Investigating Acute Gastrointestinal Inflammation Associated with Feeding a Fat-Enriched Diet in Zebrafish Larvae

Navjyot Singh Sangha,
Division of Cell and Molecular Biology,
Imperial College London

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DECLARATION OF ORIGINALITY

I declare that the work presented in this thesis is my own except where stated in the text. Where information has been taken from another source, it has been appropriately referenced or acknowledged.
ABSTRACT

Diseases of the metabolic syndrome such as obesity, type II diabetes and atherosclerosis are caused by an excess accumulation of dietary lipid and are currently a major burden on healthcare. The NLRP3 inflammasome is a molecular platform that leads to activation of caspase-1 and has received considerable attention recently regarding its role in promoting the inflammation associated with these diseases. The chronic inflammation associated with these states is well understood but the acute inflammation has not been investigated in detail and is the focus of this study. The zebrafish innate immune system functions in a similar manner to that of mammals and the uptake of dietary fat in the gastrointestinal tract is also strongly conserved. Thus, using zebrafish larvae, a model of acute gastrointestinal inflammation following feeding on a fat-enriched diet has been established and characterised. Additionally, the role of the NLRP3 inflammasome in the inflammation associated with feeding on such a diet has been investigated using pharmacological inhibitors and morpholino knockdown of components of this pathway. The results demonstrate that larvae fed a cholesterol-enriched diet for 6h develop localized inflammation in the intestine as illustrated by the recruitment of myeloid cells to this region 18h following removal from the diet. Feeding on a diet enriched in palmitic acid also achieves this effect, whilst an unsaturated fatty acid-enriched diet does not. Reverting to a standard diet following feeding on a cholesterol-enriched diet resolves the localised recruitment of immune cells to the intestine. Treatment with inhibitors targeting several components of the NLRP3 inflammasome blocks the recruitment of inflammatory cells to the intestines of larvae fed a cholesterol-enriched diet, as does treatment with a splice-blocking morpholino against ASC, which is necessary for caspase-1 activation. The results thus suggest that the acute inflammation in the intestine associated with feeding on a cholesterol-enriched diet is at least in part dependent on the NLRP3 inflammasome.
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<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>ABC</td>
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<tr>
<td>A-LPM</td>
<td>Anterior Lateral Plate Mesoderm</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>Activator Protein-1</td>
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<td>Apo</td>
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<td>Interferon-γ</td>
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<td>Monocyte Chemoattractant Protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage Inflammatory Protein-1 Alpha</td>
</tr>
<tr>
<td>mmLDL</td>
<td>Minimally Modified Low Density Lipoprotein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteases</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal Triacylglycerol Transfer Protein</td>
</tr>
<tr>
<td>Mtz</td>
<td>Metronidazole</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil Extracellular Trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-Like Receptor</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nod-Like Receptor Pyrin Domain-Containing</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1-Like 1</td>
</tr>
<tr>
<td>NTR</td>
<td>Nitroreductase</td>
</tr>
<tr>
<td>ω-3</td>
<td>Omega-3</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised Low Density Lipoprotein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBI</td>
<td>Posterior Blood Island</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline with Tween</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI</td>
<td>Posterior Intestine</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>P-LPM</td>
<td>Posterior Lateral Plate Mesoderm</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator Receptor-γ</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-Selectin Glycoprotein Ligand-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin Domain</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-Interacting Protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated Fatty Acid</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>SS</td>
<td>Single Stranded</td>
</tr>
<tr>
<td>TAB</td>
<td>Transforming Growth Factor-Beta Activated Protein Kinase Binding</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming Growth Factor-Beta Activated Protein Kinase</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-Binding Kinase-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzene Sulphonic Acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour Necrosis Factor Associated Factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T Regulatory</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Thioredoxin-Interacting Protein</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream Activating Sequence</td>
</tr>
<tr>
<td>UFA</td>
<td>Unsaturated Fatty Acid</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low-Density Lipoprotein</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>WKM</td>
<td>Whole Kidney Marrow</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Infection with pathogens, cell damage or a loss of homeostatic regulation leads to a biological response known as inflammation which is characterised by the migration of immune cells such as neutrophils and macrophages to the site of damage following the release of mediator molecules from resident immune cells. Inflammation is regulated at three different levels: Firstly, at the cellular level in terms of the composition of cells in tissues and the behaviour of these cells in response to inflammatory stimuli leading to their recruitment and activation. Secondly, at the level of control of signalling pathways and thirdly, at the level of regulating gene expression (Medzhitov and Horng, 2009). Depending on its duration, inflammation can be either classified as acute or chronic. Acute inflammation shows an immediate onset and persists for a few days, whereas chronic inflammation shows a delayed onset and lasts for weeks or months. Chronic inflammation results from a failure to resolve acute inflammation, which leads to tissue destruction over time. It is a complication arising from diet-associated diseases such as obesity, type II diabetes and atherosclerosis which constitute the metabolic syndrome and are a major burden on global health.

