophage-based drug delivery system that must still be determined is the ability of activated human macrophages to traverse the zebrafish circulatory system and extravasate at appropriate sites. Uncertainty in this regard may be mitigated when reviewing the literature on monocyte/macrophage response to a caudal fin transection.

2.5 Cross-Species Compatibility

2.5.1 Macrophage Chemotaxis and Cross-Species Compatibility: Methodological Considerations

Macrophages are the most important immune cells in the healing of wounded zebrafish larvae in both the presence and absence of pathogens (Rosowski, 2020). In this section, I will review the extensively researched roles of macrophages in the context of zebrafish larval tail transections in literature as it relates to the topic of drug delivery. Using this, I will construct a theoretical timeline of macrophage polarization and subtype contribution to the healing wound at different

timepoints. This will then be used to determine optimal intervention time points, estimate the function and fate of our human macrophage drug carriers in zebrafish larval tail transections at different timepoints, as well as serve as a comparison for future *in vivo* results.

Embryonic tail transections are usually performed in 2-3dpf zebrafish, cutting the fin from a roughly 90-degree angle to the notochord. Tail transections also occasionally include the cutting off of the distal part of the notochord to induce a stronger inflammatory response (Xie, Meijer and Schaaf, 2021).

Within 10 minutes post transection, the epithelium contracts at the lesion site due to actin-purse string formations to seal the wound (Kawakami, Fukazawa and Takeda, 2004). Simultaneously, epithelial production of H_2O_2 is initiated at the wound site, establishing a gradient extending proximally towards the nearest blood vessels within 10 minutes. Shown to be a function of the epithelial dual oxidase enzymes in response to damage induced intracellular Ca2+ oscillations, extracellular H_2O_2 concentration peaks approximately 20 minutes post wounding, extending the concentration gradient as far as 200µm away from the wound (Daly, 2010). The upstream intracellular Ca2+ oscillations are amongst the first signs of alarm in wounded tissue and are often the result of intact cells detecting extracellular ATP as a DAMP through their purinergic receptors (de Oliveira et al., 2014). Although the H_2O_2 gradient gradually decays in 1-2 hours, it first acts as a potent initial chemoattractant, recruiting the first non-resident leukocyte responders ~20 minutes post wounding (Daly, 2010). These first responders are typically neutrophils which sense and respond to wound-derived reactive ROS via the Src family kinase (SFK) Lyn (Yoo, Starnes, Deng and Huttenlocher, 2011). It is important to note, while critical for leukocyte recruitment, H_2O_2 is not the sole ROS leukocyte chemoattractant synthesized in response to zebrafish tail transections. In the context of intermediate inflammatory damage (tail transection without damage to the notochord), neutrophil number in the wound site peaks at ~6hours post wounding (HPW). In this case, the wound site is defined as the area 200µm proximally from the wound, in keeping with the H_2O_2 gradient reach. After this point, the neutrophils begin to reverse migrate from the wound site or, more rarely, undergo apoptosis (~2-3%) to resolve neutrophil inflammation (Elks et al., 2011). Although contentious in literature, mounting evidence does appear to suggest that neutrophil reverse migration during the resolution of inflammation is conserved across higher order vertebrates such as mice and humans. Resolution of neutrophil inflammation is completed by ~24HPW (Ellett et al., 2015).

From 3-6HPW, the slower monocytes/macrophages rapidly colonize the wound site, becoming the dominant immune cell type at the wound (Nguyen-Chi et al., 2017). Recently, macrophage recruitment to the wound site in zebrafish embryo tail transection models was shown to be independent of neutrophil signaling. This is in agreement with the hypothesis that neutrophils and macrophages are recruited simultaneously but the temporal delay between the two cell types' arrival is a result of different migration velocities (Sipka et al., 2021). Instead, macrophages rely on early intracellular Ca2+ oscillations at the amputation site for migration and proper M1 polarization. Also required for M1-like polarization but not migration is H_2O_2 which, as in

neutrophils, signals via SFK Lyn and to a lesser extent, SFK Yrk (Sipka et al., 2021). As wound-generated reactive oxygen species such as H_2O_2 are conserved across species, we are certain they will elicit the appropriate macrophages responses irrespective of the animal model in which they are placed (Daly, 2010). The main consideration for our study then, is the order of operations concerning cell injections and tail transections and the amount of time between them.

In steady state conditions, zebrafish do not display circulating monocytes or neutrophils. Instead, these cells are mobilized from their peripheral tissue and CHT niches following inflammatory damage (Kaveh et al., 2020). While the majority of these cells make use of the abluminal surface of the vascular endothelium on which to migrate, a smaller subset of these leukocytes enter circulation. Although both neutrophils and monocytes/macrophages do enter circulation in response to damage, macrophages are more likely to exhibit perivascular crawling than to be free flowing (Kaveh et al., 2020). This is similar to mammalian monocyte migration after monocyte capture to the endothelium and extravasation of these cells likely resembles extravasation of mammalian monocytes thereafter (section 2.4.4). This is evidence that both the abluminal and luminal surfaces of the zebrafish vascular endothelium undergo remodeling/activation in response to inflammation and can support macrophage migration. This is especially important as we do not yet know whether the human macrophages will behave dissimilarly to the zebrafish macrophages, as abluminal crawling of macrophages in humans is uncommon in literature. Indeed, so long as zebrafish and human integrins are sufficiently conserved, and human macrophages are not outcompeted by zebrafish macrophages, human macrophages should be able to migrate appropriately to sites of zebrafish inflammation. Hence, this will have to be experimentally determined.

2.5.2 Monocyte and Macrophage Form and Function in Inflammation: Cross-Species Compatibility

Studies in zebrafish often neglect to delineate between monocytes and macrophages, utilizing macrophage expressed gene 1 protein (MPEG1) as a blanket marker of macrophages despite the expression of this protein by monocytes. Direct, meaningful comparison of human and zebrafish macrophages in terms of physical characteristics and polarisation state is difficult, as human macrophages tend to be studied *in vitro* rather than *in vivo*, whereas the opposite is true for zebrafish. Scarce literature could be found describing zebrafish macrophages *ex vivo*, likely due to the obsolescence of *in vitro* work in the zebrafish model that lends itself to *in vivo* experiments.

Despite difficulty in specifically categorizing macrophage phenotypes in response to a wound in real time, *in vivo*, a number of recent studies have shown success in delineating between different macrophage states over the course of inflammation and wound healing. As mentioned previously, the main basis for this delineation is the expression, or lack thereof, of TNF-a (Nguyen-Chi et al., 2015). Interestingly, clear morphological changes can be observed in these cells over the course of a tail fin transection. It was shown that, although macrophages are recruited to the wound from 1HPW, the first TNF-a signal is observed from 3HPW. This is indicative of the early

arrival of naïve macrophages which undergo rapid polarisation to an M1 phenotype. From 5HPW, new macrophages, already expressing TNF-a, are recruited to the wound. This recruitment is impaired (until 24HPW) by the selective ablation of peripheral macrophages, normalised to total number of macrophages, compared to both wild type and zebrafish larvae with uniform ablation of CHT and peripheral macrophages. This indicates a potent role of peripheral macrophages in the recruitment of pro-inflammatory macrophages (Morales and Allende, 2019) (Nguyen-Chi et al., 2015). Therefore, if macrophage ablation is to be considered prior to injection of our human macrophages, it would be prudent to only target CHT, and not peripheral macrophages, so as to not impair recruitment of our cells to the wound site.

To date, only one known method of zebrafish macrophage ablation that does not rely on transgenic zebrafish lines (outside the scope of this thesis) exists. This involves the intravenous microinjection of liposomal chlodronate into zebrafish larvae and results in the indiscriminate ablation of all macrophage/phagocyte populations (Rosowski, 2020). Liposomal chlodronate also exhibits a long circulating half-life and requires at least 48 hours post injection to take effect (Yang, Rojas and Shiau, 2021). Free circulating liposomal chlodronate would also be toxic to our introduced human macrophages. Due to the indiscriminate nature of liposomal chlodronate macrophage ablation and the long circulating half-life of this chemical, this method of macrophage ablation is incompatible with our current study.

In accordance with M1 macrophage recruitment, blastemal cell formation is observed as early as 6HPW (Nguyen-Chi et al., 2017). Although macrophages are not dependent on TNF-a signaling for their initial recruitment to the wound, this cytokine enhances macrophage accumulation in the wound after 6HPW and is required for correct regeneration of the caudal fin (typically within 3DPW) (Nguyen-Chi et al., 2017). Taken together, this evidence appears to indicate that peripheral macrophages recruit CHT macrophages to the wound site through TNF-a, acting as a positive feedback loop.

TNF-a is a highly pleiotropic protein, expressed by a number of cells as a DAMP in response to tissue damage. Chiefly among these are macrophages which express this protein as both a paracrine and autocrine signaling molecule (Riches, Chan and Winston, 1996). CRISPR knock-out studies utilizing primers specifically designed against zebrafish TNF-a1 demonstrated early expression of this protein, secreted chiefly by macrophages at the wound site, was crucial for normal axonal regeneration in inflicted spinal cord injuries of 3dpf zebrafish larvae (Tsarouchas et al., 2018). This was further corroborated by macrophage deficient zebrafish mutants yielding a similar pathological regeneration pattern in response to the same injury model. From these data, we may safely infer that macrophage-derived TNF-a1 specifically, is a key cytokine in zebrafish tissue regeneration. Taken together with evidence demonstrating that the presence of TNFa+ macrophages at the wound site of a tail transection was insufficient to promote tissue regeneration when TNF-a receptor 1 (TNFR1) was knocked out of the larvae stromal cells, it can be safely inferred that the main pathway involved in zebrafish tail fin regeneration is governed by the interaction of macrophage TNF-a1 and stromal TNFR1 (Nguyen-Chi et al., 2017).

The structure of TNF-a1 is the best studied of the two zebrafish isoforms and, similarly to humans, is thought to be biologically active as a homotrimer. In terms of amino acid sequence identity, the zebrafish TNFa-1 is under 35% similar to human TNF-a. Structurally, the zebrafish TNFa1 trimer is unique in that the EF loop of each constituent monomer seals the central cavity present at the top of the trimer in other characterized TNF-a proteins (Duan et al., 2021). Because of this, the zebrafish TNF-a1 protein also lacks an internal coil and is shorter than other species' TNF-a. Despite obvious structural and amino acid differences, researchers digitally superimposed zebrafish TNF-a1 onto an *in silico* model of mouse TNF-a/human TNFR1 complex. In doing so, 4 putative amino acids thought to facilitate zebrafish TNF-a1 binding to zebrafish TNFR1 were elucidated – these being in the same positions as 4 bound amino acids in the mouse/human TNF-a/TNFR1 complex (Duan et al., 2021). We therefore suspect that, despite considerable structural divergence, zebrafish TNF-a1 derived from peripheral macrophages will bind to human TNFR1 on our injected macrophages, recruiting them to the wound site.

It has been postulated that due to the lack of vasculature in the larval zebrafish tail fin, circulating monocytes/macrophages are unable to contribute to the leukocyte pool at the site of amputation. The pool would therefore only comprise nearby tissue-resident cells that migrate towards the wound in a circulation independent manner. Evidence from parabiotic studies contest this hypothesis, demonstrating that macrophages originating from a conjoined embryo can be found in the wounded tail fin of its opposing 'twin' (Nguyen-Chi et al., 2017).

Recent evidence suggests that resident macrophages recruited from different tissues in the embryo may play differential roles in the inflammatory response to tail fin transection. Peripheral resident macrophages residing in the tail fin (orthologous to mammalian tissue resident macrophages in origin and function) respond fastest to the wound, likely as a result of the close proximity to the wound edge and sensitivity to inflammatory cues (Morales and Allende, 2019). These macrophages are also faster migrators in response to the wound than are CHT macrophages, being recruited at an average speed of ~73µm/h compared to the ~41µm/h average speed of CHT macrophages. This creates a temporal delay between the arrival of both macrophage subsets, with peripheral tissue macrophages arriving more rapidly to the wound site within the first 6-12HPW (Morales and Allende, 2019).

Early expression of pro-inflammatory cytokine, IL-1b at a wound site is known to be a principal effector of the inflammatory process (MacLeod and Mansbridge, 2016). Although myeloid cells are traditionally thought to be the primary contributors of this cytokine in the early stages of wound healing, tail transection studies in zebrafish larvae demonstrate that the opposite is true. Up to 3HPW, epithelial cells at the wound site rapidly upregulate IL-1b expression until the arrival of the first peripheral macrophages. This early IL-1b expression was shown to be crucial for the expression of pro-regenerative genes in the wound, but prolonged expression resulted in increased apoptosis and impaired wound healing (Hasegawa et al., 2017). Real time quantitative PCR employed at 6 and 24HPW revealed that the peripheral macrophages serve to reduce *il1b* transcripts in the zebrafish tail at 6HPW (Hasegawa et al., 2017). Targeted ablation of peripheral

macrophages impairs tissue regrowth due to sustained inflammatory damage as a result of a sustained IL-1b response (Hasegawa et al., 2017). This is in concordance with the longstanding notion that tissue resident macrophages act to regulate inflammatory damage at the wound site in mammals, as described in the macrophage typing section.

At 24HPW, peripheral macrophages express significantly more *tgfb1a* than do CHT derived macrophages (Nguyen-Chi et al., 2017). Although TGF-beta is a pleiotropic cytokine, capable of signaling many seemingly opposing functions, in the context of a zebrafish embryo tail transection, it is vital for the correct epimorphic repair of the fin (Miskolci et al., 2019). As these were the only differences in observed cytokines transcripts (also investigated *il10* and *tnfa*) between each group of macrophages, it appears peripheral derived macrophages arrive at the wound site primed towards a more regenerative phenotype, whereas CHT derived macrophages initially at the wound site are likely closer to pro-inflammatory M1-type (Morales and Allende, 2019). This further illustrates that TNF-a as the sole marker of macrophage subtype oversimplifies macrophage dynamics in injury.

This study also found that after near-complete ablation of peripheral resident macrophages, there was no significant difference in macrophage number at the wound site after 24HPW. It was also shown that the transcription of *tnfa* in the tail decreased to virtually nothing between 6 and 24HPW (Morales and Allende, 2019). This is consistent with the previously mentioned study which showed M2 (TNF-a-) macrophages being dominant in the wound from 24HPW until resolution (Nguyen-Chi et al., 2017). Taken together, this indicates that the relatively more proinflammatory macrophages derived from the CHT can undergo a phenotypic switch towards an M2-like phenotype even in the absence of most peripheral resident macrophages.

One recent study investigated the migration and survival of human naïve macrophages injected into zebrafish embryos. It was found that the exogenous macrophages preferentially associated with the head and, importantly, tail vasculature. Although this was not explicitly determined by the researchers, we suspect, based on the anatomical positioning of the exogenous macrophages in the tail region observed in this study, that from the first day post injection the macrophages preferentially associate with the CHT niche (Paul et al., 2019). This could be predictive of the response these macrophages will elicit in inflammation as being more similar to zebrafish innate CHT macrophages than peripheral tissue macrophages. This is in keeping with the presumption that CHT macrophages are orthologous to mammalian monocyte-derived macrophages. Based on the current evidence, we also cannot rule out the possibility of the CHT niche priming macrophages towards a specific response, though this has not yet been shown. Given that the CHT niche is in the abluminal space, this could also be amongst the first *in vivo* evidence for macrophages to traverse through the endothelium post monocytic differentiation.

All of the exogenous human macrophages were found closely situated to blood vessels even after 7dpi, indicating that they did not take up peripheral tissue-resident status in the fin (Paul et al., 2019). Another potential explanation for slower recruitment and differential injury response of CHT macrophages to tail fin transections as compared to peripheral tissue-resident macrophages

is the requirement of CHT macrophages, not peripheral tissue macrophages, to traverse through the basement membrane subtending the blood vessels. As previously discussed, this layer could transiently limit the migration of the macrophages as a checkpoint, to ensure they are mostly differentiated and primed to respond to an inflammatory insult (Li et al., 2020).

It is important to consider, when transplanting live immune cells as potential drug carriers in zebrafish larvae, the extent of crosstalk that can take place between the exogenous inflammatory cells and the zebrafish microenvironment.

2.5.3 Crosstalk Between Human M1 Macrophages and the Zebrafish Larval Microenvironment

Human M1, or classically activated macrophages, are considered primary actuators of acute inflammation as they secrete an array of pro-inflammatory products upon stimulation. These include cytokines such as IL-1b, IL-6, IL-12, IL-18, IL-23, TNF-a, and type I IFN; and chemokines. The first, and most important responders to damage- and pathogen-associated molecular patterns in macrophages are IL-1b, TNF-a, and Il-6 (Atri, Guerfali and Laouini, 2018). As TNF-a has already been discussed in the previous section, we will focus more on human IL-1b and IL-6 and the cross-species compatibility of these cytokines.

Cytokine synthesis and secretion play an important and tightly controlled role in mediating immune response and inflammation. Much of the control is as a result of the instability of many interleukins' mRNA, limiting the lifespan and translation window of these molecules (Brown, Lagnado, Vadas and Goodall, 1996). In both mammals and teleost fish, IL-1b is an orthologous, multifunctional protein and serves as a potent pyrogen and proinflammatory cytokine that acts primarily on T and B lymphocytes, macrophages, and endothelial cells (Frame et al., 2020). This cytokine also acts on liver tissue to stimulate the release of acute phase proteins, thereby playing a role in coordinating the adaptive immune response. In both organisms, IL-1b is translated as an immature, inactive pro-IL-1b protein that must be processed to yield the mature, active form. In humans, this processing is primarily performed intracellularly by caspase 1, cleaving the pro-protein between aspartate 116 and alanine 117 (Pyrillou, Burzynski and Clarke, 2020). In ZF, this cleavage is performed by caspase 1 orthologues, caspase A and caspase B (Frame et al., 2020) (Vojtech, Scharping, Woodson and Hansen, 2012).

Despite shared function, human and zebrafish IL-1b exhibit amino acid sequence differences. Human and ZF IL-1b only share 27% amino acid sequence identity and a conserved caspase 1 cleavage site is absent on the ZF homologue. The low sequence similarity may be inconsequential to receptor binding however, as the predicted structure of ZF IL-1b contains a conserved structural motif of a β-sheet-rich-trefoil with human IL-1b (Ogryzko et al., 2013). Cytokines containing this structure are classified as members of the β-trefoil family and can often bind to the same cognate receptor with different affinities, despite high sequence variability. For example, in humans, three β-trefoil cytokines: IL-1α, IL-1b, and IL-1Ra are all able to bind to the

IL-1RI receptor even though human IL-1α and IL-1b only share 25% sequence similarity (Fields, Günther and Sundberg, 2019). This is the molecular basis behind one of the key features of cytokines, their redundancy. Interestingly, BLASTP alignments reveal ZF Il1-β (NP_998009.2) and human pro-IL-1b (NP_000567.1) share a similarly low sequence identity of 27.27% but both the percentage identity and E value improve when the mature human IL-1b (AAC03536.1) is aligned to the ZF protein instead. This indicates that the functional structure of this protein is, in part, structurally conserved. Perhaps the most convincing evidence for human-ZF cross-species reactivity of the IL-1 cytokines and their receptors is the successful use of anakinra to reduce inflammation in 3dpf ZF larvae (Mazon-Moya et al., 2017). Anakinra is an analogue of human IL-1 receptor antagonist (IL-1Ra), only sharing 29.7% sequence homology with ZF IL-1b. This is especially important as IL-1Ra binds to site A of IL-RI and IL-1b binds to sites A and B of IL-RI, indicating that both cytokines compete for the same binding site. Human IL-Ra and IL-1b also exhibit a low root-mean-square deviation of atomic positions of only 0.90Å, despite their low sequence similarity (Fields, Günther and Sundberg, 2019). Taken together, there is good reason to believe that human IL-1b released by the macrophages will bind to and elicit effects from ZF IL-1R.

IL-6:

IL-6 is an important cytokine in mediating both the innate and adaptive immune responses and is released upon immune recognition of damage- or pathogen-associated molecular patterns. This cytokine, expressed by many cell types, is crucial during ZF development. In contrast, the primary IL-6 receptor, membrane-bound IL-6R (mIL-6R), is mostly restricted to leukocytes and liver cells, the latter of which are responsible for the coordination of the acute phase response upon IL-6 stimulation (Saito, et al., 1992)(Hibi et al., 1990).

The signaling cascade of IL-6 begins with the binding of the cytokine to IL-6R, causing the receptor to associate with transmembrane signal transducing component, glycoprotein 130 (gp130) (Schmidt-Arras and Rose-John, 2016). Homologues of these components exist in ZF. Signal initiation occurs upon subsequent homodimerization of gp130, activating PI3K, ERK, and JAK/STAT pathways. Functionally, the human and ZF homologues are highly similar in the response they elicit, including further regulation of the immune response by promoting macrophage proliferation and downregulating the expression of IL-1b and TNF-α (Costa et al., 2011). These features both exacerbate and limit inflammation respectively, which makes predicting the transient effect of additional IL-6 in ZF larvae difficult, especially considering the lack of a functional adaptive immune system at this point.

Structurally, the best categorized IL-6 protein is human IL-6, a 212 amino acid long protein with a 28 amino acid signal peptide. Human IL-6 comprises a single chain, phosphorylated glycoprotein with a tertiary structure comprising five α-helices. Four of these helices, A-D, are arranged in a bundle with A and B antiparallel to C and D. Helix E lies outside the bundle and is connected to C and D (Metcalfe, Putoczki and Griffin, 2020).

To our knowledge, zebrafish IL-6 has yet to be purified, hence the predicted structure is the current best estimate (Zou and Secombes, 2016)(Varela, et al., 2012). Zebrafish IL-6 is predicted to be a 231 amino acid long protein with a 23 amino acid signal peptide. Structurally, zebrafish IL-6 is predicted to comprise a 4-helical cytokine core with a partially conserved IL-6/G-CSF/MGF family signature. Despite low sequence similarity, the predicted 3D structure of zebrafish IL-6 is well conserved with human IL-6, suggesting conservation in function (Varela, et al., 2012). Although helpful, the extrapolation potential of evidence based on a predictive model is limited. Fortunately, two separate research groups recently published work in which human recombinant IL-6 was microinjected into zebrafish and elicited a response.

The first study showed by inducing an inflammatory response, through means of mechanical injury to the optic tectum of three-to-four-month-old zebrafish, that early IL-6/Jak-Stat pathway activation is essential for the proliferation of radial ganglia and therefore brain regeneration. Using qRT-PCR techniques, it was shown that the expression of *il6* and *stat3* peaked 6 hours post injury and coincided with macrophage migration into the optic tectum. Also demonstrated was that radial ganglia proliferation, but not macrophage migration was significantly impaired with the addition of a Stat3 inhibitor and that cerebroventricular microinjection of human recombinant IL-6 in uninjured zebrafish was sufficient to induce radial ganglia proliferation (Shimizu, Kiyooka and Ohshima, 2021). Together, this data suggests that macrophage migration and secretion of IL-6 in the early stages of inflammation is necessary for tissue regeneration and that human IL-6 (as will be secreted by our microinjected macrophages) will elicit an appropriate response in the zebrafish larval microenvironment.

The second recently published study corroborates the ability of human IL-6 to elicit physiological responses in zebrafish, demonstrating that chronic overexpression of human recombinant IL-6 in zebrafish causes hepatic steatosis. This finding contributes to our understanding of the mechanism behind IL-6 overexpression and non-alcoholic fatty liver disease, and affirms the compatibility of human IL-6 in zebrafish (Singh et al., 2021).

Based on the literature presented in this section, we have strong reason to believe that injected human M1 macrophage drug carriers would elicit predictable zebrafish responses and may partake in some degree of crosstalk with the zebrafish microenvironment during inflammation, particularly in the early stages.

2.5.4 Injected Macrophage Phenotype Plasticity

It is necessary to address whether, based on current literature, it is plausible that zebrafish cytokines will induce a phenotypic shift in the human macrophages from an M1 phenotype to an M2 phenotype. This is a more nuanced physiological process in the sense that it is entirely mediated by specific secreted signaling factors as opposed to more generalised (and interspecifically conserved) DAMP signaling that occurs during M1 polarisation. Although this phenotypic shift will not be confirmed in this study, this could pose a limiting factor in the overall usefulness of the zebrafish model for future macrophage-based drug delivery research.

As previously discussed, IL-4 and IL-13 are necessary human cytokines to induce M2 polarization of human macrophages *in vivo.* In mammals, the genes encoding these cytokines are located within the same cluster on chromosome 4 and share regulatory elements. Likely as a result of a whole genome duplication event within teleosts, two homologous cytokines for mammalian IL-4 and IL-13 within zebrafish, namely IL-4/13A and IL-4/13B, are encoded for on separate chromosomes, 9 and 14 respectively (Wang et al., 2016) (Bottiglione et al., 2020). Functionally, these zebrafish orthologues have been shown to be similarly immunosuppressive, with mutants lacking these functional genes expressing a significantly more inflammatory phenotype than wild type controls (Bottiglione et al., 2020). Very little data is available on the conservation of these cytokines between teleosts and humans besides a low level of structural conservation between human IL-4 and IL-13, and zebrafish IL-4/IL-13A and IL-4/IL-13B.

Due to the uncertainty regarding whether human macrophages would be able to undergo the entire polarisation range in response to zebrafish stimuli, future studies in this line would greatly benefit from additional *in vitro* experiments in which primary human macrophages are exposed to purified zebrafish M2 polarization stimuli.

The resolution of inflammation is complete when the macrophage presence is reduced at the wound site. As with neutrophils, this is primarily a result of reverse migration and to a much lesser extent, cell death (Miskolci et al., 2019). Macrophage death appears to be related to the severity of the inflammatory response, and therefore was not found in a less severe laser-induced pericardial damage model as compared to tail fin transection (Kaveh et al., 2020). Macrophages begin to reverse migrate around 96HPW (Miskolci et al., 2019). In conclusion, insufficient data is available to predict whether the injected human M1 macrophages will undergo the entire appropriate range of polarisation during the inflammatory response to zebrafish tail transections. We do, however, expect the macrophages to self-regulate their presence in the wound site, either by reverse migration or cell death, to prevent perpetuating inflammation.

2.6 Methodological considerations: Microinjection

The implementation of the novel macrophage drug carriers in zebrafish larvae is possible through microinjection of the drug-laden cells into the larval circulation. Microinjections are becoming more common in zebrafish research as a method of introducing foreign molecules or cells over 500nm in diameter to zebrafish, either locally or into circulation. Despite the rise in popularity of this method, however, there remains to be established a universal consensus regarding the basics, such as the maximum volumes to be injected into zebrafish larvae without significantly disrupting homeostasis and therefore the validity of the acquired data.

This has resulted in injection volumes into circulation frequently ranging from picolitres to over 20nl. At 2dpf, zebrafish larvae are thought to have a total blood volume of between 60 and 90nl (Craig, Gilday, Dabiri and Hove, 2012). There is reason to suspect the commonly injected volumes at the larger end of the spectrum could upset larval homeostasis, damage cardiovascular tissue,

and result in associated developmental defects, although has not yet been the subject of investigation.

To our knowledge, microinjection of human, non-stem, non-cancer cells into zebrafish larval circulation has only been reported once in literature. Fortunately, the research from this study demonstrated that human monocytes and macrophages, differentiated both at human and zebrafish physiological temperatures, survive for up to two weeks post injection into zebrafish (Paul et al., 2019). The data generated in this study provide further evidence for the use of our novel human macrophage-based drug delivery system in larval zebrafish models without the need for additional modification. The authors did not stimulate inflammatory conditions. Therefore, human macrophage migration and response to *in vivo* zebrafish inflammation remains to be determined.

Other factors which remain to be determined are the fates of the injected macrophages after resolution of inflammation and the extent of crosstalk between macrophage and host environment. An advantage of using macrophages as agents of drug delivery is that, unlike with foreign nanoparticles that can injuriously accumulate in tissues, there is a constant turnover: breaking down and recycling of cells in the body. Lastly, the only existing paper to report injecting human macrophages into zebrafish larvae did not provide details on the diameter of the injector tip. Technically, injection tips at the larger end of the diameter spectrum are more prone to leakage and inconsistent injection volumes, whereas tips at the finer end of the spectrum are more prone to clogging. Specifically for our purposes, this must also be optimized as a compromise between a tip size large enough for the ejection of intact macrophages with diameters of approximately 21µm, and a tip size small enough to not elicit a substantial local inflammatory reaction to which the newly injected macrophages would be recruited. Fortunately, macrophages are readily deformable cells which allows for tip diameters smaller than their maximum diameter, provided the injection pressure at least matches the minimum pressure required to for deformation of the cells. Too high an injection pressure may cause the cells to shear.

2.7 Hypothesis

We hypothesised that human M1 polarised macrophages would a) associate with the zebrafish CHT shortly after injection into 2dpf zebrafish larval circulation, b) survive for at least 3 days (until the experimental endpoint), and c) disassociate with the CHT and migrate into the inflamed zebrafish tissue within 48 hours after tail fin transections are performed, thus establishing zebrafish larvae as suitable models in which to develop and test a human macrophage-based drug delivery system.

2.8 Aims and Objectives

In order to test our hypothesis, the following aims and objectives were formulated:

Aims:

1. To optimise microinjection protocols for the introduction of human macrophages into zebrafish larval circulation
2. To optimise cell visualisation parameters for the long-term visualisation of the injected cells *in vivo*
3. To determine whether the injected human macrophages respond appropriately, through adequate survival (over 72 hours post injection) and migration, to acute zebrafish inflammation

Objectives:

1. Optimise microinjector needle tip size
2. Determine appropriate injection volumes
3. Optimise cellular fluorescent stain protocol
4. Introduce labelled human macrophages into 2dpf larval circulation via microinjection, induce a localised inflammatory response, by means of tail fin transections, and assess the response of the injected cells to the inflammation

Chapter 3: Materials, Methods, and Results

3.1 Introduction

Due to this thesis describing a model development project, I have deviated from the traditional discrete sections of materials and methods, and results. Instead, I have integrated these sections to better reflect the nature of the project and provide a more logical, chronological and easier to follow account of research done. Briefly, the layout of this section is information on ethical aspects, followed by a section outlining the most basic techniques that were similar in all experiments. This introductory part is then followed by a chronological account of each experiment, which describes the experiment-specific methods and results.

3.2 Ethical considerations

Ethical approval was granted by the Animal Committee of Stellenbosch University (ref # ACU-2019-11820) for zebrafish work using larvae up to <5 days (<120 hours post fertilization (dpf)). Ethical clearance exemption was also granted by the Health Research Ethics Committee (HREC) of Stellenbosch University for the use of donated blood (buffy coat) for isolation and preparation of primary monocyte cultures (ref #: X15/05/013).

3.3 General Zebrafish Methodology

Zebrafish Maintenance and Breeding: Adult zebrafish were housed under standard husbandry conditions (14:10 light/dark cycle, tank water temperature 28°C, pH 7.2-7.8 and conductivity 500-800uS/cm^3). After spawning, fertilized eggs were sorted into petri dishes with embryo media (E3: 5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$, $10^{-5\%}$ methylene blue, made up in dH2O) at maximum density of 50 eggs per dish and media refreshed daily.

General Anesthesia Protocol: Healthy, hatched 2 days post fertilization (2dpf) larvae were selected for the microinjection procedure and anaesthetized with 0.168g/L tricaine (3-amino benzoic acid ethyl ester also called ethyl 3-aminobenzoate) (Sigma Cat# A5040) in E3 for 20 minutes prior to tail fin transection and/or injection.

General Euthanasia Protocol: All larvae used in experimentation were euthanized before they reached the age of 5dpf. This was performed by combined means of tricaine overdose (immersion in 300mg/L tricaine solution in E3, buffered to pH7) and hypothermia (freezing in -80°C freezer). Euthanasia was always performed by a South African Veterinary Council authorised individual.

Tail Fin Transections: In order to create a natural chemotactic signal to attract macrophages to a site for drug delivery, anaesthetized larvae were individually placed into each well of a 24-well plate with 1ml tricaine supplemented E3 and set under a stereo light microscope (World Precision Instruments, Sarasota, Florida, Model No. 504941). Using a pair of stainless steel Vannas scissors

with 5mm blades (WPI, 14003), caudal fin tissue was transected perpendicularly to the notochord (Fig. 3.1), taking care to not clip the notochord itself.

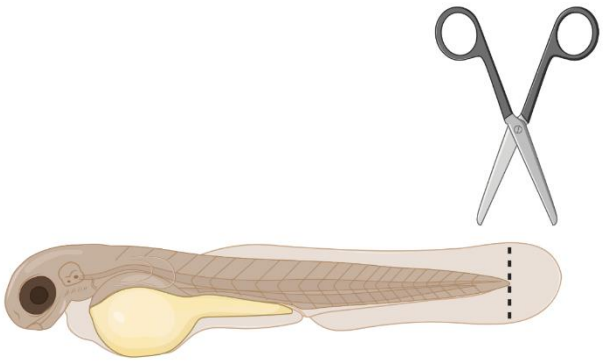


Figure 3.1: Diagram representing a typical tail fin transection.

Microinjection Preparation: Using Eppendorf Microloader tips, ~3µL methylene blue or triturated cell suspension was pipetted into a pulled borosilicate capillary tube (microinjector needle). The filled capillary tube was fitted to a M3301-M3 micromanipulator (WPI) and PV830 PicoPump (WPI) for immediate calibration and injections.

Microinjector Calibration: To calibrate the microinjector, pressure and time period were adjusted to expel droplets of consistent diameter into oil. This was assessed using a micrometer slide upon which the oil was placed, under a WPI Model No. 504941 stereomicroscope. The diameter was used to calculate the volume of the droplets (Volume= (π x diameter^3)/6). For 10nl, a consistent diameter of ~267µm was achieved (Figure 3.2). These results indicate that this method of volume calibration offers an acceptable approximation of the ideal volumes with high consistency.

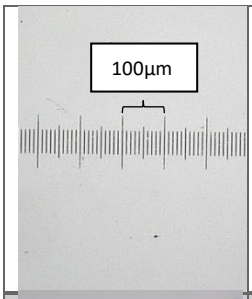
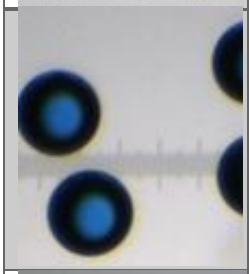
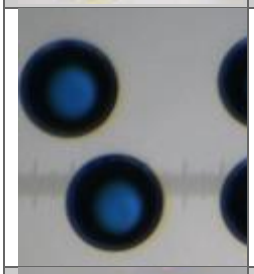
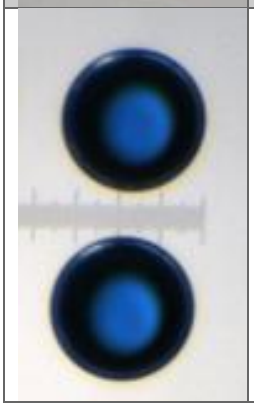
	**Expected Diameter (µm)**	**Average Calculated Diameter (µm)**	**Expected Volume (nl)**	**Calculated Average Volume (nl)**
	212.19	210.08±0.96	5.00	4.85±0.05
	267.30	263.33±1.77	10.00	9.56±0.19
	305.99	294.13±5.50	15.00	13.33±0.76
	336.78	336.64±0.55	20.00	19.97±0.10

Table 3.1. Digitally measured real droplet sizes and volumes compared to ideal, calculated droplet sizes and volumes (±SD). *The scale without droplets is pictured in the top left of the table. Distance between the smallest gradations on the scale are 10µm in length.*

General Zebrafish Microinjection procedure: Anaesthetized zebrafish larvae were individually plated onto a mold consisting of 1% low melting-point agarose and excess media dabbed off with paper towels, taking care to not touch the larvae. Under a stereo light microscope (WPI Model No. 504941), the larvae were aligned for direct access of the microinjector needle to larval circulation via the Duct of Cuvier. Once the needle had broken the epidermis and entered the duct, the required volume was injected and the needle retracted. Successful injection was confirmed by observation of the slight expansion of the duct and the heart immediately after bolus injection. Occasionally, individual injected cells were visible, but this proved an unreliable parameter, as it was dependent on contrast, depth effects, and lack of obfuscation.

The successfully injected larvae were returned individually into a well of a 24 well plate with 1ml fresh tricaine anesthetic solution to be immediately visualized. In cases where a delay prior to visualization was unavoidable, wells were refreshed with fresh E3 and returned to the incubator. For subsequent imaging, larvae were re-anesthetized for 20 minutes prior to visualization as per the general zebrafish anaesthesia protocol in section 3.3. Given the novelty of intravenous cellular injections into 2dpf zebrafish larvae, the following methodological considerations were addressed.

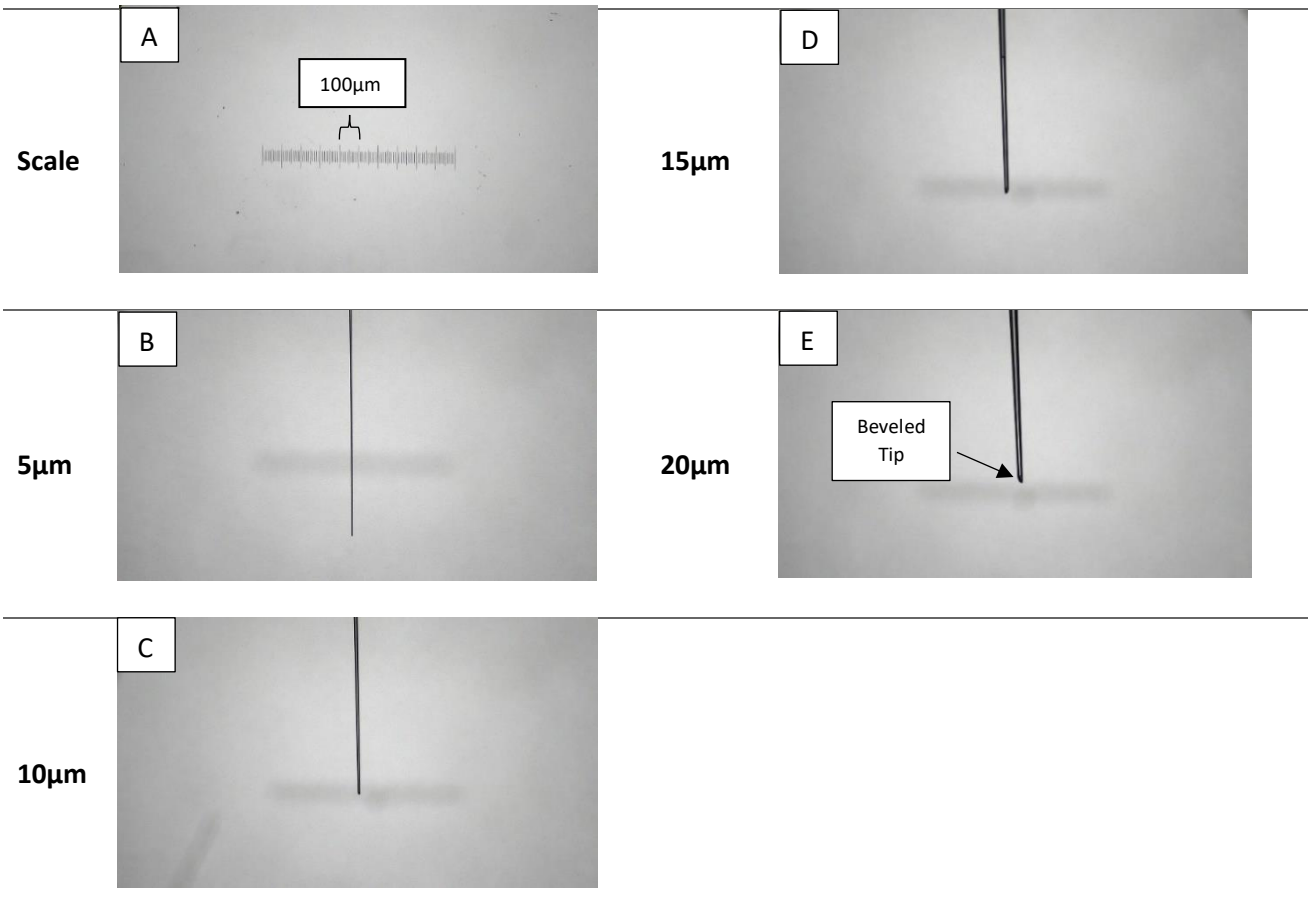


Table 3.2: Images of tip sizes at different diameters under 10x magnification*. In the scale image, the distance between two of the smallest gradations is 10µm. All images were captured at the same magnification with the scale in the background. Image E clearly shows the tip was cut at an angle to create a bevel allowing for smoother breaking of the zebrafish epidermis.*

Tip Diameter Optimization: Both monocytes and macrophages are highly deformable cells and can theoretically be ejected out of a microinjector tip with a smaller diameter than the cells themselves (21-23µm). A relatively narrow tip would be beneficial in avoiding tissue damage but may cause clogging of the tip by cells, hence risking inconsistent injection volumes. Microinjector needles with different tip diameters, starting at 5µm and increasing by 5µm per tip were used experimentally until clogging ceased and droplets of consistent diameters were expelled. The needles were pulled from, 100mm length borosilicate capillary tubes with internal/outside diameter 0.58mm/1.0mm (1B100F-4 WPI) using a PUL-1000 micropipette puller (WPI) at the following settings - Heat Index: 650; Force: 250g; Distance: 8.00mm; Delay: 0s to yield needles with long graduated tapers. Tip diameter was established by placing the needle on a micrometer slide under a stereomicroscope (WPI Model No. 504941) and cutting along the graduated taper at the desired diameter, using a pair of stainless steel Vannas scissors with 5mm blades (WPI, 14003) (Figure 3.3). Our most successful tip diameter proved to be between 15 and 20µm, as cells could be consistently ejected without clogging, once practical issues (discussed later) were accounted for.