1.1 Acute Inflammation

1.1.1 Inflammatory Stimuli and their Recognition

Stimuli that incite inflammation are classified into two categories based on their origin. The first class are infectious inflammatory stimuli, known as pathogen-associated molecular patterns (PAMPs), which are conserved structures on microorganisms necessary for their survival (Akira et al, 2006; Kumar et al, 2011). Examples include lipopolysaccharide (LPS) in Gram negative bacteria, lipotechoic acid (LTA) in Gram positive bacteria, and lipoproteins and peptidoglycans present in both Gram negative and Gram positive bacteria. Viruses also contain PAMPs in the form of DNA, RNA or glycoproteins (Akira et al, 2006). PAMPs are recognised by pattern recognition receptors (PRRs), which are evolutionarily conserved,
germline-encoded receptors that are constitutively expressed in macrophages and other non-professional immune cells. Examples of PRRs include the transmembrane (TM) Toll-like receptors (TLRs) and the cytoplasmic Nod-like receptors (NLRs) (Akira et al, 2006; Takeuchi and Akira, 2010). Upon recognition of PAMPs by PRRs, signalling pathways are activated leading to inflammatory responses that result in elimination of the infecting pathogen (Kumar et al, 2011). The second class of inflammatory stimuli are non-infectious or sterile stimuli, known as damage-associated molecular patterns (DAMPs), which are also recognised by PRRs (Takeuchi and Akira, 2010). These can take the form of pro-inflammatory mediators released from necrotic cells following loss of integrity of the plasma membrane (PM), or various irritant particles (Matzinger, 2002; Rock et al, 2010). Irritant particles can be either inorganic (asbestos), organic (amyloid beta (β)) or amorphous (alum). The inflammatory response initiated against DAMPs can often fail to remove them and several types of DAMPs may actually be harmless (Rock et al, 2010). Thus, the resulting collateral damage to the host’s tissues does more harm than good.

1.1.2 Mediators of Acute Inflammation

Cytokines and Chemokines

Cytokines are low molecular weight proteins that are secreted from a variety of cells and activate intracellular signalling pathways following binding to their receptors. Cytokines that play a role in promoting inflammation are known as pro-inflammatory cytokines and examples include interleukin-1 (IL-1), IL-6, IL-12, interferon-gamma (IFN-γ) and tumour-necrosis factor-alpha (TNF-α). Cytokines display synergy and redundancy in their function, and examples of their roles in acute inflammation are activating effector functions of immune cells, inducing expression of other cytokines or chemokines and enhancing expression of adhesion molecules on endothelial cells (ECs) (Goldsby et al, 2003). Chemokines are small peptides that play a role in the migration of leukocytes from the circulation to tissues. They possess a cysteine (C) motif and are classified into four categories depending on the amino acid sequence of the cysteine motif: the first are CXC chemokines, where two cysteine residues are separated by an amino acid (X). The second are CC chemokines, where two cysteine residues are next to one another (Dinarello, 2000). In the third class, there is a single
cysteine residue and in the fourth, two cysteine residues are separated by three amino acids (CX\textsubscript{3}C) (Mantovani, 1999). Chemokines for neutrophils are of the CXC families, whereas monocyte chemokines are of the CC families (Yadav et al., 2010). Chemokines show functional redundancy in that no chemokine acts specifically on one leukocyte population, and thus every leukocyte type is affected by at least one chemokine. Additionally, chemokines bind to multiple receptors and the receptors are capable of binding to multiple ligands. The receptors for chemokines are 7-TM domain G-protein-coupled receptors (GPCRs) (Mantovani, 1999). Examples of chemokines include IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1\textalpha). The sources and major functions of several cytokines and chemokines involved in acute inflammation are summarised in **Table 1.1**:
1. Introduction

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Source(s)</th>
<th>Function(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Cytokine</td>
<td>Monocytes, macrophages, neutrophils (IL-1α and β). ECs (IL-1α only)</td>
<td>Enhancing neutrophil survival, adhesion, respiratory burst and protease release. Enhancing cytokine production and phagocytosis by macrophages. Increasing ICAM-1 and VCAM-1 expression on endothelial cells. Inducing chemokine production.</td>
<td>Lord et al, 1991; Dinarello, 2009</td>
</tr>
<tr>
<td>IL-6</td>
<td>Cytokine</td>
<td>ECs, macrophages</td>
<td>Promoting the acute phase response. Inducing myeloid cell differentiation.</td>
<td>Fiers, 1991; Heinrich et al, 2003; Kishimoto, 2005</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Cytokine</td>
<td>Activated macrophages</td>
<td>Activates macrophages and increases their cytotoxicity. Activates neutrophils. Increases adhesion molecule expression on ECs. Induces chemokine expression.</td>
<td>Fiers, 1991; Goldsby et al, 2003</td>
</tr>
<tr>
<td>IL-8</td>
<td>Chemokine</td>
<td>Macrophages, neutrophils, ECs</td>
<td>Neutrophil chemoattractant</td>
<td>Harada et al, 1994</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Chemokine</td>
<td>Monocytes, macrophages, ECs</td>
<td>Monocyte chemoattractant</td>
<td>Yadav et al, 2010</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Chemokine</td>
<td>Monocytes, macrophages, ECs</td>
<td>Monocyte and macrophage chemoattractant</td>
<td>Cook, 1996</td>
</tr>
</tbody>
</table>

Table 1.1: Summary of the Sources and Major Functions of Several Cytokines and Chemokines Involved in Acute Inflammation.
Lipid Mediators of Acute Inflammation