Optimisation of Injection site: Conventionally, intravenous injections into zebrafish larvae are performed from 1-3dpf, in either the caudal vein or the Duct of Cuvier (Veinotte, Dellaire and Berman, 2014) (Duggan and Mostowy, 2018). Experimentally we achieved the highest rates of successful injections (i.e., resulted in the introduction of an adequate number of cells into circulation) in 2dpf zebrafish larvae, in the Duct of Cuvier. At 2dpf, this venous sinus is most apparent (as compared to other timepoints in development) under a stereo light microscope and the epidermis is still sufficiently thin for smooth entry of the needle into the duct without damaging the needle or blunting it too fast. Duct of Cuvier injections at 3dpf were also investigated for our purposes but proved less efficient due to epidermal thickening and anatomical remodeling making the duct more difficult to visualise.

We also investigated the caudal vein as potential injection site, evaluating injections at 1 and 3dpf. The former proved difficult as the vein was insufficiently wide to allow for injections with our relatively large (required) tip diameters. The aforementioned injection sites are represented visually in Figure 3.4.

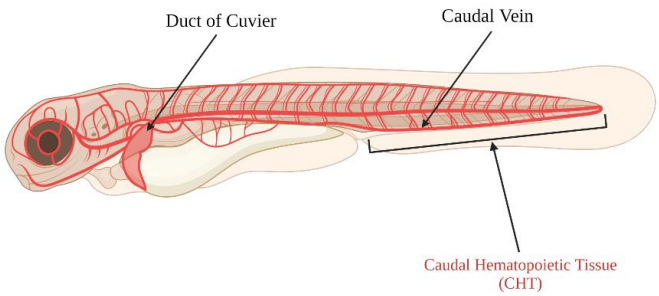


Figure 3.4. Diagram demonstrating investigated microinjection sites

3.4 Experiment 1: Injection Volume Optimisation

3.4.1 Purpose

In literature, injection volumes administered into the circulation of 2dpf larvae frequently range from below 1nl to above 20nl (Cianciolo Cosentino, et al., 2010) (Fehr et al., 2016) (Osmani et al., 2019) (Paul et al., 2019) (Zhu et al., 2018) (Lan et al., 2019). This variability is primarily due to lack of convention, as similar studies investigating metastasis of intravenously microinjected cancer cells vary substantially in injection volumes, with some reportedly injecting volumes of 60nl (Berens, Sharif, Wellstein and Glasgow, 2016) (Teng et al., 2013). Due to this large reported range of variability in injection volumes, we sought to optimise injection volumes for our purposes. As our aim was to inject functional human macrophages - which rely on adequate circulation for chemotaxis - into zebrafish larval circulation, and, as the upper limit of tolerable intravenous injection volumes in 2dpf zebrafish larvae has not yet been established, we injected a range of volumes and assessed parameters pertaining to cardiovascular function: heart rate, expressed in beats per minute (BPM), and swim bladder development, as well as qualitative morphology (pericardial oedema, yolk sac edema). Zebrafish larvae with significantly impaired circulation/heart failure, fail to inflate their swimbladders within 5dpf (Winata et al., 2010) (Yue, Peterson and Heideman, 2015). Because macrophages are relatively large cells, a cell suspension on the upper limits of tolerable injection volume was deemed most desirable for our purposes.

3.4.2 Methods

Healthy, hatched 2dpf zebrafish larvae were selected, placed individually into wells of a 24 well culture plate, and anaesthetised as described in 3.3 (General Anesthesia Protocol) for intravenous injections of 0.05% methylene blue solution of 5; 10; 15; and 20nl (n=18 larvae per group), as previously described in section 3. 3 (General Zebrafish Microinjection Procedure). This

solution was selected due to its stark colour, allowing visualisation of successful injections. Methylene blue is a commonly used anti-fungal agent in zebrafish E3 and has been shown previously by our group not to cause adverse effects in zebrafish larvae immersed in the concentrations of this chemical that was used in this study (J Conradie, BSc Hons thesis, 2020). The larvae were then randomly divided into heart rate monitoring groups (n=8 per dose volume) and morphological assessment groups (n=10 per dose volume) and monitored once a day over 3 days (up to 5dpf). Daily images were taken using a stereomicroscope (WPI Model No. 504941).

3.4.3 Results

A two-way factorial ANOVA comparing the effects of both age and dose volume shows that that there is no interaction between these variables. One-way ANOVAs (Figure 3.5) of each independent variable show that both dose volume (Figure 3.5 A; $p<0001$) inverse-proportionally, and age (Figure 3.5 B; $p<0.01$) proportionally, have significant effects on heart rate. Bonferroni post-hoc tests indicate that the statistical differences in heart rate between injection volume groups lies between the 20nl injection group and the 0nl ($p<00001$), 5nl ($p<0.00001$), and 10nl ($p<0001$) injection groups but not the 15nl ($p>0.9999$) injection group.

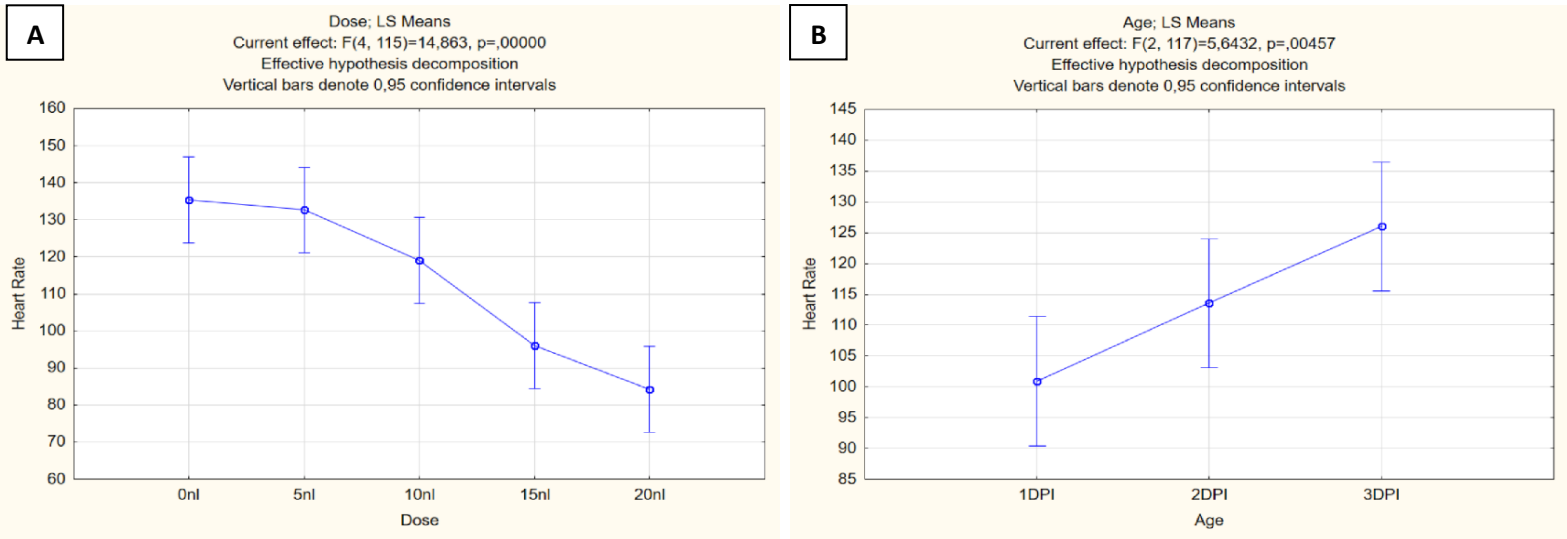


Figure 3.5: ANOVA comparing the effects of A: methylene blue dose volume; and B: age on the heart rate of injected zebrafish larvae*. Total zebrafish sample of n=40 was used for all statistical analyses.*

From the data presented in Table 3.3 and pictured in Figure 3.6, it is clear that cardiovascular abnormalities occur in a dose-dependent manner, with 5nl injections incurring the lowest number of larvae with morphological cardiovascular defects and 20nl being most harmful to larval morphology.

dpi	Group	Cardiac Edema	Yolk Edema	Bent Tail	Uninflated Swim Bladder
1	5nl	0	0	0	
	10nl	0	0	0	
	15nl	4	4	0	
	20nl	4	1	0	
2	5nl	1	1	0	
	10nl	1	1	0	
	15nl	3	3	0	
	20nl	8	7	0	
3	5nl	0	2	1	0
	10nl	2	3	2	1
	15nl	3	4	2	3
	20nl	5	5	4	5

Table 3.3: Recorded morphological defects of larvae with increasing intravenous methylene blue dose volume (n=10 per dose volume)*. The swim bladder typically inflates at the larval age of 5dpf (3*dpi *in this case), hence an uninflated swim bladder at 1 and 2*dpi *is not considered a morphological defect.*

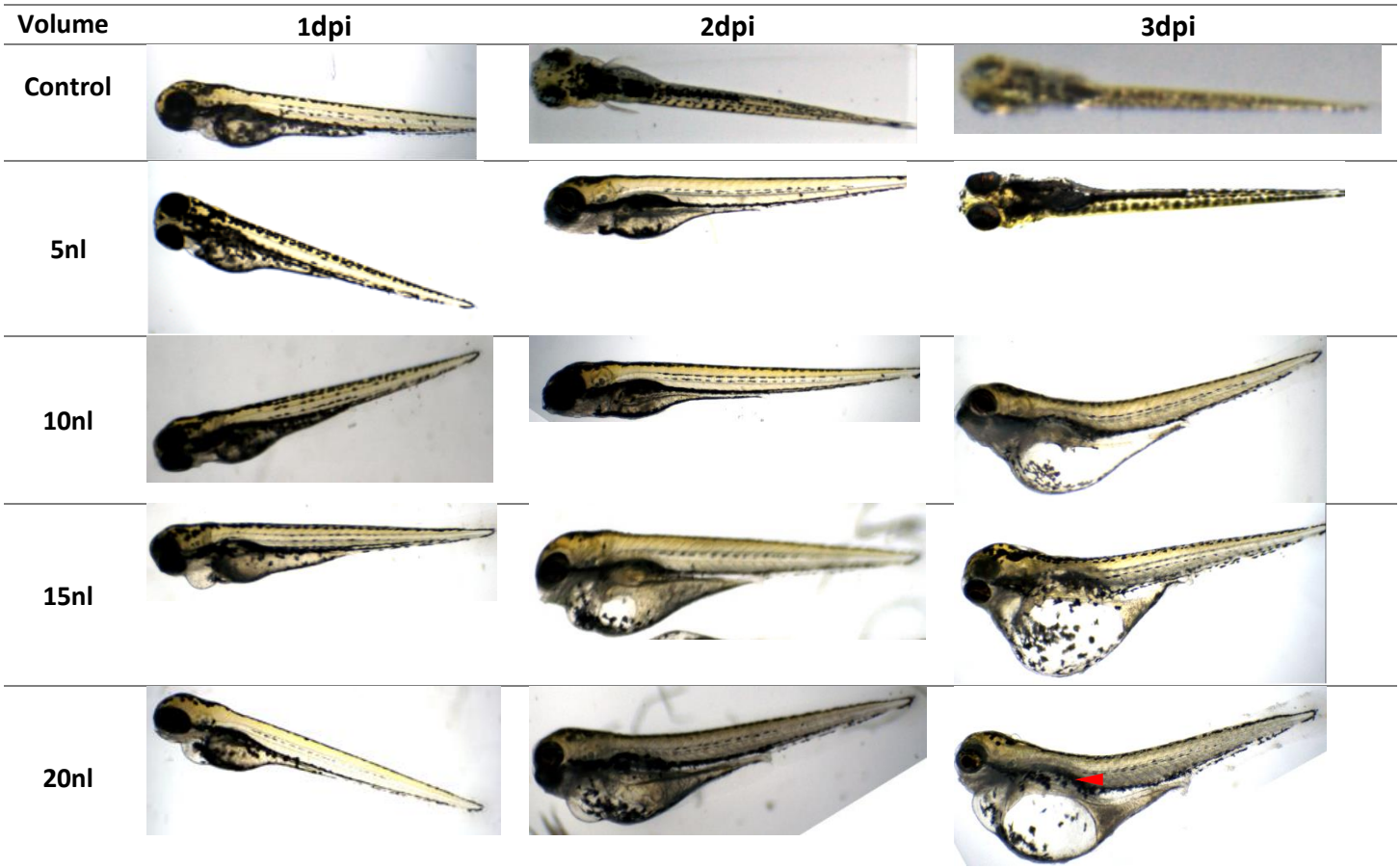


Figure 3.6: Morphological effects of increasing injection dose volume of methylene blue into zebrafish larval circulation over 3dpi***.*** *One larva per each dose volume group (n=10) is pictured, not necessarily representative of the group (refer to table 3.3 for distribution of abnormalities per*

group). As the healthy larval swimbladders inflated between 2-3 dpi*, the natural orientation of healthy larvae in the water became upright (control at 2*dpi *and 3*dpi *and 5nl at 3*dpi*). Red arrow indicates example of an uninflated swimbladder. As the swimbladder develops and inflates, larvae rotate to be perpendicular to the surface (e.g. Control 2*dpi*, 3*dpi*, and 5nl 3*dpi*).*

3.4.4 Interpretation

The ultimate aim with volume optimisation for cell-based injections for our purposes, is to introduce the largest volume with the least associated cardiovascular risk. Data suggest the use of 10nl as optimal injection volume in subsequent experiments, with at least n=10 larvae per experiment to mitigate the low cardiovascular risk associated with this volume, from being a significant confounding factor. Although 5nl is the most conservative volume in terms of associated morphological defects in larvae, this volume would necessitate a cell suspension of high density to allow for the microinjection of an adequate number of cells into larval circulation, significantly increasing the risk of needle clogging/inconsistent number of injected cells. Of further interest, the density used in prior work in literature ($1x10^7$ cells/ml) (Paul et al., 2019), to inject human macrophages into zebrafish larval circulation, proved adequate.

One apparent complexity of cell-based microinjections, as compared to more conventional microinjections, which was a challenge in the current experiment, was the effect of gravity acting on the cells in suspension over time; the longer the experiment (i.e., the greater the number of larvae to inject), the greater the risk of the cells settling towards the tip and adhering to the borosilicate needle due to its approximately 45-degree downward angle. This can result in either fewer cells than anticipated being ejected with PBS or too many cells dislodging with a pulse at once. This was mitigated by rotating the needle approximately 90 degrees and recalibrating injection volume between every injection.

3.5 Experiment 2: Verification of *in vivo* Cell Tracking

3.5.1 Purpose

Prior to investigating the *in vivo* behaviour of THP-1-derived macrophages in zebrafish larvae, we aimed to verify whether intravenous cell injections were feasible with our optimised microinjection protocols, and whether the dye concentration used allowed for long term visualisation of the cells without imparing cellular viability. However, given the relatively longer protocol required for preparation of macropohages, we opted to use undifferentiated THP-1 monocytes for this experiment. To achieve these aims, undifferentiated THP-1 cells were labeled and injected into the circulation of 2dpf zebrafish larvae via the Duct of Cuvier. Tail fin transections were subsequently performed on the larvae to determine the effect of acute inflammation on the undifferentiated cells.

3.5.2 Methods

THP-1 Cell Maintenance: Human monocytic (THP-1) cells were thawed and suspended at an initial density of 2-3x10^5 cells/ml in pre-warmed complete THP-1 standard growth medium (SGM; 10% FBS v/v + 1% v/v PenStrep + 25 mM HEPES in RPMI 1640 (with L-glutamine and Phenol Red)) in a T-75 culture flask and incubated at 37.5°C. Cells were refreshed every 2-3 days by either adding new SGM to the cell suspension (upon the first refreshing) or – to prevent cells from reaching a density of 1x10^6 cells/ml or greater - by centrifuging the cells at 130*g* for 7 minutes and resuspending the pellet in SGM. The THP-1 cells in a flask were generally split when a density of 8x10^5 cells/ml was reached.

Cell Labelling: Cell suspensions were centrifuged at 130*g* for 7 minutes. The resultant supernatant was aspirated from the pellet and replaced with pre-warmed FBS-free SGM containing 10uM CellTracker Violet fluorescent dye (Thermo Fisher, Massachusetts, USA, cat # C10094). This suspension was incubated for 30-45 minutes, spun down at 130*g* for 7 minutes, supernatant aspirated off and pellet resuspended in pre-warmed PBS to yield a density of 1x10^7 cells/ml (Paul et al., 2019). At this point, the cell suspension was ready to be injected into zebrafish.

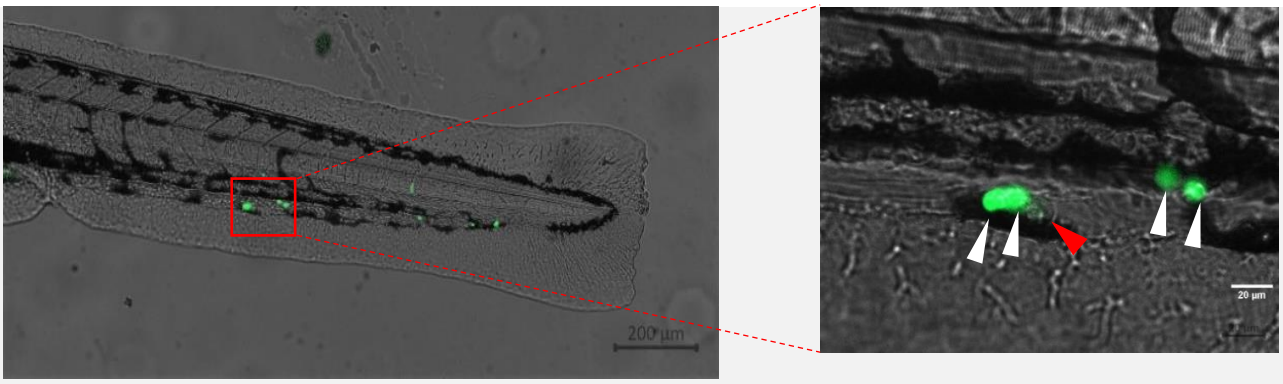
Healthy, hatched 2dpf zebrafish larvae (n=10) were selected to be anesthetised, mounted, and microinjected with undifferentiated THP-1 cells as described in section 3.3. Tail fin transections were then performed on the injected larvae as described in section 3.3. Images were captured using a Zeiss Axio Observer 7 inverted fluorescent microscope at 5x, 20x, and 40x magnification.