Lipid mediators, or eicosanoids, also play an important role in the early stages of acute inflammation. There are two types of such mediators: the prostaglandins and the leukotrienes. Prostaglandins enhance vascular permeability and act in either an autocrine or paracrine manner. They are synthesised from arachidonic acid (AA) following its release from the nuclear or endoplasmic reticulum (ER) membrane by type IV cytosolic phospholipase 2 (cPLA₂). Cyclooxygenase (COX) then converts AA into an intermediate prostaglandin, PGH₂ (Funk, 2001). There are two isoforms of COX: COX-1 and COX-2. COX-1 is responsible for the constitutive synthesis of basal levels of PGH₂, whereas COX-2 synthesises PGH₂ during an inflammatory response. PGH₂ is then metabolised to mature prostaglandins by a range of downstream enzymes, depending on the cell type (Funk, 2001). Leukotrienes are produced by inflammatory cells such as neutrophils and macrophages, and are also synthesised from membrane-released AA. 5-lipoxygenase catalyses the synthesis of leukotriene A₄ (LTA₄) from AA and LTA₄ hydrolase converts LTA₄ to LTB₄, which is a potent neutrophil chemoattractant (Funk, 2001). The receptors for LTB₄ are GPCRs of which there are two: B-LT1 and B-LT2. B-LT1 is a high affinity receptor expressed on leukocytes, whereas B-LT2 is a low affinity receptor with a more diverse expression (Tager and Luster, 2003). In addition to promoting neutrophil chemotaxis, LTB₄ stimulates reactive oxygen species (ROS) production and release of granule enzymes by neutrophils. It also enhances phagocytosis of bacteria by neutrophils (Sumimoto et al, 1984; Mancuso et al, 2001; Tager and Luster, 2003).

1.1.3 The Role of Macrophages and Neutrophils in Acute Inflammation

Neutrophils

Neutrophils are granulocytic immune cells which are recruited early in the inflammatory response and have a relatively short half-life. However, their lifespan is prolonged by microbial products such as LPS, and cytokines including IL-1β, IL-6, TNF-α and IFN-γ. This increased survival occurs through interference of apoptosis in neutrophils and is
necessary for them to carry out their function in eliminating damaging agents (Colotta et al., 1992; Mantovani et al., 2011).

Neutrophils present in the circulation infiltrate sites of infection or injury following activation of resident macrophages. Upon sensing danger signals, macrophages produce pro-inflammatory cytokines such as TNF-α and IL-6, and chemokines such as IL-8 and CXCL1, which recruit neutrophils. Macrophages also release matrix metalloproteases (MMPs), which cleave chemokines and enhance their potency (Soehnlein and Lindbom, 2010). In response to TNF-α, IL-1β and IL-17 produced by resident macrophages, ECs express E and P-selectins on their surface. These molecules bind to cell surface markers constitutively expressed on neutrophils and allow the immune cells to adhere to ECs. (Borregaard, 2010). E and P-selectins bind to P-selectin glycoprotein ligand-1 (PSGL-1) expressed on neutrophils and this binding mediates the initial contact between neutrophils and ECs. The interaction between E-selectin and CD44 expressed on neutrophils mediates the slower rolling of neutrophils, and E-selectin ligand-1 (ESL-1) co-operates with both PSGL-1 and CD44 to allow both the initial contact and slow rolling to occur (Hidalgo et al., 2007). Chemokines bound to the EC surface then bind to their respective receptors expressed on neutrophils and this triggers the activation of the β2 integrins, lymphocyte function associated antigen-1 and Mac-1. These interact with intracellular cell adhesion molecule (ICAM)-1 and 2, expressed on ECs, allowing firm adhesion to occur (Borregaard, 2010; Williams et al., 2011). Newly recruited neutrophils can roll on adherent neutrophils through interactions between L-selectin on the circulating neutrophils and PSGL-1 on the adherent neutrophils (Bargetze et al., 1994; Sperandio et al., 2003). Following firm adhesion, extravasation of neutrophils through the endothelial layer occurs by which the immune cells can either pass between cell-cell junctions of ECs (paracellular migration) or directly through ECs (transcellular migration) into the underlying tissue (Zen and Parkos, 2005).

A well-defined characteristic of neutrophils is their ability to generate ROS through a respiratory burst; an abrupt process by which molecular oxygen is reduced to more reactive forms (Nathan, 2006). This is catalysed by the phagocyte NADPH oxidase (phox), which is an enzyme complex composed of five components; three localised in the cytosol and two at the PM. Phox functions at the PM upon translocation of the cytosolic components to the PM, leading to assembly of the functional complex (Babior et al., 2002).
Neutrophils exert their effector functions through three subsets of granules, which each contain characteristic proteins. Primary (azurophil) granules are characterised by myeloperoxidase (MPO) content, secondary (specific) granules by lactoferrin and tertiary (gelatinous) granules by gelatinase (Borregaard, 2010). During extravasation, gelatinous granules undergo exocytosis and the released MMPs degrade the extracellular matrix (EM), facilitating the further entry of immune cells (Faurschou and Borregaard, 2003). Upon encountering bacteria in the underlying tissue, exocytosis of specific granules occurs. This leads to release of proteins such as lactoferrin, which sequesters iron required for bacterial survival, and LL37, which has antimicrobial properties (Nathan, 2006). This is followed by exocytosis of azurophil granules resulting in the release of MPO. MPO converts $H_2O_2$ produced by phox into more potent ROS, such as HOBr, HOI and HOCl, which have enhanced microbicidal properties (Nathan, 2006). Exocytosis of azurophil granules leads to the release of bacterial permeability increasing (BPI) protein, which specifically targets Gram-negative bacteria affecting their growth and causes damage to their inner membrane (Faurschou and Borregaard, 2003). The N-terminal domain of BPI protein binds to LPS in the outer membrane of Gram-negative bacteria, whereas the C-terminal domain promotes attachment of bacteria to neutrophils, facilitating phagocytosis (Ooi et al, 1987; Iovine et al, 1997). Furthermore, azurophil degranulation results in the release of broad-specificity antimicrobial agents, termed seprocidins (Faurschou and Borregaard, 2003). There are three such proteins: proteinase 3, elastase and cathepsin G, which have microbicidal properties and also degrade the EM. Additionally, elastase induces the release of cytokines and chemokines from endothelial cells (Owen and Campbell, 1999). Neutrophils also form extracellular traps (NETs) which are composed of proteins from all three types of granule, as well as chromatin. NETs are produced by activated neutrophils and kill bacteria extracellularly and degrade virulence factors (Brinkmann et al, 2004).