3.5.3 Results

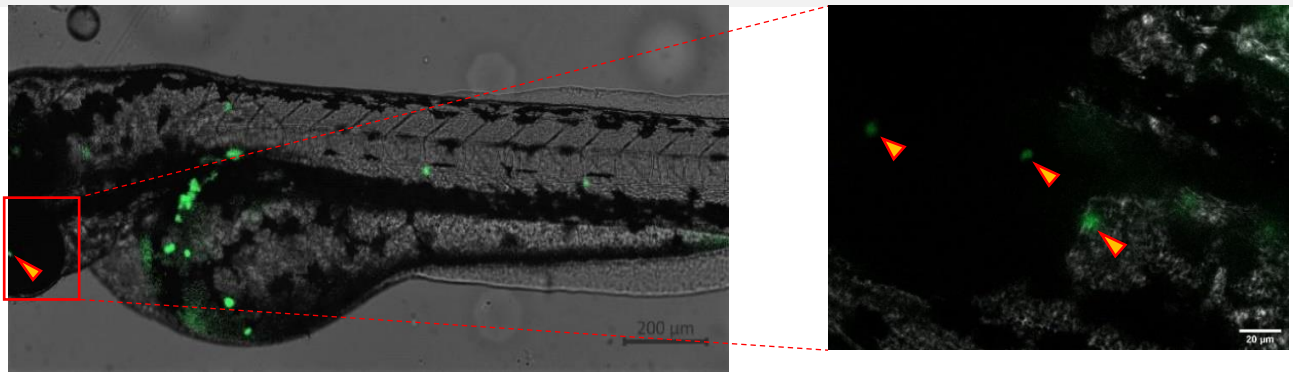
Undifferentiated THP-1 cells appeared to adhere to vascular endothelium, specifically in the distal tail and rapidly proliferate, seemingly significantly increasing in number within 24 hours post injection (Figure 3.7). The fluorescent stain (CellTracker Violet) retained sufficient intensity after 24 hours for the unambiguous identification of the human cells *in vivo* (Figure 3.7; monocytes are indicated with white arrows). Undifferentiated THP-1 cells do not undergo differentiation into macrophage-like cells upon exposure to traditional DAMPs and PAMP signals, instead relying on *in vitro* stimulation with PMA. Hence, these cells likely remained undifferentiated for the duration of the experiment. Unstained polymorphonuclear cells (indicated with red arrow in Figure 3.7) of similar size were visualised adjacent to injected stained cells immediately post transection. We speculate these unstained cells may be endogenous zebrafish neutrophils similarly trapped in the CHT. Specific staining to confirm this was not performed in this experiment and would be beneficial to perform in future studies.

Immediate (5x) **Immediate (40x)**

Transected 1

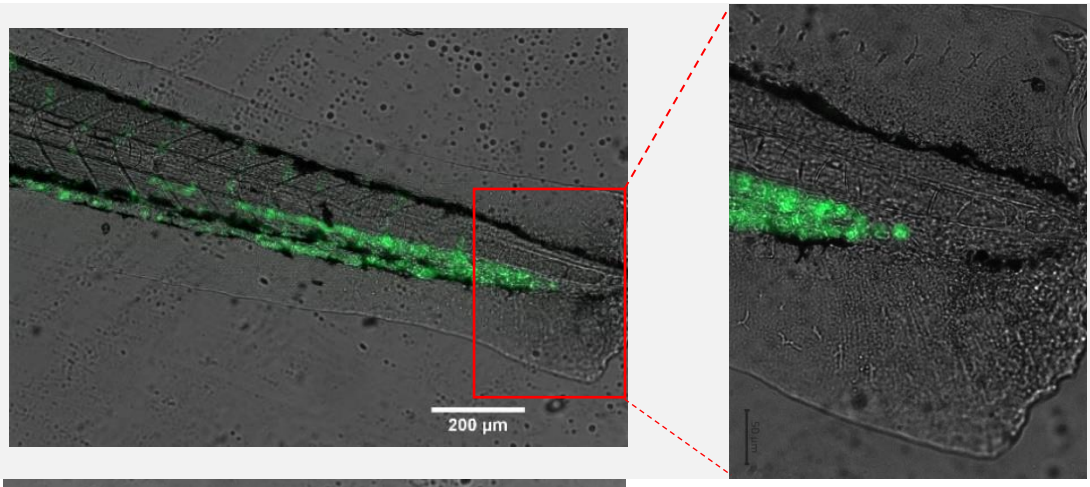


Transected 2



24HPW (5x) **24HPW (20x)**

Transected 1



Transected 2

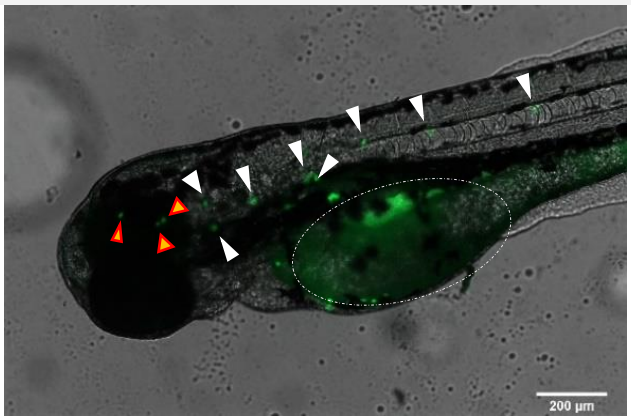


Figure 3.7: Undifferentiated THP-1 cells rapidly expand, both systemically (in circulation) (Transected 1) and locally (Transected 2) – although to a lesser extent – within 24 hours post tail fin transection. *Red boxes indicate the area that is enlarged in following image at the same timepoint (e.g., red box in Transected 1, immediate (5x) is area shown under 40x magnification in Transected 1, immediate (40x). Red arrow indicates unstained polymorphonuclear cell adjacent to similarly sized stained cells. We suspect this to be an endogenous zebrafish neutrophil trapped in the CHT niche and indicates that the same is happening to the injected human THP-1 cells. The area enlarged in Transected 2, 40x magnification shows THP-1 cells (orange arrows) unexpectedly associating with the eye of the zebrafish larvae and surviving there for at least 24 hours (Transected 2, 24HPW, 5x). White dotted circle indicates area of zebrafish larval autofluorescence (yolk sac). Monocytes not associating in the eye of the Transected 2 larva (white arrows, 24HPW) were found to disperse is an apparently random pattern in the larva.*

As indicated in Transected 2, THP-1 monocytes were also found to migrate into the small blood vessels within the eye of the larvae, and survive there for at least 24HPW. Of interest, the monocytes were not found to migrate out of circulation, evidenced in Transected 1 at 24HPW. They were, however, found in the most distal portion of circulation, nearest to the wound.

3.5.4 Interpretation

We showed that our microinjection protocols were sufficient in introducing cells into the circulation of 2dpf zebrafish larvae. The injected cells retained sufficient stain intensity for unambiguous identification of our injected cells over the course of 24 hours, despite the rapid cellular division. Taken together, the cells retained sufficient dye intensity without incurring of loss of cellular viability, indicating an appropriate concentration of CellTracker Violet being used. Interestingly, the fact that monocytes were found at the most distal end of circulation, closest to the wound, without leaving circulation (Figure 3.8, Transected 1, 24HPW) could indicate that they have the capacity for migration towards an inflammatory site in circulation, but not transendothelial migration. It is also possible that the rapid proliferation of these cells, indicating their activation in response to zebrafish inflammation, was more pronounced closest to the site of inflammation due to exposure of DAMPs and PAMPs, thus migration towards the inflammatory site is not the only possible explanation. Activation of the human THP-1 cells upon exposure to zebrafish inflammatory cues is a promising early result and differentiation of these cells into macrophages may enable their ability to undergo diapedesis towards the inflamed tissue.

3.6 Experiment 3: Survival and Migratory Capacity: M1 THP-1-Derived Macrophages

3.6.1 Purpose

As postulated in literature and confirmed by our group, M1 macrophages are the most suited macrophage subtype to the role of drug delivery vehicles due to their responsiveness to early inflammatory cues and ability to efficiently undergo transendothelial migration after being laden

with cargo. We therefore set out to assess whether THP-1-derived M1 macrophages would behave similarly in zebrafish larvae, by migrating into inflamed tissue.

3.6.2 Methods

THP-1 cells were cultured and maintained as described in 3.5.2.

THP-1 Differentiation: A calculated volume of cell suspension was removed from the flask, centrifuged at 130*g* for 7 minutes, and resuspended in a calculated volume of fresh, pre-warmed differentiation media (dSGM; SGM + 25nM phorbol 12-myristate 13-acetate) to yield a suspension density of $5x10^5$ cells/ml. In each well of 6-well plates, 3ml cell suspension in dSGM was added and the plates were incubated at 37.5°C for 48 hours without refreshing media again.

THP-1-Derived Macrophage Polarization and Lifting: After 48 hours of incubation, the dSGM was aspirated off the cells and replaced with 3ml pre-warmed polarization SGM (pSGM; SGM + 10ng/ml LPS) per well. The plate was incubated for a further 4 hours at 37.5°C. The pSGM was replaced with fresh, pre-warmed SGM. The SGM was subsequently aspirated from the cells and each well was washed twice with 1ml PBS per wash before being replaced with 1ml TryPLE Express (Thermo Fisher) and incubated at 37.5°C for 30 minutes. The cells were gently and thoroughly scraped from the growth surface of the wells using a sterile cell scraper. The TryPLE Express was neutralised by the addition of 2ml pre-warmed SGM in each well and the combined cell suspensions were added to a 15ml centrifuge tube. The cell suspension was triturated using a pipette for a cell count employing a Fuchs-Rosenthal hemocytometer under a Zeiss Primovert inverted light microscope.

The cells were then labeled and prepared for microinjection as described in section 3.5.2. Healthy, hatched 2dpf zebrafish larvae (n=10) were selected, anesthetised, and prepared for microinjection as already described in section 3.3. Terminally differentiated and M1 polarised THP-1 cells were injected into circulation of 10 larvae which were randomly divided into two groups of n=5. One group was subjected to tail transections immediately post injection. After 1 hour, successfully injected larvae were visualised under a Zeiss Axio Observer 7 inverted fluorescent microscope at 5x, 20x, and 40x magnification. Larvae potentially displaying injected macrophages were imaged again 24 hours later.

3.6.3 Results

Macrophages could be located in 4 of the 10 larvae which were then imaged immediately and 24 hours later. In most cases, the macrophages were found localised in the caudal vasculature of the larvae. No macrophages were located in any larvae after 24 hours, suggesting a potential loss in viability over time, although no assays were performed to determine whether apoptosis had occurred. Representative images are provided in Figure 3.8.

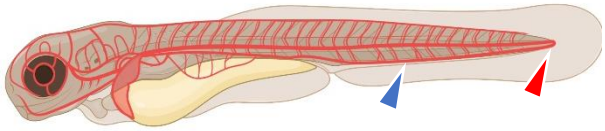
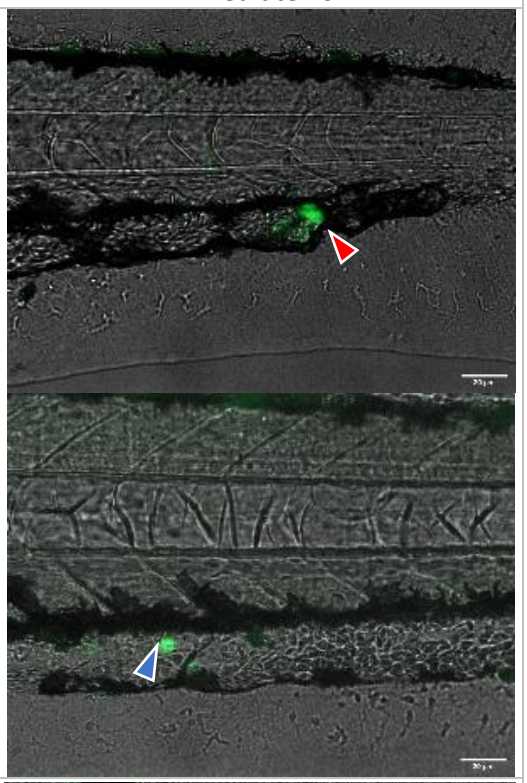
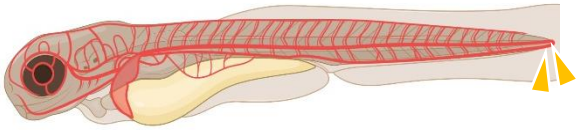
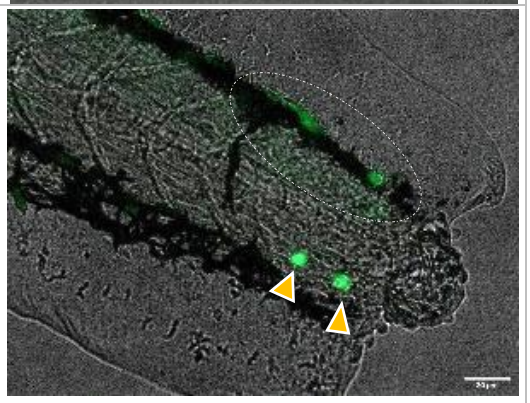
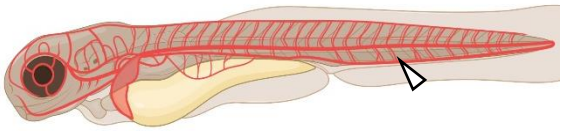
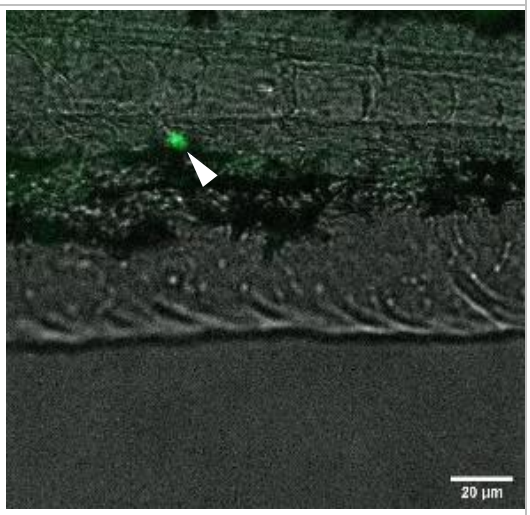
Sample	Location	Immediate 20x
Control 1		
Transected 1		
Transected 2		

Figure 3.8: M1 polarised macrophages in zebrafish larval circulation immediately post transection and 24 hours post injection. *As convention, all larvae are pictured with head to the*

left and tail to the right, dorsal side up. Illustrations of zebrafish (with visible blood vessels) are provided to demonstrate the anatomical positioning of the injected cells shown in the immediate 20x magnification images. Triangular colour-matched arrows indicate the same macrophages across images. Dotted white circle indicates areas of green autofluorescence as exhibited by the zebrafish pigment cells.

3.6.4 Interpretation

To our knowledge, this experiment was the first recorded instance of THP-1-derived macrophages being introduced into the circulation of zebrafish larvae and the first to report a rapid loss in human macrophage viability in zebrafish larvae. The cause of the loss in viability was not determined in this experiment and may be as a result of numerous factors including mechanical damage caused by injection or possibly a culturing intervention such as differentiation or polarization rendering these cells more fragile in the zebrafish microenvironment. Repeating this experiment with a larger zebrafish cohort and unpolarized, naive THP-1-derived macrophages is necessary to elucidate whether THP-1-derived cells are suitable for drug delivery research in larval zebrafish models.

3.7 Experiment 4: Survival and Migration Capacity: Naïve THP-1-derived Macrophages

3.7.1 Purpose

To repeat the previous experiment with a greater larval sample-size using undifferentiated, naïve THP-1-derived macrophages and determine whether these cells possess adequate migrational and survival capacity within the circulation of zebrafish larvae to feasibly model drug carriers in our proposed drug delivery system. This is also a necessary step to determine if/which cell-culturing interventions occurring between the undifferentiated THP-1 monocyte-based experiment (Experiment 2) and differentiated and polarized THP-1-derived macrophage experiment (Experiment 3) resulted in loss of long-term viability of the transplanted cells in the zebrafish larvae.

3.7.2 Methods

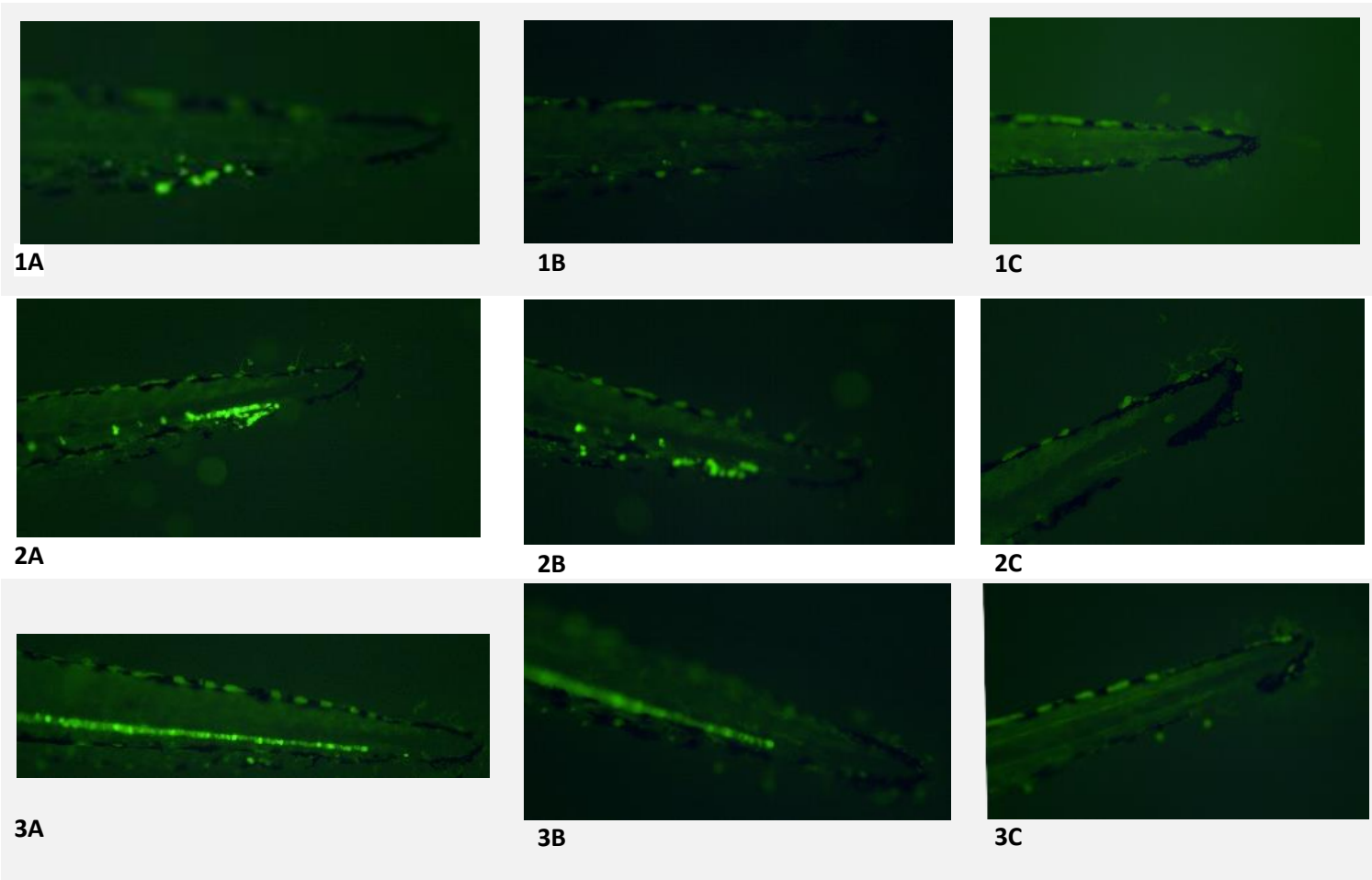
THP-1 cells were maintained, differentiated and lifted as described in section 3.6.2, before being fluorescently labeled as described in section 3.5.2. Healthy, hatched 2dpf zebrafish larvae (n=20) were selected, anaethetised, and prepared for microinjection as described in section 3.3. Larvae were injected with naïve THP-1-derived macrophages. At 1 hour post injection, larvae were randomly divided into two groups, with one group being subjected to tail transected (n=10) while one group remained uninjured controls (n=10). Successfully injected zebrafish larvae (n=7) were imaged 1 hour-(prior to transections), 3 hours-, 24 hours-, and 48 hours post injection on an ECLIPSE Ti2 inverted microscope (Nikon, Tokyo, Japan). Practically, the identification of the macrophages using this microscope posed a challenge for the researchers, as zebrafish larval

pigment cells autofluoresce strongly in the green spectrum, similarly to the CellTracker Violet stain used in the experiment. Delineation between endogenous pigment cells and exogenous primary macrophages involved identification of movement (macrophages are significantly more mobile than pigment cells), positioning (macrophages are located within or adjacent to blood vessels), shape (macrophages are relatively deformable cells), and comparison to bright field images (pigment cells appear black and opaque under bright field, whereas macrophages are more translucent).

3.7.3 Results

Due to the larger number of larvae per group to be injected in this experiment, the effects of gravity acting on the cells in suspension over time was most evident here. This resulted in a discrepancy in the number of cells injected, yielding some injections of higher densities (>100 cells). Images in Figure 10 are approximately arranged in order of increasing cell number (per group) for ease of comparison for the reader. Although discrepancy of cell numbers was a potential confounder in our interpretation of data, it at the same time allowed us to investigate the effects of cell density on cell survival and behaviour *in vivo,* albeit with low statistical power. Furthermore, due to reagent limitations, more specific cell labelling was not possible, so that absolute cell counts were not feasible. More comprehensive labelling of macrophages in future will enable more comprehensive assessment of cell numbers and ultimate fate of injected cells. Nevertheless, a number of quantitative observations were possible. To mitigate the discrepancy in number of injected cells in future experiments, the cell suspension was freshly aspirated into the capillary tubes before every injection. Prior to tail transections – at 1 hour after injection, most identified macrophages were observed to be associated with the zebrafish tail vasculature, in what is considered to be the CHT region thereof, despite our inability to distinguish between luminal and abluminal space at this magnification and resolution (Figure 3.9). This is consistent with our previous data (Figure 3.7, Figure 3.8) and literature (Paul et al., 2019), and likely shows compatibility and recognition between human macrophages and zebrafish endothelial cells, as the exogenous cells are sequestered from circulation in a manner similar to that reported for endogenous zebrafish HSCs (macrophage precursors) (Tamplin et al., 2015). At 1 hour post transection (3hpi in transected group), a relative decrease in macrophage number in the CHT area is observed compared to the controls and a greater proportion of these cells are found in circulation. The successfully injected zebrafish larvae (n=7) were monitored and imaged over 48 hours from the time of injection. The images at 48hpi revealed the complete absence of macrophages across all injected larvae, independent of group. These images are not reported in Figure 3.9 as they provide no further information after the absence of macrophages demonstrated in the 24hpi images. Although some THP-1-derived macrophages were observed to enter circulation shortly following fin transections (white arrows in Figure 3.9, 3hpi), none of the injected macrophages were observed to have migrated into the inflamed tissue within 48hpi. Given that the injected macrophage distribution along the tail vasculature also did not shift noticeably towards the site of inflammation 2 hours after tail transections were performed

(Figure 3.9, 3hpi) in the transection group compared to the uninjured controls, we infer that abluminal crawling of these cells towards the injection site, as is described for native zebrafish macrophages in response to tissue damage in literature, occurred minimally, if at all. Native zebrafish leukocytes were not visualized during this experiment. From 24-48 hours post injection (Figure 3.9, 24hpi), THP-1-derived macrophages could not be located in any of the injected larvae, irrespective of the number of cells injected.



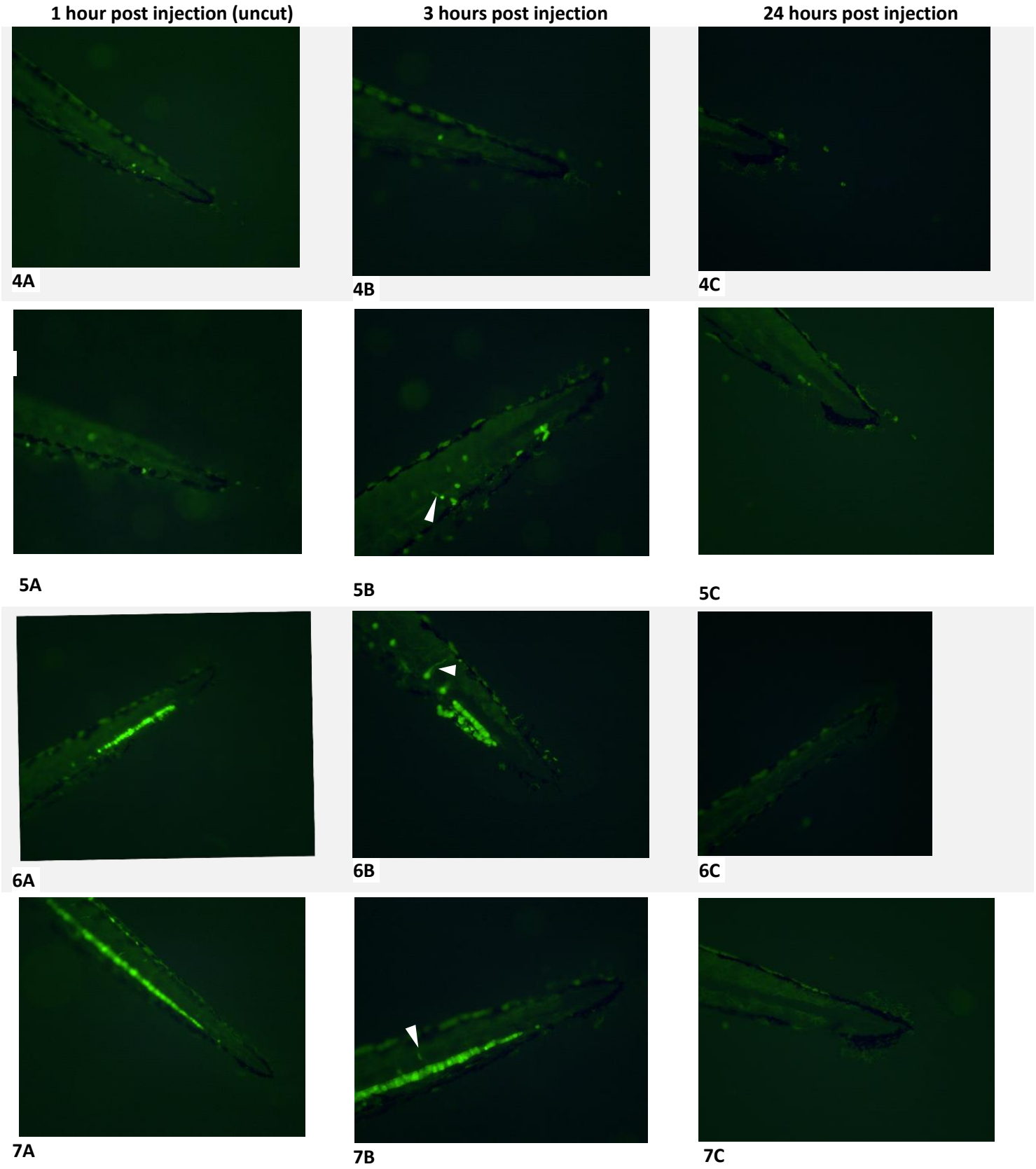


Figure 3.9: Unpolarised THP-1-derived macrophage behaviour in 2dpf zebrafish larvae over 24 hours post injection, in uninjured controls (1A-3C) vs tail transected larvae (4A-7C). *As*

convention, all larvae are pictured head to tail dorsal side facing up. White arrowheads indicate motion blur as a result of capturing images of fast-moving macrophages in transit.

3.7.4 Interpretation

This experiment successfully demonstrated that neither cell density nor LPS-dependent polarization of the THP-1-derived macrophages likely accounted for the poor survivability of THP-1-derived macrophages in zebrafish larvae.

Given the lack of evidence generated in this experiment demonstrating that THP-1-derived macrophages possess the capacity to crawl towards inflamed zebrafish tissue or undergo transendothelial migration, together with the data generated from this and the previous experiment, both demonstrating an *in vivo* loss in THP-1-derived macrophage viability in the short term, it is evident that THP-1-derived macrophages are not adequate candidates with which to investigate cell-based drug delivery in zebrafish larvae. These experiments are the first recorded incidence of viable THP-1 cells, in undifferentiated, differentiated, and M1 polarised state being transplanted into zebrafish. Given the reported survivability of human primary macrophages in zebrafish larvae, our experiments demonstrate clear differences in behavior between THP-1 macrophages and primary human macrophages in zebrafish larvae as reported in literature. We suspect the immortilisation of the THP-1 cell line may have resulted in an alteration of migratory/adhesion proteins and may be the underlying cause of the discrepancy in behaviour between human primary macrophages in zebrafish larvae (as described in literature) and the THP-1-derived macrophages in zebrafish larvae (as described here). Hence the decision was made that future experiments should preferentially make use of human primary macrophages.

3.8 Experiment 5: Survival and Migration Capacity: Primary Human M1 Macrophages

3.8.1 Introduction

This experiment essentially repeated the previous experiment, but utilised labelled primary human M1 polarised macrophages as opposed to THP-1-derived macrophage cells, to evaluate whether primary human macrophages may be better candidates with which to develop cell-based drug delivery systems in zebrafish.

3.8.2 Methods

Monocyte isolation from whole blood: Whole blood was donated by a normally healthy individual and immediately transferred from 4ml EDTA vacutainers into sterile 15ml centrifuge tubes containing room temperature 2ml Ficoll/Histopaque solution (1.077 g/ml) each, taking care to do so slowly to prevent mixing of the layers. The tubes were then centrifuged at 400*g* for 30 minutes at room temperature before pipetting 1ml 1mM PBS-EDTA (1xPBS with 0.5M EDTA) into each tube. After each centrifugation, the white ring layer of peripheral blood mononuclear cells

(PBMC) was transferred into a new sterile 15ml centrifugation tube using a plastic Pasteur pipette and aspirated with PBS-EDTA until 7ml total volume was reached. The tubes were then centrifuged at 300*g* for 10 minutes at room temperature. The supernatant was aspirated from the pellet and the pellet was washed in equivalent volume of fresh PBS-EDTA. Because all of the available blood for the experiment was donated by a single donor, the pellets were pooled and resuspended in 6ml RPMI-1640 without phenol red, supplemented with 10% FBS. In a new 15ml centrifuge tube, 6.939ml room temperature Percoll solution was added with 0.561ml 10x FBS. 6.9ml of this solution was then mixed with 8.1ml RPMI-1640 with phenol red and 10% FBS to obtain a 46% iso-osmotic Percoll solution in a new 15ml centrifuge tube. Using a pipette, 7.3ml Percoll solution was added to a new 15ml centrifuge tube and the prepared 6ml cell suspension was gently layered on top, taking care not to mix the solutions. The tube was then centrifuged at 550*g* for 30 minutes at room temperature. The PBMC layer was again retrieved and transferred into a 15ml centrifuge tube with PBS-EDTA with total volume of 7ml and centrifuged at 400*g* for 10 minutes at room temperature. The supernatant was then aspirated from the pellet which was resuspended in complete monocyte media comprising RPMI 1640 with 10% Human Serum from AB patient (CAT# H4522 Sigma-Aldrich), and 1% Penicillin-Streptomycin (100 U/ml) for immediate seeding.

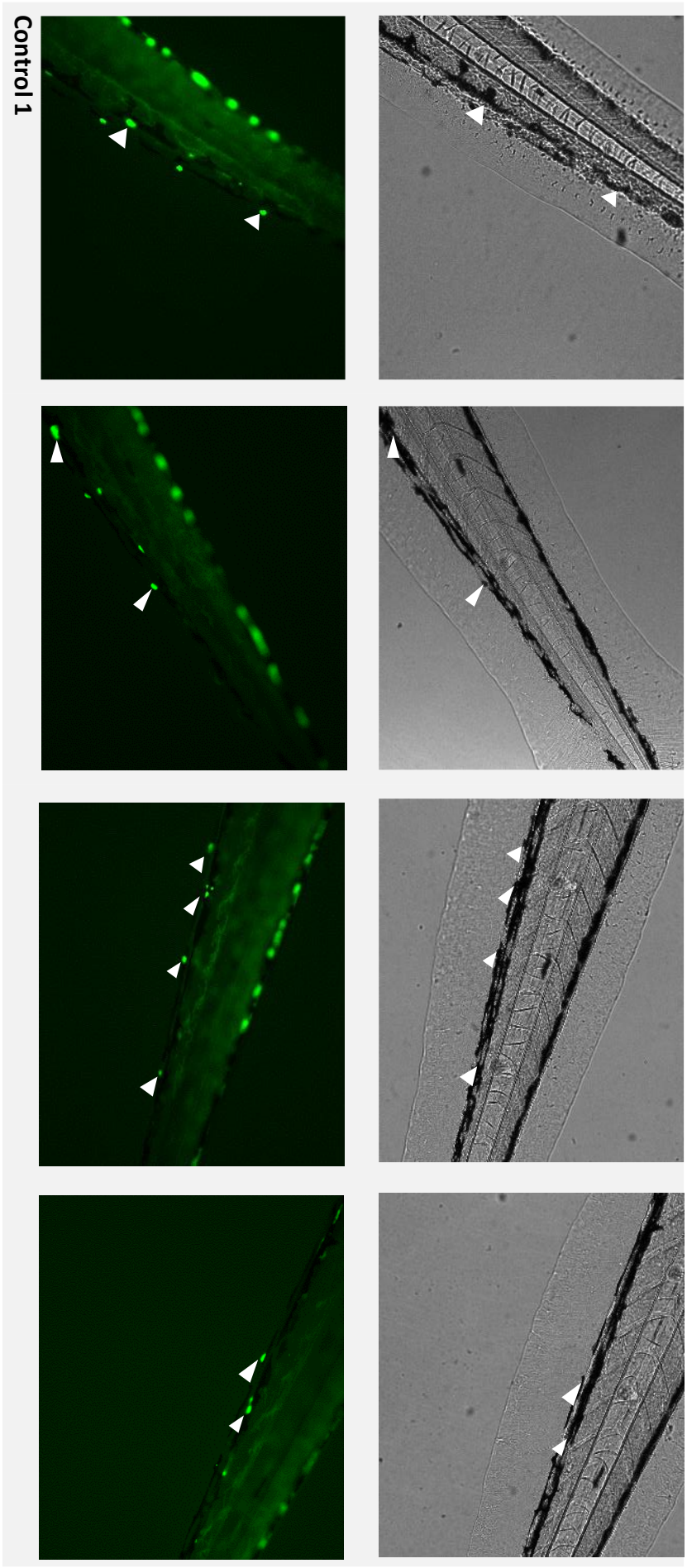
General primary monocyte cell culture: Primary human monocytes were seeded into wells of a polystyrene 12-well dish with complete monocyte media and immediately treated with 50ng/ml GM-CSF. The cells were left to adhere for 24 hours before the spent media was aspirated off and the cells washed with pre-warmed PBS. Fresh complete monocyte media supplemented with 50ng/ml GM-CSF was added to the cells. Media was refreshed every three days following additional PBS washes for a total of 5 days. For polarization, cells were treated with 50 ng/ml LPS and 20 ng/ml IFN-γ for 24h in the presence of GM-CSF, on day 6. After 24 hours, the polarisation media was aspirated off and cells washed once with pre-warmed PBS. Lifting of the cells was accomplished using 1ml Accutase per well and incubated at 37°C for 40 minutes. The growth surfaces of the cells were gently scraped for the complete lifting thereof. The cell suspensions were combined in a 15ml falcon tube and the Accutase was neutralised with the addition of pre-warmed complete monocyte media to the volume of 10ml. At this point, a cell count was performed. The suspension was then centrifuged for 10 minutes at 400*g* to pellet the cells. Spent media was aspirated off and the pellet resuspended in a calculated volume of pre-warmed serum-free media containing 10uM CellTracker Violet and incubated at 37°C for 30 minutes. Once 30 minutes had elapsed, the cells were centrifuged once more as described and resuspended in a pre-calculated volume of RPMI-1640 to yield a concentration of $1x10^7$ cells/ml to be injected.

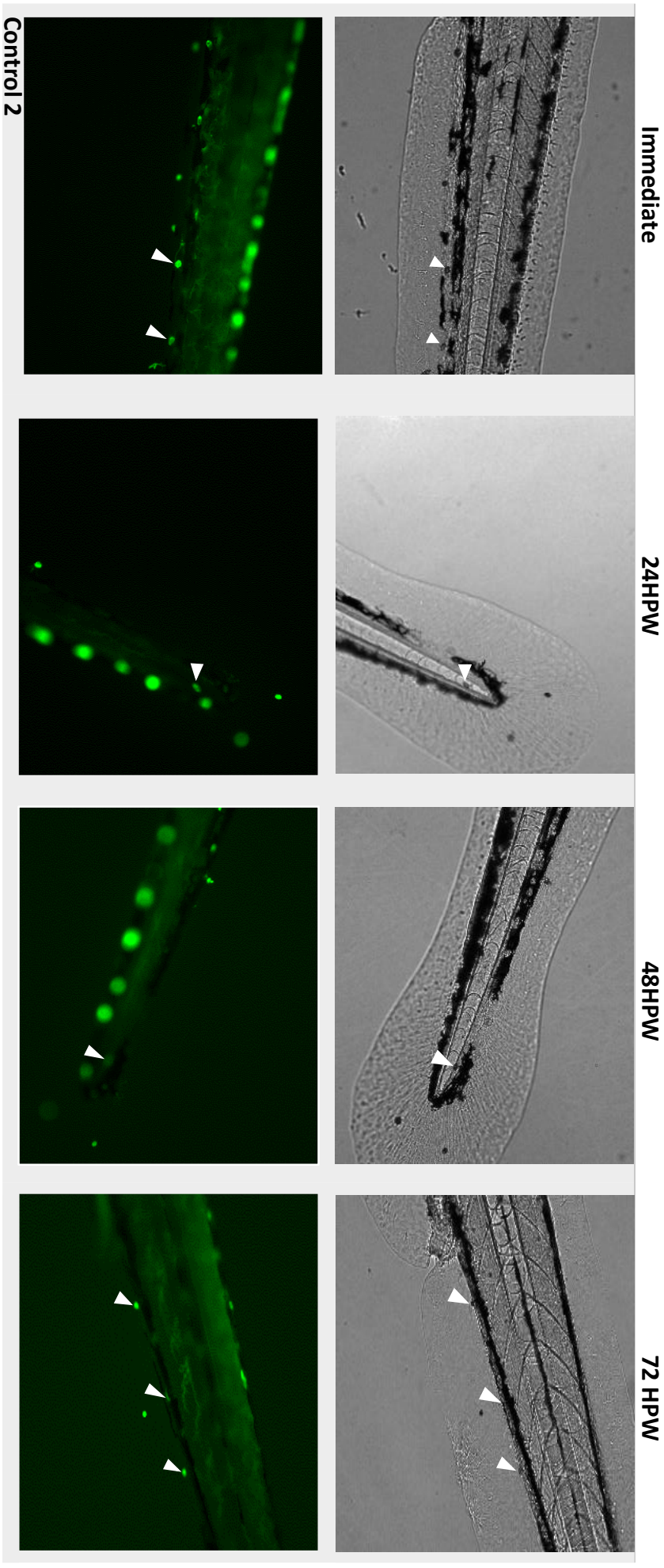
Healthy, hatched 2dpf zebrafish larvae (n=32) were selected, anaesthetized, and prepared for microinjection as described in 3.3. Successfully injected larvae (n=12) were randomly divided into tail transection (n=6) and uninjured control (n=6) groups and monitored over 3 days. Images were captured at 24-, 48-, and 72-hours post transection, using objectives of 4x and 10x magnification, with a Nikon ECLIPSE Ti2 fluorescent microscope.