**Macrophages**

Macrophages are mononuclear phagocytes which function as a bridge between innate and adaptive immunity through their ability to phagocytose foreign particles and present antigenic peptides to lymphocytes. Macrophages are present throughout the body and play an important role in surveying the surrounding tissue for damage and the presence of invading microorganisms (Murray and Wynn, 2011). They also remove dead cells and toxic materials,
and respond to danger signals through the recognition of PAMPs and DAMPs by their PRRs. Macrophages possess phagocytic receptors that enable them to bind to and internalise opsonised particles (Murray and Wynn, 2011).

Macrophages differentiate from precursor cells, termed monocytes, upon the entry of monocytes from the circulation into tissue. Monocytes possess chemokine receptors and adhesion molecules that allow them to extravasate from the circulation into tissues during an inflammatory response in much the same way as neutrophils do (Geissmann et al, 2010). Selectin–mediated weak adhesion initially occurs between monocytes and ECs, followed by integrin-mediated firm adhesion as a result of chemokine stimulation and then transmigration. The granule components of neutrophils contained within secretory vesicles are involved in the attraction of monocytes to the inflamed tissue by increasing CAM expression on ECs or through activation of monocytes (Soehnlein et al, 2009). One such protein is azurocidin (CAP37/Heparin-binding protein), which is involved in increasing expression of ICAMs and vascular cell adhesion molecule-1 (VCAM-1) on ECs, thereby enhancing the adhesion of monocytes to ECs (Lee T et al, 2003). Azurocidin is also deposited on ECs and promotes monocyte arrest, as well as inducing production of IL-8, MIP-1α and TNF-α by monocytes (Heinzelmann et al, 2001; Soehnlein et al, 2005). Another granule protein responsible for monocyte recruitment is the seprocidin, proteinase-3, which increases ICAM-1 expression on ECs and increases production of MCP-1 by ECs, thereby enhancing monocyte chemotaxis (Taekema-Roelvink et al, 2001). An additional seprocidin, cathepsin G, cleaves CCL15 which enhances its binding to its receptor and therefore increases monocyte chemotaxis (Berahovich et al, 2005). Thus, the initial recruitment of neutrophils to a site of infected or damaged tissue is followed by a second wave of monocyte recruitment in response to release of neutrophil granule proteins.

Monocytes in mice are classified into two types based on their expression of CX3CR1, Gr1 (LY6C) and CCR2. Monocytes which have a low expression of CX3CR1, are Gr1 and CCR2 positive (CX3CR1Lo Gr1+ CCR2+) are termed inflammatory monocytes. However, monocytes that have a high expression of CX3CR1, are Gr1 and CCR2 negative (CX3CR1Hi Gr1- CCR2-) are termed anti-inflammatory or patrolling monocytes (Geissmann et al, 2003; Geissmann et al, 2010). Both types of monocytes in mice are equally abundant, whereas in humans, 90% of the monocytes are of the inflammatory phenotype and only 10% of the patrolling, non-inflammatory phenotype. Patrolling monocytes give rise to the non-
Inflammatory tissue resident macrophages, known as alternatively activated (M2) macrophages, and replenish the population of these cells in the steady state (Geissmann et al., 2003; Geissmann et al., 2010). Inflammatory monocytes are rapidly mobilised for inflammation in response to MCP-1, the ligand for CCR2, and upon entry into tissues, differentiate into inflammatory, classically activated (M1) macrophages (Murray and Wynn, 2011). During differentiation from monocytes to M1 macrophages, expression of CCR2 is initially downregulated and then following increased levels of MCP-1 is downregulated further, possibly through ligand-induced downregulation (Fantuzzi et al., 1999). CCR1 and CCR5 levels are also increased during differentiation together with increased responsiveness to MIP-1α (Kaufmann et al., 2001). M1 macrophages are activated by LPS and IFN-γ, and upon activation, secrete pro-inflammatory cytokines such as TNF-α, IL-1 and IL-12 (Mantovani et al., 2004). Furthermore, they produce ROS, such as nitric oxide (NO) and superoxide, which are toxic to microorganisms. M1 macrophages also secrete MMP2 and MMP9, which degrade the EM, facilitating further entry of monocytes (Mosser and Edwards, 2008; Murray and Wynn, 2011). A list of inflammatory mediators produced by neutrophils and macrophages is summarised in Table 1.2:
<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactoferrin</td>
<td>Sequesters iron required for bacterial survival</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>LL37</td>
<td>Antimicrobial properties</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>MPO</td>
<td>Converts $H_2O_2$ into more potent ROS</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>BPI</td>
<td>Affects bacterial growth, causes damage to their inner membrane and promotes phagocytosis of bacteria by neutrophils</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>Microbicidal properties, degrades EM, increases expression of ICAM-1 on ECs and enhances production of MCP-1 by ECs</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>Microbicidal properties, degrades EM and cleaves CCL15, enhancing monocyte chemotaxis</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Elastase</td>
<td>Microbicidal properties, degrades EM and induces release of cytokines and chemokines from ECs</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Azurocidin</td>
<td>Increases ICAM and VCAM-1 expression on ECs, promotes monocyte arrest, induces production of IL-8, MIP-1α and TNF-α by monocytes</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>TNF-α</td>
<td>See Table 1.1</td>
<td>Macrophages</td>
</tr>
<tr>
<td>IL-1</td>
<td>See Table 1.1</td>
<td>Neutrophils/Macrophages</td>
</tr>
<tr>
<td>IL-12</td>
<td>See Table 1.1</td>
<td>Neutrophils/Macrophages</td>
</tr>
<tr>
<td>NO</td>
<td>Microbicidal properties</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Superoxide</td>
<td>Microbicidal properties</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MMP2</td>
<td>Degrades EM</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MMP9</td>
<td>Degrades EM</td>
<td>Macrophages</td>
</tr>
</tbody>
</table>