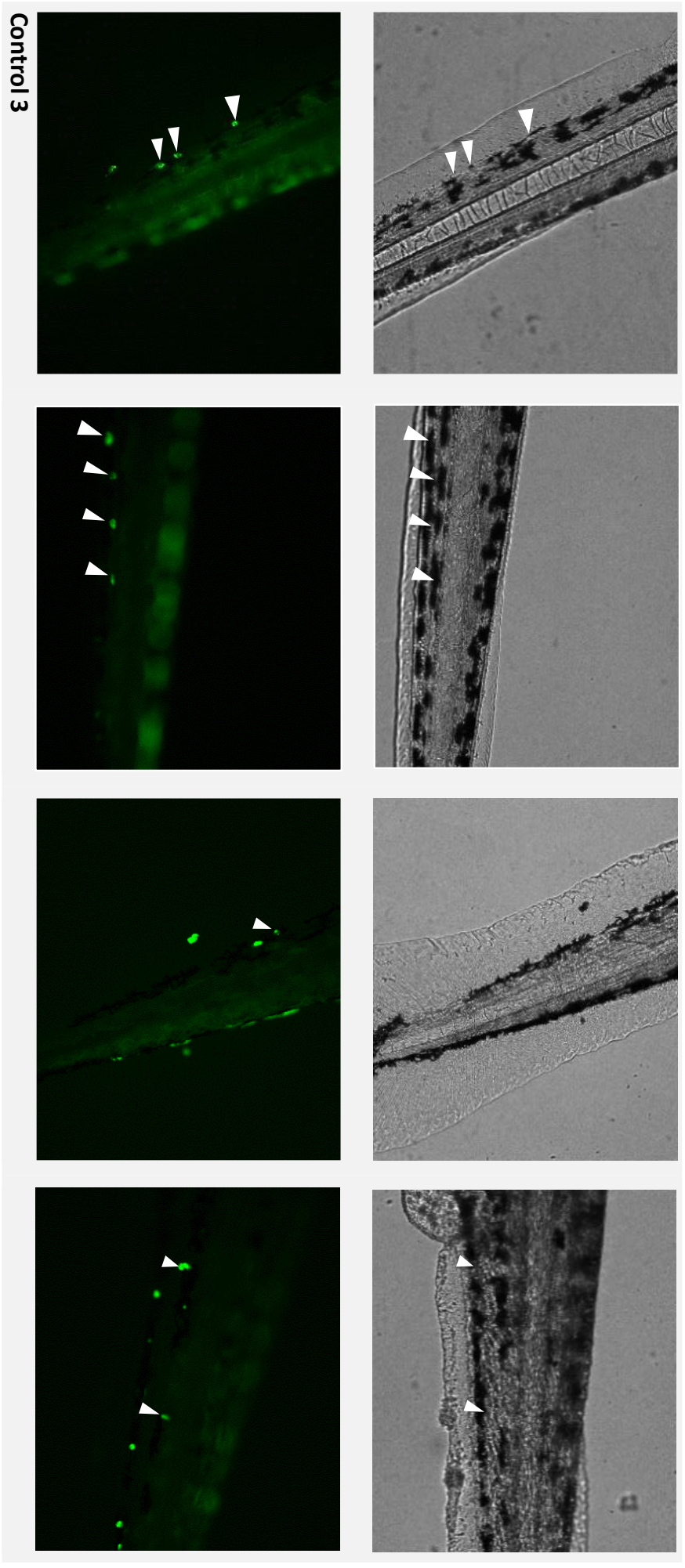
3.8.3 Results

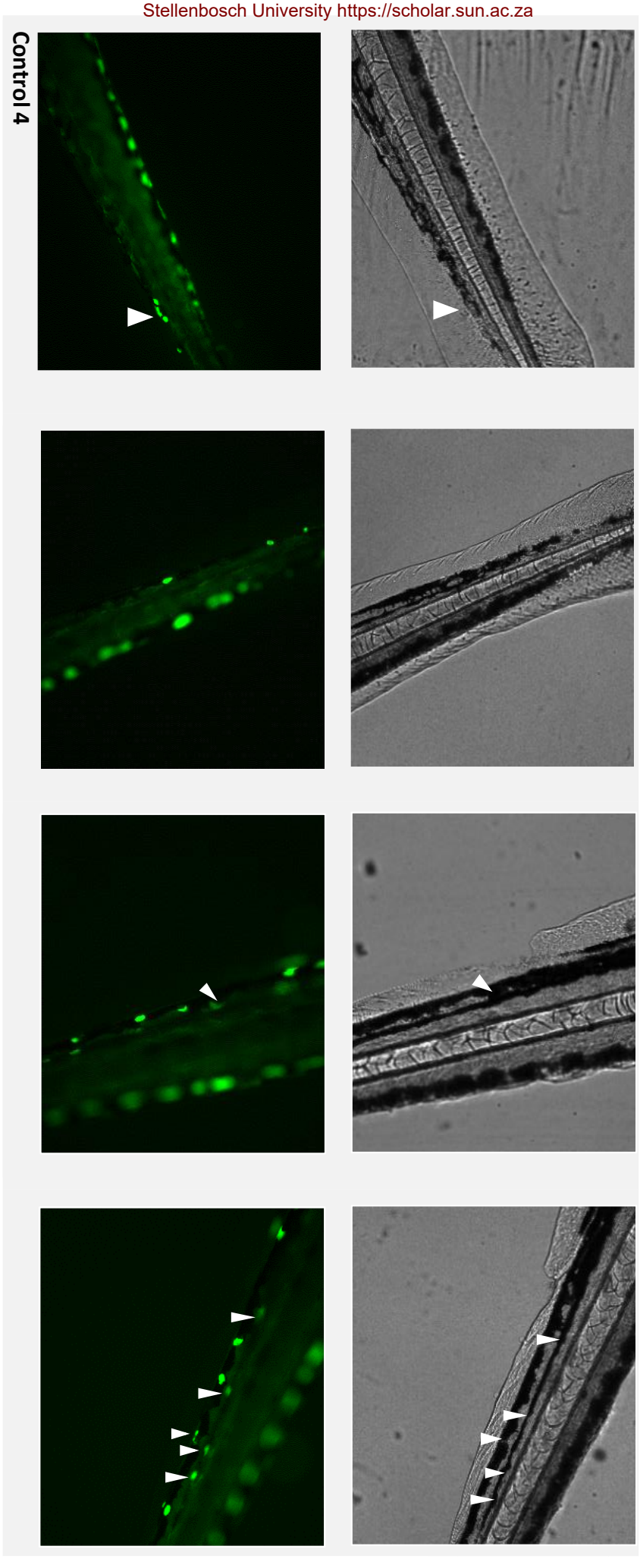
Consistent with what has been described in literature (Paul et al., 2019), the primary human macrophages survived up to the experimental endpoint at 72 hours post injection (Figure 3.10). This contrasts what was observed when using THP-1-derived macrophages in our previous experiments, as the THP-1-derived macrophages did not possess the capacity to survive beyond 24 hours post injection into the zebrafish. As with the THP-1-derived macrophages, the primary cells were predominantly found adhering to the CHT region of the caudal vein, from 1 hour post injection until the experimental endpoint (72HPW) in some cases (Figure 3.10). Fewer cells were observed to become free of the CHT after tail fin transection in this experiment than in the THP-1 experiments (Figure 3.10). This was only qualitatively observed, as no primary macrophages were pictured to be free-flowing at any observed timepoint in this experiment, whereas some THP-1-derived naïve macrophages were clearly shown to be free-flowing after inflammatory insult (Figure 3.9).

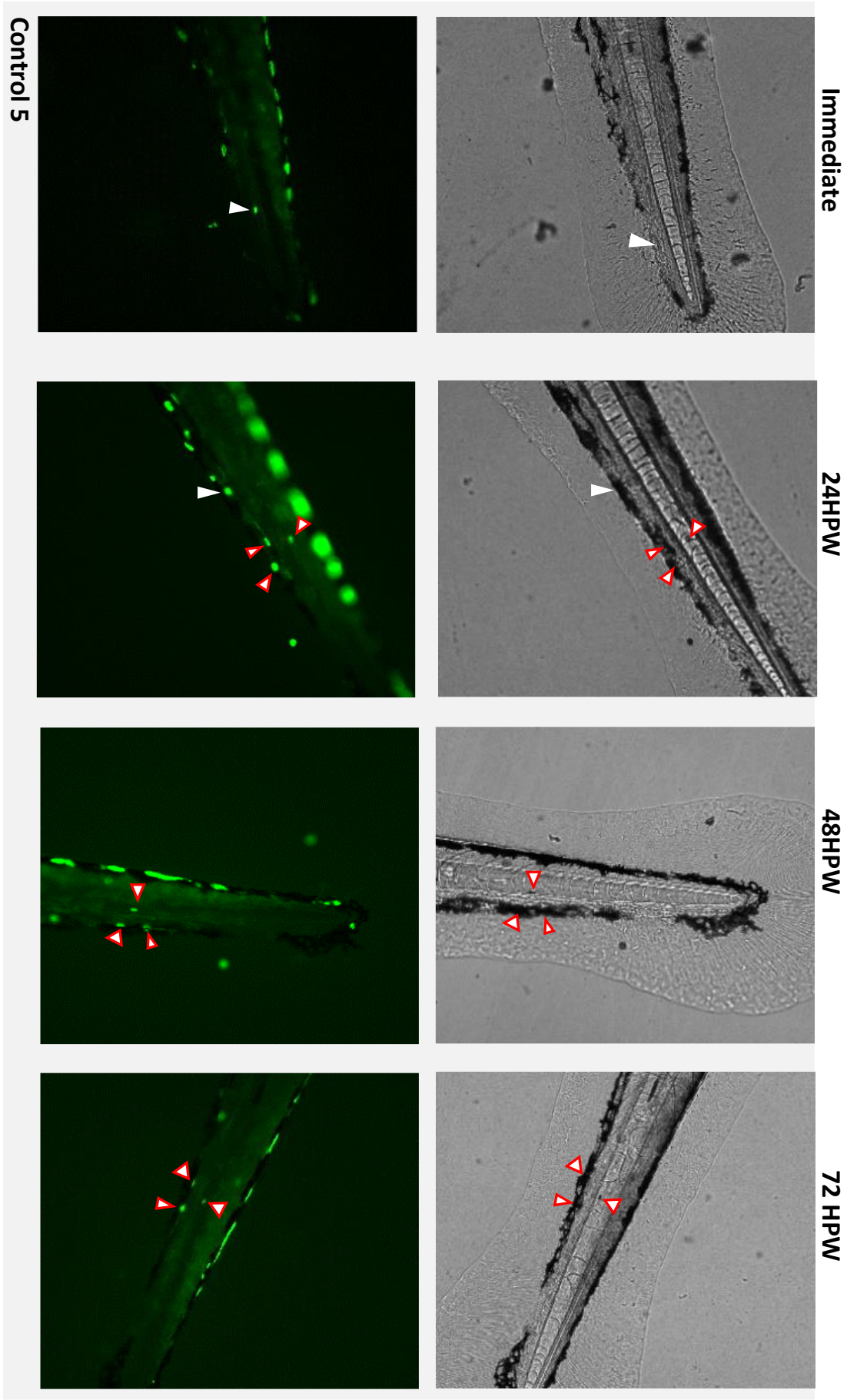
Despite improved long-term survivability over THP-1-derived macrophages, consistent with the literature describing the survival of primary human macrophages in zebrafish circulation, the primary macrophages pictured in Figure 3.10 do not appear to migrate towards the inflamed tissue in the transected samples (n=6) or appear to be more mobile than the macrophages in the uncut samples (n=6) over the course of 72 hours. This can be seen in the images in Figure 3.10 with white arrows indicating primary human macrophages. These cells are shown to be relatively stationary over time, with no clear shift in dispersion towards the site of inflammation in the tail transected group compared to the uninjured controls.