Table 1.2: Summary of the Inflammatory Mediators Produced by Neutrophils and Macrophages.
1. Introduction

1.1.4 Signalling Pathways Involved in Acute Inflammation

The Toll-Like Receptor (TLR) Signalling Pathway

TLRs are a family of PRRs expressed on macrophages, dendritic cells, B cells, T cells and non-immune cells such as fibroblasts and epithelial cells. They recognise a range of microbial and viral PAMPs, and upon this recognition, expression of genes involved in antimicrobial and antiviral defence is induced (Akira et al., 2006). TLRs are type-1 TM proteins that possess extracellular leucine-rich repeats (LRRs) for PAMP recognition and a cytosolic Toll/IL-1R (TIR) domain for intracellular signalling (Kumar et al., 2011).

In humans, 10 TLRs have been identified and 12 in mice, with TLRs 1 - 9 being conserved between the two species (Kumar et al, 2011). TLRs 1, 2, 4, 5 and 6 are expressed at the cell surface and recognise a diverse range of microbial PAMPs. TLRs 3, 7, 8 and 9 are expressed in intracellular vesicles such as the ER, endosomes and lysosomes and recognise nucleic acids from bacteria and viruses (Kawai and Akira, 2010). TLR2 recognises components from bacteria, such as peptidoglycan, LTA and lipopeptides, and specificity for recognition of lipopeptides is conferred through formation of a heterodimer with either TLR 1 or 6 (Takeuchi and Akira, 2010). Each heterodimer recognises a distinct set of ligands, with TLR2/TLR1 recognising tri-acylated lipopeptides and TLR2/TLR6 recognising di-acylated lipopeptides (Jin et al, 2007; Kang et al, 2009). TLR4 is responsible for the recognition of LPS and forms a complex with MD2. Two complexes of TLR4-MD2-LPS then come together to form a functional homodimer (Park et al, 2009). TLR5 binds to flagellin from Gram positive and Gram negative bacteria (Hayashi et al, 2001). TLR3 recognises double stranded (ds) RNA from viruses and binding of dsRNA to the LRRs of TLR3 results in dimerisation of TLR3, leading to activation of signalling (Liu et al, 2008). TLR7 and TLR8 bind to single stranded (ss) RNA from RNA viruses, and TLR9 to bacterial genomic DNA (Hemmi et al, 2000; Heil et al, 2004). TLRs 1, 2, 5 and 6 activate signalling pathways leading to induction of expression of inflammatory cytokines, whereas TLRs 7 and 9 activate pathways leading to induction of expression of type I interferons. TLRs 3 and 4 are capable of activating pathways leading to induction of expression of both types of cytokines (Kawai and Akira, 2010; Kumar et al, 2011). Table 1.3 summarises the localisation of TLRs and the origin of their recognised ligands:

Table 1.3 summarises the localisation of TLRs and the origin of their recognised ligands:
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<table>
<thead>
<tr>
<th>Name of TLR</th>
<th>Localisation</th>
<th>Recognised Ligands</th>
<th>Origin of Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1/2</td>
<td>Cell surface</td>
<td>Tri-acylated lipopeptides</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR2</td>
<td>Cell surface</td>
<td>Peptidoglycan, LTA</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR3</td>
<td>Intracellular vesicles</td>
<td>dsRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR4</td>
<td>Cell surface</td>
<td>LPS</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR5</td>
<td>Cell surface</td>
<td>Flagellin</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR6/2</td>
<td>Cell surface</td>
<td>Di-acylated lipopeptides</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR7</td>
<td>Intracellular vesicles</td>
<td>ssRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR8</td>
<td>Intracellular vesicles</td>
<td>ssRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR9</td>
<td>Intracellular vesicles</td>
<td>DNA</td>
<td>Bacteria and Viruses</td>
</tr>
</tbody>
</table>

Table 1.3: Summary of the Localisation of TLRs and the Origins of their Recognised Ligands.

There are four adaptor proteins that associate with the TIR domain of TLRs following ligand binding and an induced conformational change in the domain allowing recruitment to occur: MyD88, TRIF, TIRAP and TRAM (Akira et al, 2006; Kumar et al, 2011). All TLRs require MyD88 for their signalling except for TLR3, which recruits only TRIF. TLRs 1, 2, 4 and 6 also recruit TIRAP in addition to MyD88, and TLR4 recruits TRIF and TRAM in the absence of MyD88. Thus, TLR4 recruits all four adaptor proteins and can signal in a MyD88-dependent and MyD88-independent manner (Kumar et al, 2011).

In MyD88-dependent signalling, MyD88 is either directly recruited to the TIR domain of TLRs or in the case of TLR2 and TLR4 signalling, is recruited following prior recruitment of TIRAP to the TIR domain (Yamamoto et al, 2002; Akira et al, 2006). MyD88 then interacts with IL-1 receptor-activated kinase (IRAK) -4, which then sequentially activates IRAK-1 and IRAK-2 through phosphorylation. Following this activation, the IRAKs then dissociate from MyD88 and interact with the E3 ubiquitin ligase, tumour necrosis factor associated factor (TRAF) 6 (Kawagoe et al, 2008; Takeuchi and Akira, 2010). TRAF6 forms a complex with the E2 ubiquitin-conjugating enzyme complex, Ubc13 and Uev1A, which results in self-ubiquitination of TRAF6 (Deng et al, 2000; Takeuchi and Akira, 2010). Transforming growth factor-beta (TGF-β) activated protein kinase binding (TAB) 2 and TAB3 are also ubiquitinated and form part of a complex together with TAB1 and TGF-β activated protein kinase (TAK1), which leads to activation of TAK1. TAK1 then activates a signalling cascade involving the mitogen activated protein (MAP) kinases extracellular signal-regulated kinases (ERK) 1 and 2, p38 and c-Jun N-terminal kinases (JNK). This leads to activation of
transcription factor activator protein-1 (AP-1) and induction of expression of genes encoding pro-inflammatory cytokines (Kawai and Akira, 2010). TAK1 also phosphorylates IκB kinase (IKK)-β, which is part of a complex along with IKK-α and nuclear factor kappa B (NF-κB) essential modulator, and this leads to phosphorylation and ubiquitin-mediated degradation of IκB. NF-κB is released and translocates to the nucleus to induce expression of genes encoding pro-inflammatory cytokines, such as IL-1, IL-6, IL-12 and TNF-α (Medzhitov et al, 1997; Kaisho et al, 2001; Takeuchi and Akira, 2010).

In MyD88-independent signalling, TLR3 directly recruits TRIF to its TIR domain, whereas TLR4 requires recruitment of TRAM prior to recruitment of TRIF (Yamamoto et al, 2003a; Yamamoto et al, 2003b). TRIF then associates with TRAF3 and TRAF6 through its N-terminal TRAF-binding motifs (Takeuchi and Akira, 2010). TRIF also has a C-terminal receptor-interacting protein (RIP) homotypic interaction motif which is involved in interacting with RIP1 (Meylan et al, 2004). RIP1 is then ubiquitinated and together with TRAF6, lead to NF-κB activation in a similar manner to MyD88-dependent signalling. TRAF3 undergoes self-ubiquitination and activates TANK-binding kinase-1 (TBK1) and IKK-i, which are associated with NAK-associated protein-1 and SINTBAD (Sasai et al, 2005; Ryzhakov and Randow, 2007; Takeuchi and Akira, 2010). This leads to activation of IRF3 and IRF7 which results in their translocation to the nucleus and induction of expression of genes encoding type 1 interferons (Takeuchi and Akira, 2010).

The NLRP3 Inflammasome

NLRs are a family of highly evolutionarily conserved cytoplasmic PRRs that are primarily expressed in immune cells such as macrophages and dendritic cells, but also in other non-immune cells such as epithelial cells (Chen et al, 2009). One member of this family that has gained a lot of attention recently is NLRP3, which forms part of a molecular platform known as the NLRP3 inflammasome that results in the activation and secretion of mature IL-1β. It is composed of three components: NLRP3, the adaptor protein apoptosis-associated speck like protein containing a caspase activation and recruitment domain (ASC) and the cysteine protease, caspase-1 (Fig 1.1; Agostini et al, 2004). The NLRP3 inflammasome is expressed in monocytes, neutrophils, dendritic cells, T and B lymphocytes, as well as epithelial cells, along the gastrointestinal tract. It is also expressed in the oral cavity and oesophagus.
In addition to the NLRP3 inflammasome, there are other inflammasomes that are involved in the processing of caspase-1, which are each activated by different danger signals. The NLRP1 inflammasome and the IPAF inflammasome, which do not require ASC, are activated by anthrax toxin and secretion signals of Gram negative bacteria, respectively. The AIM2 inflammasome, which does require ASC, is activated by bacterial and viral DNA (Schroder and Tschopp, 2010).