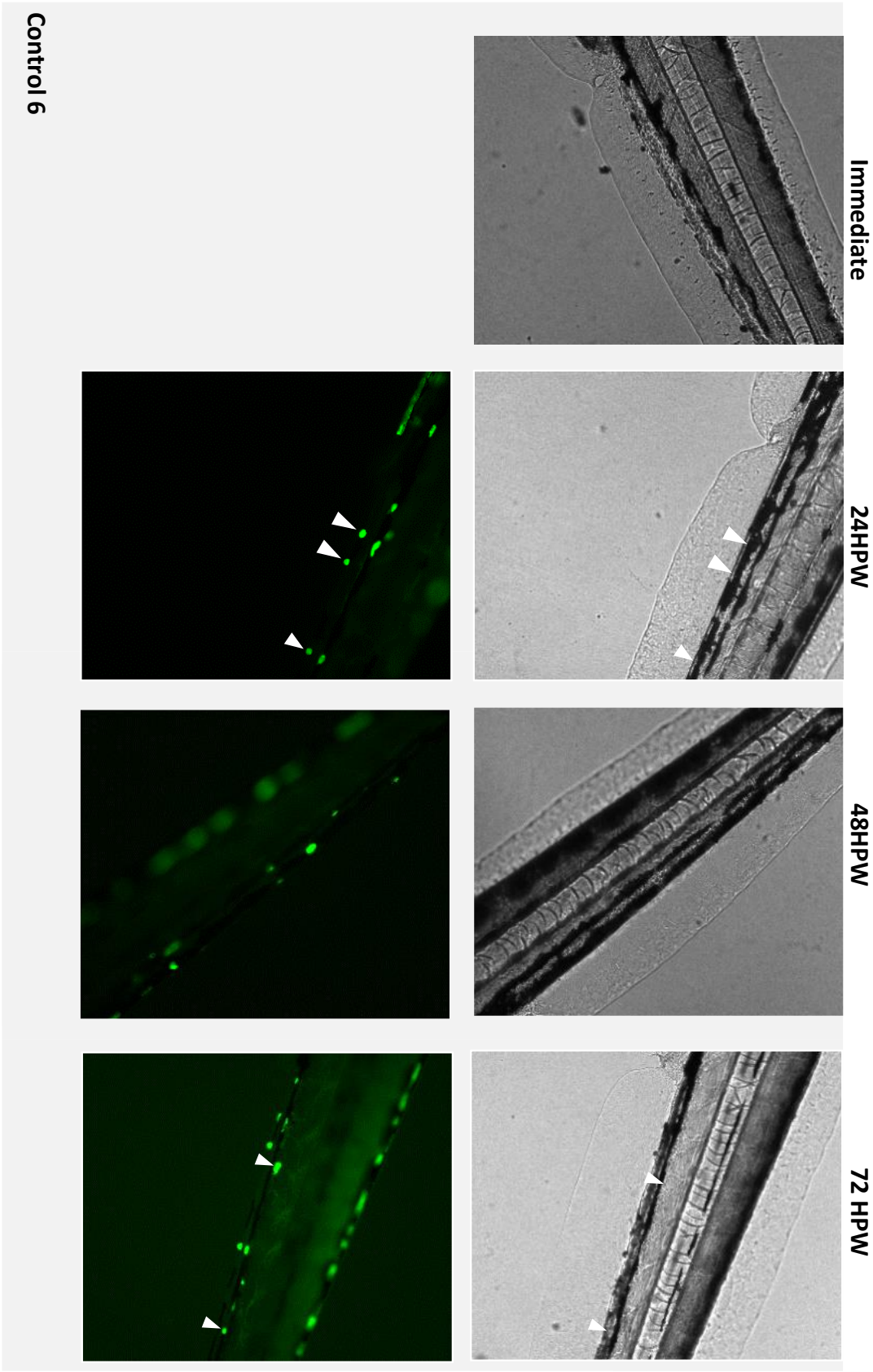


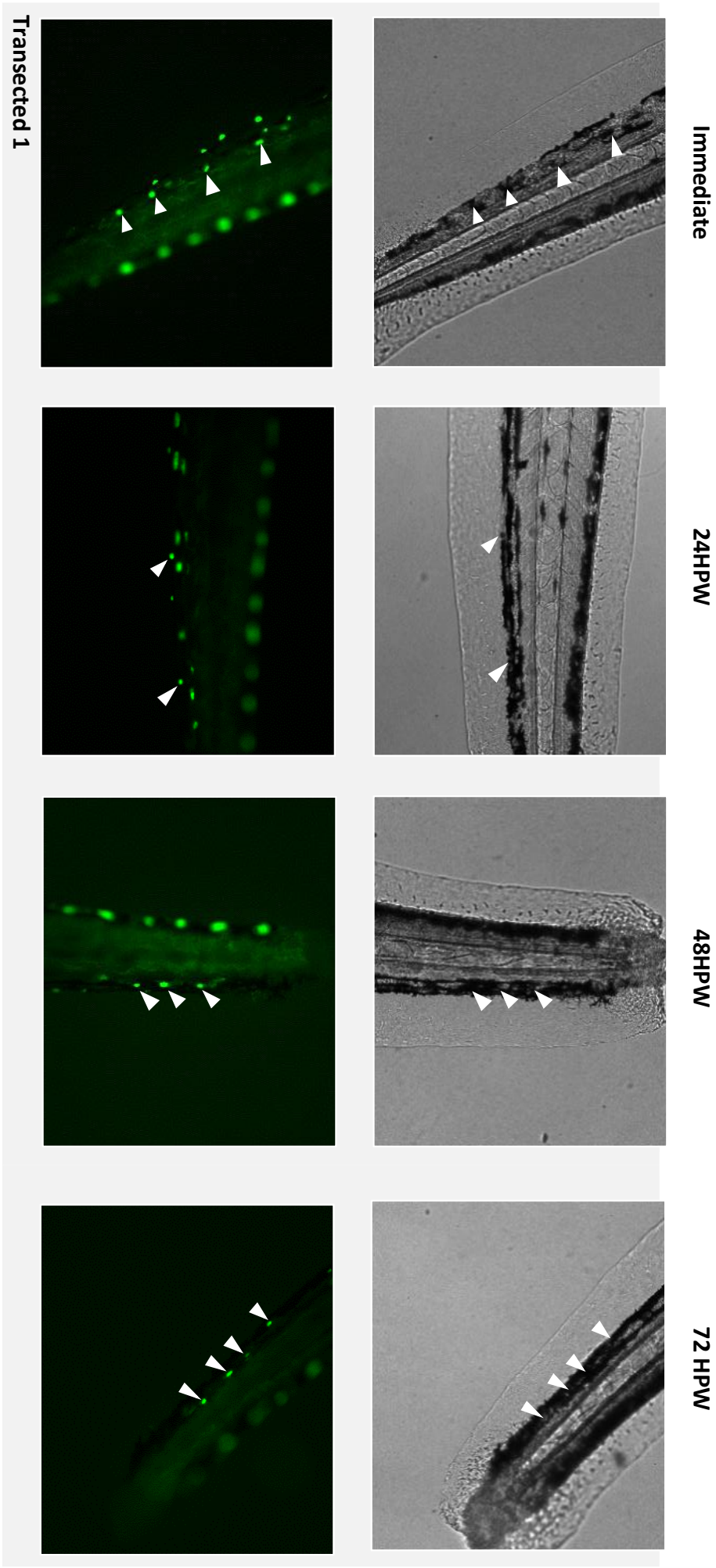


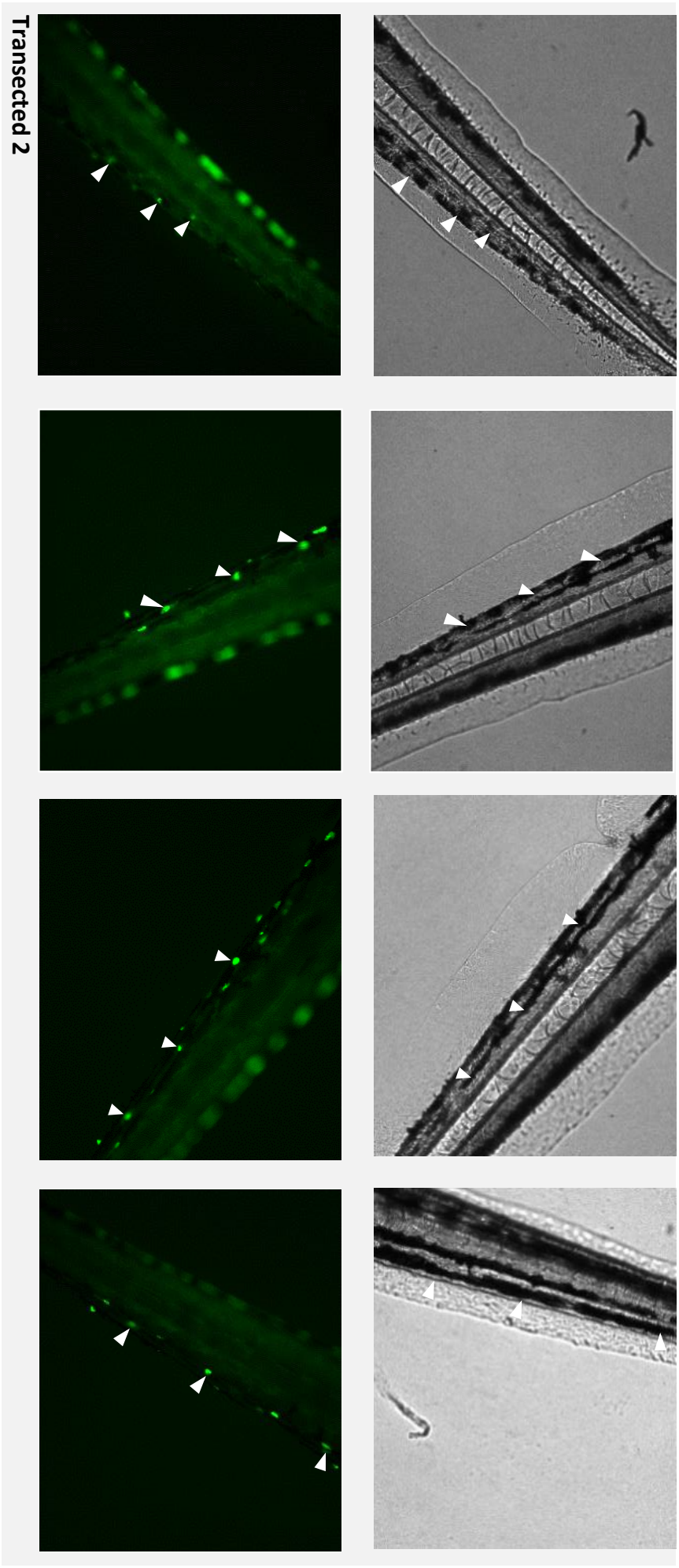


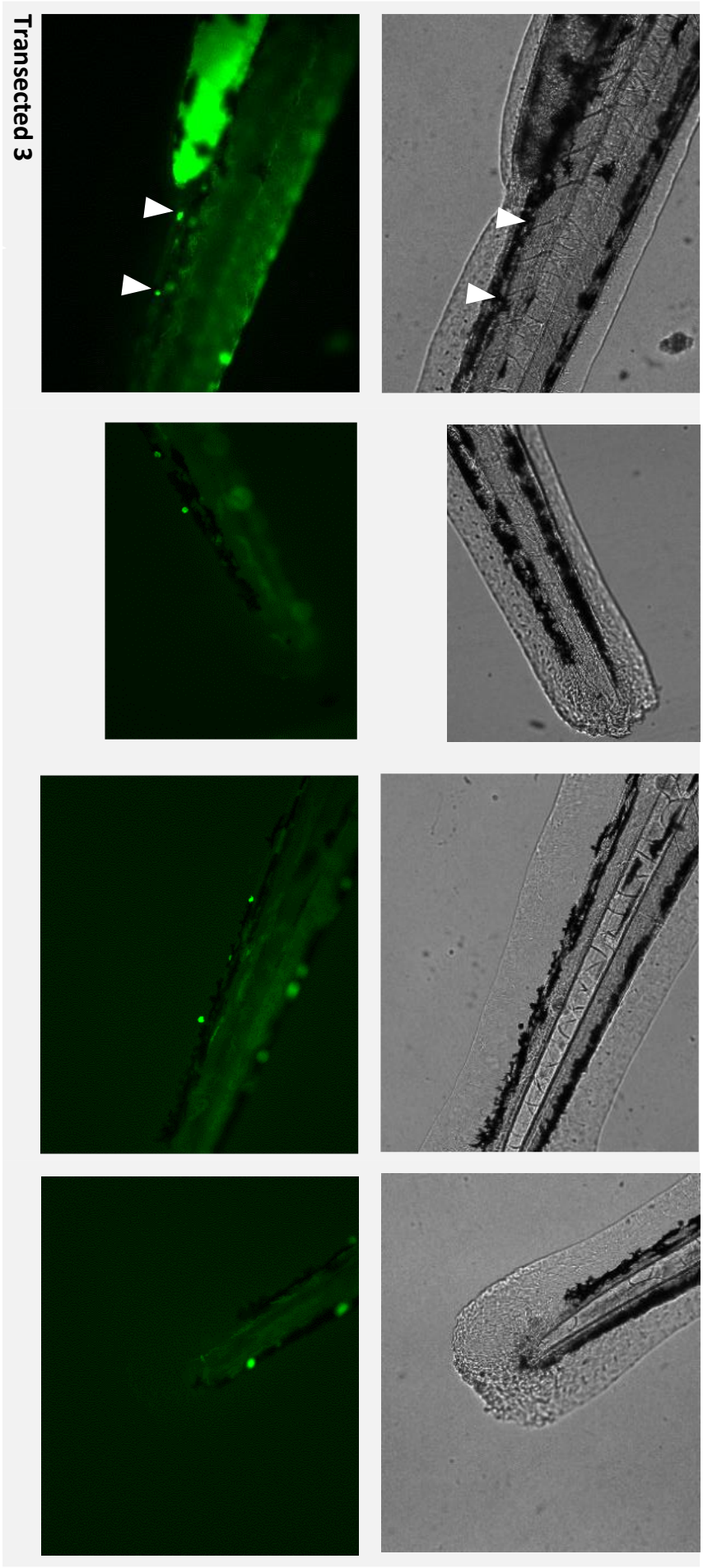


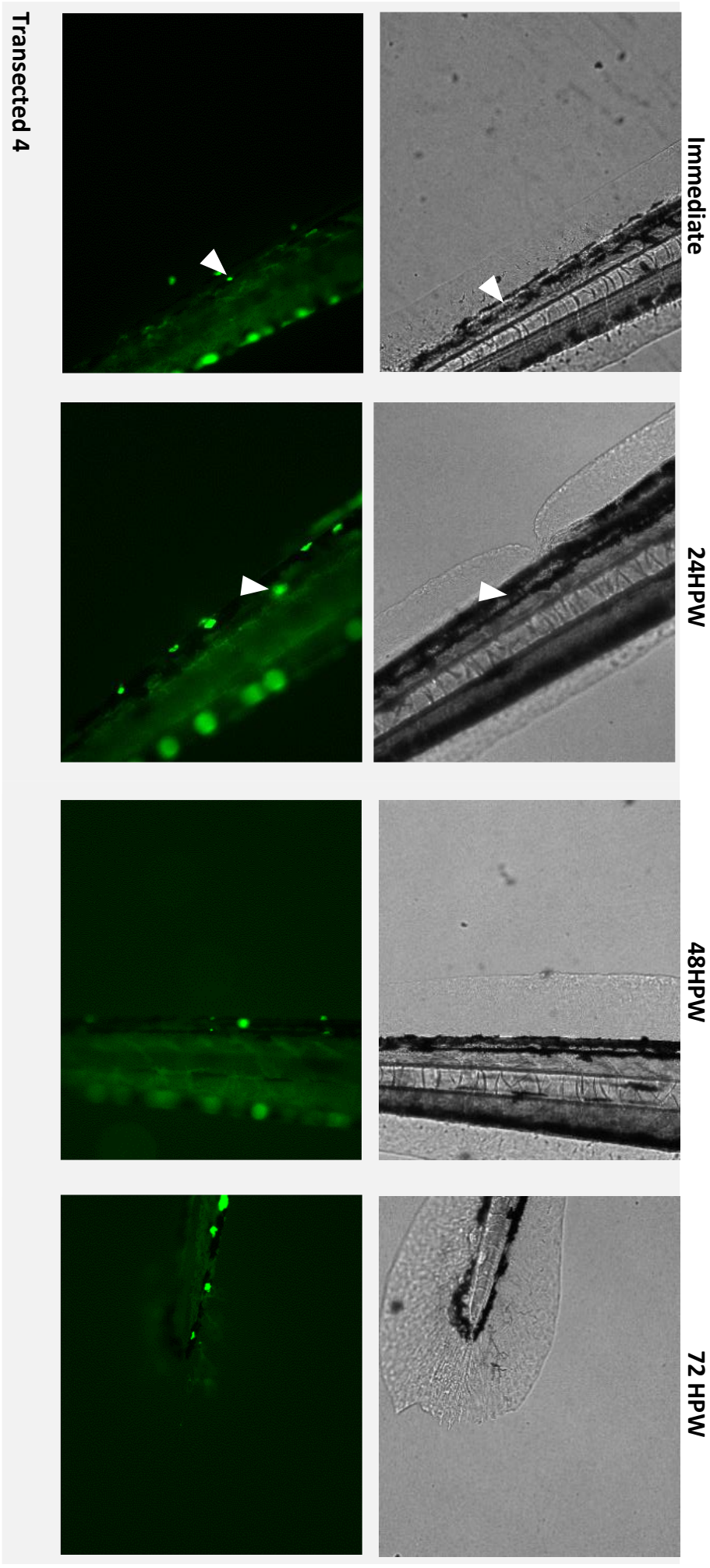


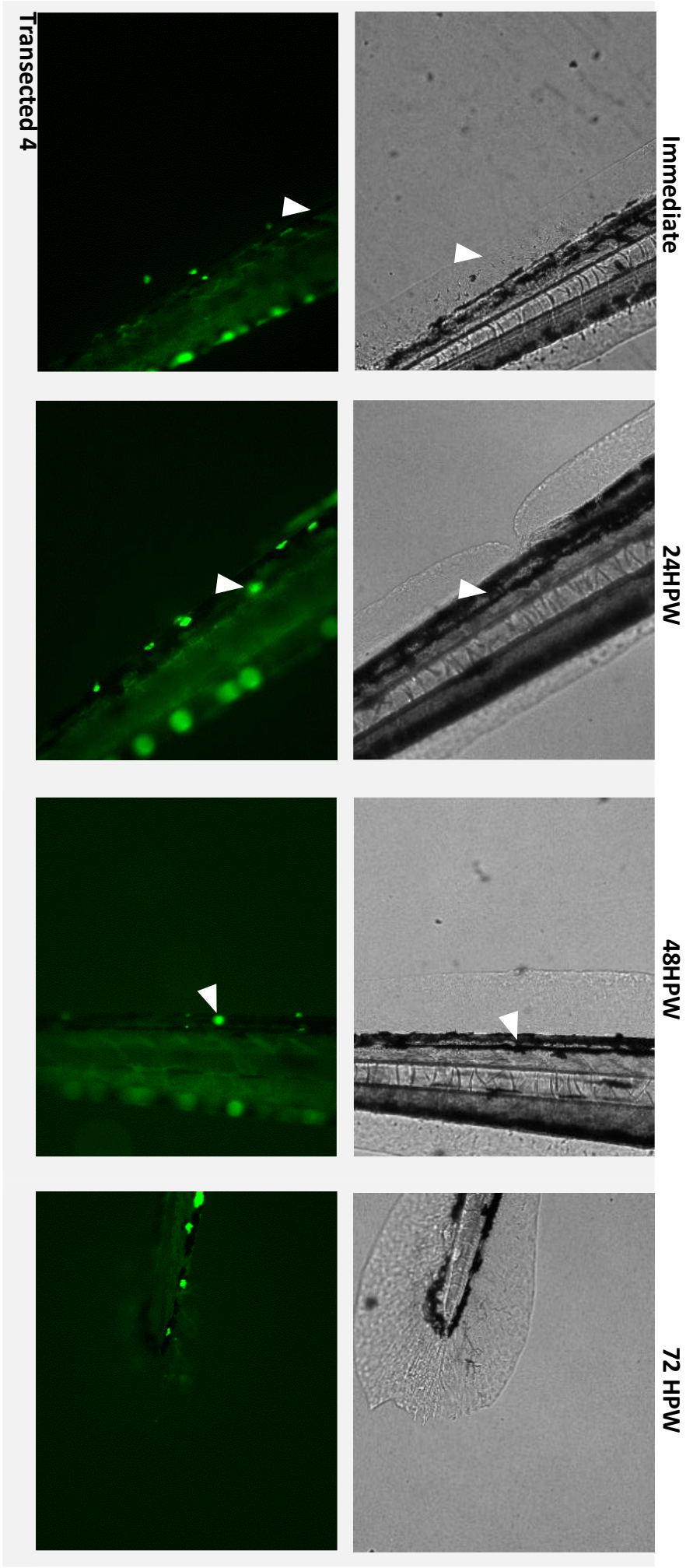


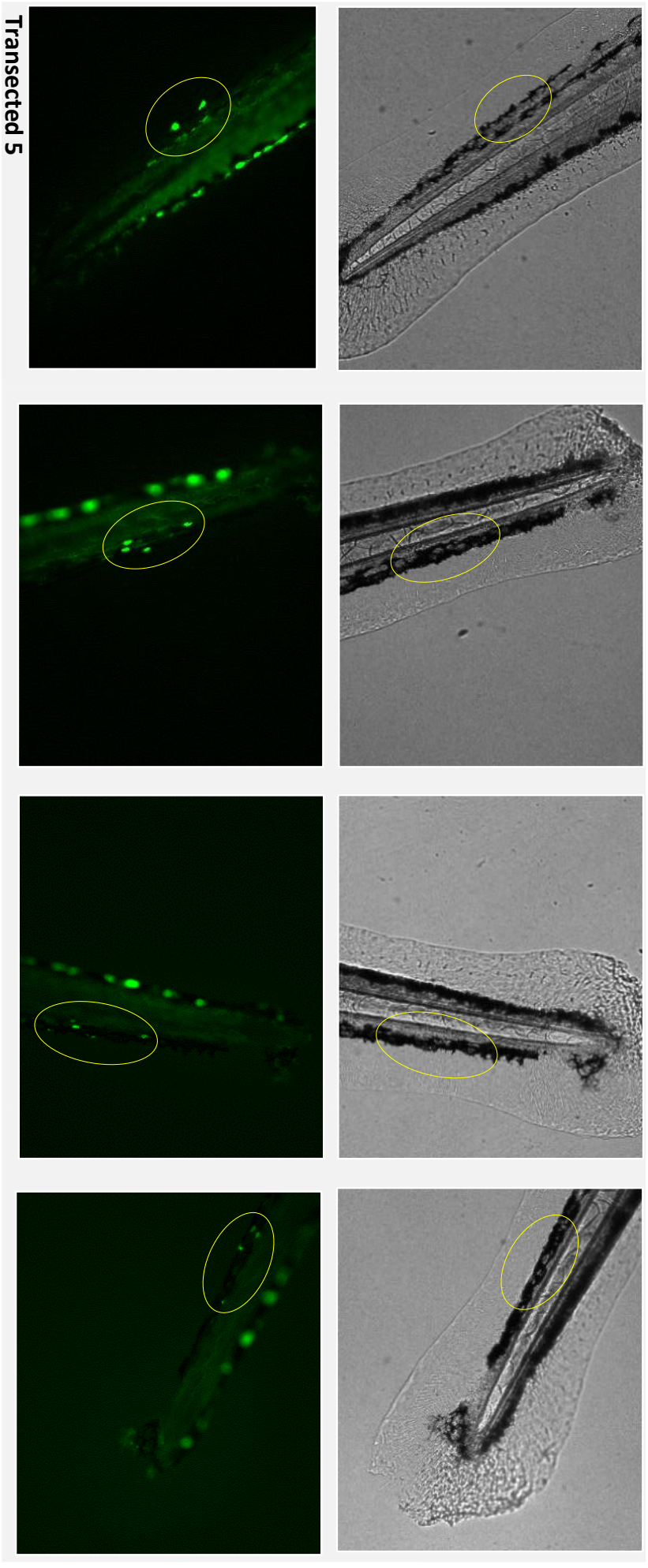












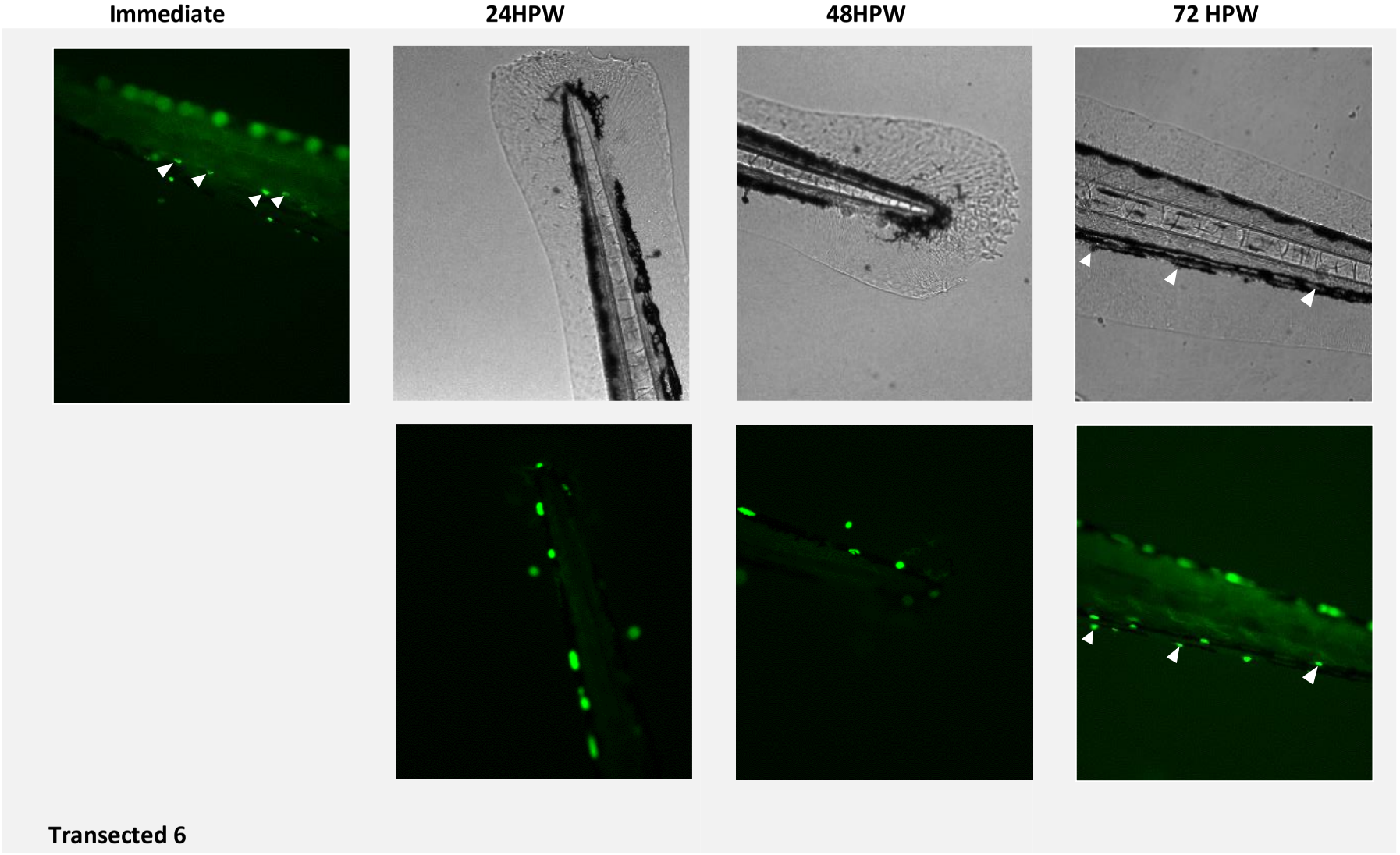


Figure 3.10: Injected primary M1 polarised human macrophages (green, indicated by white arrows) survive in zebrafish larval circulation for the entire duration of the experiment (72HPW) in both tail fin transected and uncut larvae. *Brightfield images taken using a 10x objective are pictured above (to the right in landscape) the corresponding fluorescent images. Arrows and circles indicate macrophages to differentiate between exogenous injected cells and endogenous autofluorescent pigment cells in fluorescent images.*

3.8.4 Interpretation

The improved survival capacity of the primary macrophages over the THP-1-derived macrophages, despite the injection parameters remaining consistent between the experiments utilizing both cell types, indicates either superior robustness of the primary macrophages, able to withstand the mechanical stress of microinjection better than the THP-1-derived cells, or that mechanical stress was not a factor in the poor survivability of the THP-1-derived cells. Either explanation confirms the inadequacy of THP-1-derived macrophages in zebrafish larval models as drug carriers, and supports the use of primary human macrophages instead.