Various danger signals can activate the NLRP3 inflammasome, such as PAMPs, asbestos, ATP and crystalline particles (Tschopp and Schroder, 2010). Several of these activators are internalised at the cell surface and once in the lysosome, cause destabilisation of the lysosomal membrane. This leads to release of the lysosomal protease, cathepsin B, which activates NLRP3 through an unknown mechanism (Hornung et al., 2008; Tschopp and Schroder, 2010). However, not all of these danger signals activate cathepsin B, such as ATP, indicating an activation mechanism independent of the lysosome (Hornung et al., 2008). All known activators of the NLRP3 inflammasome, however, appear to lead to the production of ROS although the source of ROS is currently a matter of controversy (Schroder et al., 2010; Tschopp and Schroder, 2010). Studies by Dostert et al have shown that inhibition of NADPH oxidase with diphenylene iodonium or apocynin result in blocking production of mature IL-1β. However, the mitochondrial complex II inhibitor TTFA, which prevents mitochondrial ROS production, had no effect on production of mature IL-1β (Dostert et al., 2008). It was also shown that knockdown of the p22phox subunit of NADPH oxidase decreased IL-1β secretion (Dostert et al., 2009). Thus Dostert et al propose that the source of ROS is NADPH oxidase and not mitochondria (Dostert et al., 2008; Dostert et al., 2009). In contrast, another study has shown that IL-1β secretion is independent of the NADPH oxidases Nox 1 – 4 (van Bruggen et al., 2010). Zhou et al propose that the source of ROS species is derived from the mitochondria and this is shown by inhibition of complex II with TTFA resulting in a block in IL-1β secretion. Furthermore, inhibition of complexes I and III, which results in ROS production, leads to IL-1β secretion (Zhou et al., 2011). Complexes I, II and III are electron-transporting complexes that play a sequential role in the formation of ROS from oxygen through a series of redox reactions (Raha and Robinson, 2000). Thus, it appears that whilst ROS are required for the activation of the NLRP3 inflammasome, the source is still debatable.
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The generation of ROS results in thioredoxin dissociating from thioredoxin-interacting protein (TXNIP), allowing TXNIP to bind to NLRP3, activating it (Zhou et al., 2010). Upon activation, NLRP3 oligomerises through homotypic interactions between NACHT domains and uses its pyrin domains (PYDs) to interact with PYDs of ASC. ASC then recruits pro-caspase-1 and they interact through their caspase activation and recruitment domains (CARDs). This leads to auto-activation of pro-caspase-1, which then cleaves IL-1β from its pro-form to its active form (Schroder et al., 2010; Tschopp and Schroder, 2010). The induction of expression of the pro-form of IL-1β occurs via signalling through PRRs, such as TLRs which generate IL-1β through NF-κB signalling (Chen et al., 2009; Stutz et al., 2009). Thus, two signals are required for full activation of IL-1β: the first is a priming signal necessary for inducing expression of the pro-form of IL-1β and a second signal resulting in activation of the inflammasome leading to cleavage of the pro-form of IL-1β to its active form. A schematic of NLRP3 inflammasome activation is shown in Fig 1.1:
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Figure 1.1: Schematic Representation of the Two Signal Hypothesis of NLRP3 Inflammasome Activation. A priming signal (Signal 1) that activates TLR signalling leads to induction of expression of pro-IL-1β in a NF-κB-dependent manner. Various danger signals (Signal 2) can lead to the production of ROS (1), which activates the NLRP3 inflammasome (2). Several danger signals are internalised at the cell surface and once in the lysosome, cause rupture of the lysosomal membrane, leading to release of cathepsin B. Cathepsin B activates the NLRP3 inflammasome through an unknown mechanism (3). The end result of activation of the inflammasome is production of mature, active IL-1β from the pro-form in a caspase-1 dependent manner (4). Inset indicates colour-coded domains of the core components of the inflammasome (Figure adapted from Tschopp and Schroder, 2010).

Following its release, mature IL-1β binds to its receptor IL-1RI and a heterotrimeric complex is formed with the co-receptor IL-1RAcP. MyD88 is then recruited to the TIR domain of IL-
1RI and IL-1RAcP, and recruits IRAK 4. This leads to auto-phosphorylation of IRAK4, resulting in recruitment of IRAKs 1 and 2 (Weber et al., 2010; Dinarello, 2011). The ubiquitin E3 ligase TRAF6 is then recruited and ubiquitinates TAB2, TAB3 and TAK-1 (Weber et al., 2010). Following this, TAK-1 associates with TRAF6 and is phosphorylated leading to activation of signalling through p38 kinase and JNK. This results in activation of AP-1 and its translocation to the nucleus. TAK-1 also activates IKKB, which then phosphorylates IxB, leading to activation of NF-κB and its translocation to the nucleus. AP-1 and NF-κB then cooperatively induce expression of a variety of pro-inflammatory cytokines and chemokines including IL-6, IL-8, MCP-1 and IL-1β itself (Weber et al., 2010; Stienstra et al., 2012).