Despite adequate long-term survivability of human primary macrophages in zebrafish larval circulation, the macrophages appear relatively unreactive to zebrafish inflammatory stimuli at all assessed time points. This may be due to the relatively high αDβ2 integrin expression of M1 macrophages as opposed to monocytes or naïve macrophages, potentially resulting in stronger tethering to the CHT and therefore rendering these cells more static than would be suitable for drug delivery. The ability of unmodified human leukocytes to undergo transendothelial migration into the inflamed tissue in zebrafish remains unconfirmed after this series of experiments, and appears to be unlikely from the data generated herein.

Chapter 4: Discussion

The two primary objectives of this thesis were to develop a novel method for cell-based zebrafish larvae microinjections and to assess the suitability of zebrafish larvae as a model for human macrophage-based drug delivery research. The former objective was completed in a stepwise manner. First, tip diameters were optimised to allow for the expulsion of an appropriate number of intact macrophages per pulse, while minimising larval tissue damage. In this regard, optimal tip diameters were determined to be between 15-20µm. Subsequently, injection volumes were optimised to allow for an appropriate number of cells to be introduced without detrimental homeostatic effects to the larvae. This was necessary due to a lack of consensus or rational for intravenous microinjection volumes into zebrafish larvae in literature. To our knowledge, this experiment offers the first comprehensive assessment of potentially adverse cardiovascular effects of different intravenous injection volumes in zebrafish larvae. In terms of heart rate and morphology, 5- and 10nl were determined to be the least detrimental to the larvae, with 10nl being selected as the optimal injection volume to allow for a less dense cell suspension while injecting a sufficient number of cells, and hence a reduced shear force experienced by the cells, compared to 5nl. Finally, the optimal larval injection site, allowing for a needle of diameter 15-20µm into circulation was determined as being in the Duct of Cuvier at 2dpf, as the anatomically broad duct and thin epidermis at this time point allowed for smooth entry of the injection needle and accurate delivery of the cells. We suspect these data may help in providing a benchmark for future studies involving zebrafish larval microinjections to aid in determining the choice of intravenous dosage volume. Given the relative lack of suitable literature – or lack of methodological detail in those we did access – contextualization of these data is not possible. However, as optimization of methods is likely specific to a laboratory and/or individuals, this is not seen as a limitation in the current context.

To determine whether zebrafish larvae are suitable *in vivo* models in which to study and develop a human macrophage-based drug delivery system, a series of experiments using both THP-1-derived macrophages (in undifferentiated, differentiated, unpolarised, and polarised states), and primary human M1 polarised macrophages were conducted. In the experiments, these cells were microinjected into the circulation of 2dpf zebrafish larvae, with, and in the absence of tail fin transections and observed over the course of at least 24 hours.

The THP-1 cell line was utilised due to ease of culturing for high throughput and, according to literature, functional conservation with primary macrophages after PMA-dependent differentiation. First, undifferentiated THP-1 monocytic leukemia cells were first injected into the circulation of 2dpf zebrafish larvae as a pilot study to verify optimal dye concentration and observe behaviour/viability of these cells in zebrafish larvae with tail fin transections. Shortly post injection, the cells were observed to adhere to the endothelium of the caudal vein, in the CHT region thereof, and Duct of Cuvier. In the larva where CHT-region endothelial adherence of the

THP-1 cells was apparent, we also observed a stationary, unstained polymorphonuclear cell directly adjacent to the similarly sized injected, stained cells. This is likely an endogenous zebrafish neutrophil and demonstrates shared behaviour and adhesion between the endogenous zebrafish immune cells and the injected exogenous human monocytes (undifferentiated THP-1 cells) in circulation. This observation is similar to those reported by Paul et al., 2019, and supports our hypothesis that the injected macrophages will indeed associate with the CHT endothelium of the zebrafish larvae, as this is the niche in which endogenous zebrafish endogenous neutrophils and macrophages also associate.

Rapid cellular division was observed within 24 hours after tail fin transections, the rate of which far exceeded the generally accepted doubling time of 26-36 hours in optimal growth conditions in cell culture. This is especially unexpected given the optimal temperature for THP-1 cell growth is 37°C ("THP-1 (ATCC TIB-202)" 2021), whereas the poikilothermic zebrafish were maintained at 28.5°C, almost 10°C cooler than what is optimal for human leukocytes. We therefore postulate that this expansion is primarily driven through zebrafish endogenous inflammatory signals; this data thus suggests some level of cross-species compatibility and conservation between zebrafish and human inflammatory ligands/receptors (Zanandrea, Bonan and Campos, 2020). Immediately post wounding, the injected THP-1 cells were not observed to follow a distinct distribution pattern in where they associated in the CHT-region of the caudal vein. After 24HPW, however, the cells were clearly more densely situated at the distal-most end of the caudal vein, without clearly undergoing diapedesis out of the caudal vein. This either demonstrates that migration along the 'circulatory highway' towards the inflamed tail tissue was achieved by the injected THP-1 cells, or that the cells which were already situated at the distal end of the caudal vein were most exposed to high concentrations of inflammatory cues (DAMPS, predominantly H_2O_2) and therefore were signaled to undergo more rapid expansion than the injected cells which originally situated further from the transection site.

This pilot was also useful in demonstrating that the optimisation of the microinjection, cell labelling, and visualisation parameters was successful and allowed for long term tracking of the injected THP-1 cells. The exogenous cellular response to zebrafish inflammation is a promising initial result and supports the use of human immune cells in modeling drug delivery systems in zebrafish larvae. The subsequent step in the development of the drug delivery model was to differentiate these cells into suitable candidates for drug delivery research, THP-1-derived, M1 polarised macrophages. Undifferentiated THP-1 cells themselves are not optimal drug carriers, given their rapid cellular expansion in response to an inflammatory insult, reducing the relative number of exogenous cells containing therapeutic cargo and potentially obstructing the migration of the cells. Cellular expansion to the observed extent also elicits adverse cardiovascular effects by overpopulating the primary blood vessels and increasing resistance to blood flow, thus straining the heart of the injected larvae.

PMA-differentiation of THP-1 cells results in the generation of naïve macrophage-like cells, mostly unable to undergo mitosis. Due to the opacity of literature on the subject of when, or if,

zebrafish endogenous monocytes undergo differentiation to macrophages, it is possible that differentiation is required to facilitate transendothelial migration in the zebrafish inflammatory reaction. As discussed in Chapter 2, the general consensus in literature regarding the use of macrophages for drug delivery is that M1 polarised macrophages are the optimal subtype to use due to their superior early inflammation-sensing and migratory capacity as compared to other macrophage subtypes (Liang et al., 2021) (Pang et al., 2018). For these reasons, the next experiment included LPS-induced M1 polarised THP-1 macrophage-like cells to be injected into 2dpf zebrafish circulation. Interestingly, these cells were found to either adhere to CHT-region of the caudal vein endothelium, primarily at the most distal point in circulation (vascular loop where caudal artery becomes the caudal vein) or adjacent to the CHT, in smaller vessels.

We postulate two possible explanations for this: 1) In terms of constant circulatory shear force, by virtue of being an anatomical loop, small eddies are created, which are sites of lower shear force resulting from turbulent flow. These eddies would require less adhesion strength to keep cells trapped within them than in sites where laminar flow dominates (throughout the length of CHT). This is also true for the macrophages located in the smaller adjacent vessels; and 2) partial cellular migration to the inflammatory site, without leaving circulation. The latter is unlikely due to similar cellular positioning in circulation between the control and tail transected larvae.

In stark contrast to the previous pilot, a complete absence of cells was observed in all larvae after 24 hours, indicating a potential loss of cellular viability. This was an unexpected outcome, given that the (sparse) literature available on the behaviour of human macrophages in zebrafish circulation does suggest that the cells should survive for at least the duration of our experiment, assuming the conservation of function and behaviour between THP-1-derived M1 macrophages and primary human macrophages (Paul et al., 2019). Given the fact that both the control and transected groups experienced a complete loss of injected macrophages within 24HPW, we conclude that negative inflammatory feedback or the outcompeting of endogenous macrophages in an inflammatory response was likely not the cause of the seemingly poor long-term survival of the injected M1 THP-1-derived macrophages. We suspect two potential reasons for the apparent loss of viability in the short term: 1) Cell culturing interventions (I.e., PMA-based differentiation or LPS-based polarisation may stress the cells and result in a less robust cell type in the foreign organism of zebrafish larvae), or 2) mechanical damage from microinjection, resulting in the poor survivabililty of these cells in zebrafish larval circulation. One limitation of this experiment was the conservative sample size resulting in lower than optimal statistical power, hence subsequent experiments utilized a larger n.

To determine whether the pre-treatment of LPS was a primary factor in the poor survival of the M1 THP-1-derived macrophages, the next experiment included naïve THP-1-derived macrophages and a larger zebrafish larval cohort. Due to the increased sample size and therefore longer experiment, gravitational effects acting on the cells in suspension were more pronounced and resulted in occasional unexpectedly high cell numbers being ejected. Although not optimal, because of the large sample size, we were retrospectively able to add cell density as a qualitative

parameter in which to evaluate potential role players affecting THP-1 cell survival in the larvae. This is a worthwhile parameter, as THP-1 cells in culture require relatively high cell densities to survive and grow effectively ("THP-1 (ATCC TIB-202)" 2021).

In this experiment, we observed macrophages along the CHT-region of the caudal vein, as observed in the undifferentiated THP-1 pilot immediately post tail transection: unlike the M1 macrophages primarily found in the circulatory loop. Shortly after tail fin transections in the naïve THP-1 macrophage experiment (3HPW), some macrophages were found to be released from the CHT into circulation. This was not observed in the uninjured control zebrafish group and may indicate an appropriate response of these cells to zebrafish inflammatory cues, most likely in response to the damage-induced H_2O_2 gradient generated from the site of the wound. Despite the release of these cells, none were ultimately observed to migrate into the inflamed tissue, instead remaining in circulation. Due to the rapid movement of these cells in circulation, it is unlikely that they exhibited the characteristic vascular rolling of migratory macrophages towards a site of inflammation as would be expected in both zebrafish and humans.

As this action is dependent on integrin/selectin interactions between immune cells and vascular endothelium, it is likely that, as discussed in Chapter 2, the level of conservation of adhesion molecules between species is insufficient to allow for efficient migration of the human cells on the zebrafish endothelium. Hence, it is unlikely that these cells will be able to undergo extravasation and into the inflamed tissue. Similarly to the M1 THP-1-derived macrophages, but in opposition to the behaviour of the undifferentiated THP-1 cells which displayed a similar initial distribution pattern, the naïve THP-1-derived macrophages reduced in number after 24 hours, with zebrafish becoming completely devoid of THP-1-derived macrophages after 48 hours. This demonstrates that neither LPS nor cell density are primary factors in the poor survivability of these cells in zebrafish over time. It is also unlikely that the PMA in these experiments directly reduced the survivability of these cells, as the concentration used in our experiments falls below the non-cytotoxic "low" concentrations used in other studies demonstrating the cytotoxic/hyper LPS-sensitive effects of only "high" concentrations of the chemical (Starr, Bauler, Malik-Kale and Steele-Mortimer, 2018). We suspect that the immortalisation of this cell line may have resulted in the alteration of adhesion/migratory proteins and thus, rendered THP-1-derived cells unsuitable for drug delivery research, at least in the context of zebrafish.

In the experiments up to here, specific markers of apoptosis were not tested for, and the mechanisms of THP-1-derived macrophage cell death remains unknown. Although loss of injected macrophage viability in the short term (<48 hours post injection) could be beneficial in a cell-based drug delivery system so as not to perpetuate inflammation, the success of such a system would rely on adequate transendothelial migration of the drug carrier to ensure accurate, target-specific delivery of the therapeutic agent. A limiting factor for transendothelial migration of our relatively large immune cells (21-23µm diameter) could potentially be the zebrafish endothelial pore size relative to that of humans, thus restricting the diapedesis of the cells. No

literature capable of elucidating this topic was available, so this remains to be elucidated by super-resolution microscopy.

Given the demonstrated limitations of THP-1 cells in this context, we next set out to replicate the previous experiment, but employing human primary M1 polarised macrophages instead of the THP-1 cells. Our group has previously shown that, of polarised primary macrophage subtypes, M1 macrophages are the only subtype capable of efficiently transmigrating through human endothelial cells with pore size of 8um, after being loaded with cargo (4.5 um polystyrene beads) (Visser and Smith, 2017). The data generated in this final study was consistent with the prior published study demonstrating the long-term survivability of human primary macrophages in zebrafish larvae, as fluorescent macrophages were observed in larvae up to our experimental endpoint (72hpi).

No qualitative differences in macrophage behaviour were observed between zebrafish larvae with or without tail fin transections, unlike the observed dislodging of naïve THP-1-derived macrophages in zebrafish larvae with tail fin transections only. The primary human M1 macrophages were not observed to behave differently in the presence or absence of inflammation. This is a surprising finding as M1 macrophages should theoretically be primed to respond to inflammatory stimuli. This potentially indicates a role for the macrophage integrin αDβ2 - which is relatively highly expressed in the M1 macrophage subtype as compared to naïve macrophages (Cui, Ardell, Podolnikova and Yakubenko, 2018) - in the endothelial cuddling reaction and may explain the inability of these macrophages to migrate towards the site of inflammation. Despite this, the primary human macrophage dispersion throughout the CHT region was shown to be relatively dynamic over time, but not confined to the distal caudal vascular loop unlike the observed positioning of the M1 THP-1-derived macrophages. Together, these data may indicate fundamental differences in integrin expression between primary macrophages and THP-1-derived macrophages (Daigneault et al., 2010).

Unlike primary monocytes/macrophages, THP-1 cells do not express endogenous L-selectin (Rzeniewicz et al., 2015), the adhesion protein responsible for the secondary tethering and rolling of leukocytes on activated endothelial protein PSGL-1 in humans, as well as for the transendothelial migration of leukocytes (Ivetic, Hoskins Green and Hart, 2019). The absence of this protein could be a prevalent factor in preventing the efficient migration of the THP-1-derived cells. Although the primary human macrophages do express L-selectin, these cells appeared to exhibit the opposite problem to the circulating THP-1-derived cells in that they remained relatively static with reference to the inflamed tissue site (transected tails). No primary human macrophages were found in the inflamed tissue at any timepoint in the experiment, with larval tail fins often fully regenerating without the macrophages migrating to the distal end of the CHT vasculature, as seen in Figure 3.10, Transected 6. Because the macrophages failed to even reach the distal end of the caudal vein, we find it unlikely that the endogenous zebrafish leukocytes are preventing the egress of the injected primary macrophages through negative feedback or physical blocking, hence endogenous macrophage ablation prior to injection would likely not

solve this issue. We do, however, offer the future recommendation of partially inhibiting integrin αDβ2 on primary M1 macrophages, e.g., using blocking antibodies, prior to injection. This could enable macrophage migration, allowing for less stationary behaviour, while maintaining the benefits of utilizing primary M1 macrophages for the application of drug delivery.

4.1 Conclusion

In conclusion, a significant limitation of this study was the COVID pandemic of 2020-2021, which imposed limitations not only on laboratory availability, but also on availability of reagents such as highly specific antibodies, e.g. such as would be required for blocking integrins on M1 macrophages. However, although the data presented in this thesis do not conclusively answer the question of whether zebrafish larvae are suitable models in which to develop a human macrophage-based drug delivery system, they do cover substantial ground as a starting point, delivering several optimised protocols and answering some key questions on appropriate cell types to be used (or rather excluded). To summarise, the key novel contributions to development of a zebrafish model for cell-based drug delivery, were as follows: firstly, data confirmed that human immune cells can/do respond to zebrafish inflammatory stimuli (experiment 2); secondly, THP-1-derived cells were shown to likely be unsuitable in zebrafish models for the application of drug delivery due to their demonstrated poor migrational and survival capacity (experiments 3 and 4); thirdly, human macrophages, when introduced into zebrafish circulation, were illustrated to effectively associate with the same tissues as do endogenous zebrafish macrophages; where they remain, primed to respond to inflammation (experiments 3, 4, and 5); and fourthly, unaltered human primary M1 macrophages possess adequate survival, but not sufficient migrational, capacity within the circulation of zebrafish larvae.

At this point, we can confidently make some recommendations for future research to comprehensively (and perhaps definitively) determine whether zebrafish larval models are suitable for the simulation and study of human macrophage-based drug delivery.

Firstly, given the positive findings in the context of human primary macrophages, future research focused on enhancing the migratory capacity of macrophages, should be a focus in the development of this model, as current data suggest this factor to be the main limitation. This may be done either by modification of adhesion molecule expression, or the experimental modification of the zebrafish immune system itself. Secondly, the use of transgenic zebrafish larvae can assist significantly with microscopic investigations: e.g. larvae with fluorescent endothelial cells may assist visualization of diapedesis, to determine whether diapedesis of human primary macrophages in zebrafish is indeed possible, and larvae with fluorescent macrophages and/or neutrophils may offer more information on the potential confounding role of the zebrafish endogenous inflammatory immune system in this context. Finally, although unlikely, the inflammatory insult of fin transection may not have been severe enough to elicit a chemotactic signal sufficiently strong to attract the human macrophages. Future studies may thus benefit from inclusion of bacterial infection/LPS models in tandem with tail fin transections

to elicit a greater inflammatory reaction (or microbe-associated PAMPs that are perhaps more likely to activate human macrophages than endogenous zebrafish chemotactic molecules) that provides a greater chance of recruiting injected human macrophages.

Thus, although a fully developed zebrafish model for human macrophage-based drug delivery remains elusive at this point, the limitations that remain do not appear insurmountable. We are confident that in future, such a model will enable individualized medicine approaches for treatment-resistant conditions.

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Appendices

UNIVERSITEIT
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Approved with Stipulations

14 April 2020

PI: Prof Carine Smith

REC: ACU Reference #: ACU-2019-11820

Title: Use of Zebrafish larvae (<5dpf) for drug screening

Dear Prof Carine Smith

Your Notification ACU-2019-11820 was reviewed on 25 March 2020 y the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is valid for a period of five years. A new application must be submitted when the source of the material changes.

Stipulations for clarification:

1. How will it be ensured that the larvae do not come in direct contact with the ice?
2. What is the treatment that will be done to/on the larvae?

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered paraveterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your REC: ACU reference number: # ACU-2019-11820 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: ACU

policy and procedures. Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Animal Care and Use)