*Mitogen Activated Protein Kinase (MAPK) Signalling Pathways*

MAPKs are a family of Ser/Thr kinases that play an important role in inflammatory responses by affecting cell differentiation, proliferation and apoptosis. MAP kinases themselves are activated by phosphorylation of Thr and Tyr residues by dual specificity MAP kinase kinases (MAPKKs), which are in turn activated through phosphorylation by MAP kinase kinase kinases (MAPKKKs). There are three types of MAPKs: ERKs, JNKs and p38 (Hommes et al., 2003). ERKs are activated by growth factors and mitogens, and to a lesser extent by cytokines and environmental stresses, whereas JNKs and p38 are involved in pathways activated by cytokines such as TNF-α or IL-1, or through cellular or environmental stresses (Ip and Davis, 1998; Zarubin and Han, 2005; Nishimoto and Nishida, 2006). Signalling through ERKs results in phosphorylation of transcription factors such as Elk-1, c-Myc and c-fos, and this results in induction of expression of genes involved in cell growth and proliferation (Nishimoto and Nishida, 2006; Cargnello and Roux, 2011). Signalling through JNKs results in their binding to c-Jun, a member of the AP-1 family of transcription factors, and phosphorylating it (Hibi et al., 1993). As well as c-Jun, JNK is also capable of phosphorylating other AP-1 members such as JunB, JunD and ATF2, which form part of the AP-1 transcriptional complex (Ip and Davis, 1998; Davis, 2000). Phosphorylation leads to translocation of the AP-1 complex to the nucleus and induction of expression of genes encoding pro-inflammatory cytokines such as IL-1 and IL-6, as well as chemokines such as MCP-1 (Vukic et al., 2009). Signalling through p38 results in activation of a range of transcription factors including those of the AP-1 family (Ono and Han, 2000; Zarubin and
Han, 2005). Thus, several of the genes whose expression is induced through p38 signalling are common to that of JNK signalling, such as IL-1\(\beta\), IL-6, TNF-\(\alpha\), MCP-1 and IL-8 (Garcia et al, 1998; Miyazawa et al, 1998; Goebeler et al, 1999; Marie et al, 1999).
1.2 Resolution of Acute Inflammation

Resolution of inflammation is an active process that is required to prevent further tissue damage to the host once the inflammatory stimulus has been eradicated. This is necessary to avoid chronic inflammation and potentially disease from occurring (Serhan et al, 2008; Soehnlein and Lindbom, 2010).

As well as their role in promoting acute inflammation, lipid mediators play an important part in the resolution of inflammation and undergo a ‘class switch’ during the change from promotion of inflammation to its resolution (Levy et al, 2001). This results in an alteration in production of pro-inflammatory lipid mediators such as prostaglandins and leukotrienes to anti-inflammatory mediators (Soehnlein and Lindbom, 2010). One of these groups is the lipoxins, which are derived from AA (Serhan et al, 2008). Lipoxins are produced by neutrophils following their interaction with other cells, such as platelets and epithelial cells, and their production by macrophages occurs following uptake of apoptotic neutrophils in a TGF-β-dependent manner (Freire-de-Lima et al, 2006; Soehnlein and Lindbom, 2010). Lipoxin A4 inhibits neutrophil entry to inflammatory sites, reduces ROS production by neutrophils, decreases NF-κB activation and production of pro-inflammatory cytokines and chemokines by neutrophils, monocytes and macrophages (József et al, 2002). Whilst lipoxins reduce the transmigration of neutrophils, they promote that of monocytes (Maddox et al, 1997).

Another group of anti-inflammatory lipid mediators are the resolvins, which are derived from the omega (ω)-3 unsaturated fatty acids (UFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). E-resolvins are derived from EPA and D-resolvins from DHA (Serhan et al, 2008). E-resolvins have been shown to block neutrophil transmigration to inflammatory sites and inhibit TNF-α-mediated NF-κB activation (Tjonahen et al, 2006; Arita et al, 2007; Serhan et al, 2008). D-resolvins also inhibit neutrophil transmigration (Soehnlein and Lindbom, 2010). A third group of lipid mediators involved in the resolution of inflammation are the protectins, which are derived from DHA (Schwab et al, 2007). Protectin D1, together with resolvin E1, increases expression of CCR5 on apoptotic neutrophils which results in binding of CCL3 and CCL5, thus preventing further neutrophil...
influx (Ariel et al, 2006). Protectin D1 and resolvin E1 also promote phagocytosis of neutrophils (Schwab et al, 2007).

The resolution of acute inflammation is dependent upon the removal of apoptotic cells by phagocytes. This is to prevent cell death by necrosis from occurring and exacerbating inflammation further through the release of inflammatory mediators from dying cells. To enable their recognition by phagocytes, apoptotic cells display ‘eat-me’ signals on their surface. One of these is phosphatidylserine which binds to a phosphatidylserine receptor expressed on phagocytes, as well as other receptors, such as scavenger receptors and integrins (Grimsley and Ravichandran, 2003; Li et al, 2003). This receptor-ligand interaction is required for engulfment of apoptotic cells by phagocytes. Another ‘eat me’ signal is annexin A1, which is constitutively expressed in the cytoplasm of neutrophils, monocytes and macrophages but upon activation, translocates to the PM. Annexin A1 then engages its receptor, ALXR, and activates signalling in an autocrine, paracrine or juxtacrine manner (Perretti and D’Acquisto, 2009). Annexin A1 reduces neutrophil transmigration, promotes apoptosis of neutrophils and their phagocytosis by macrophages (Lim et al, 1998; Solito et al, 2003; Scannell et al, 2007).

A second type of signal involved in the recognition of apoptotic cells by phagocytes are ‘find-me’ signals which are released by apoptotic cells and have chemoattractive effects on phagocytes (Grimsley and Ravichandran, 2003). An example of this is lactoferrin, which is released by neutrophils and a variety of other apoptotic cells, and reduces neutrophil transmigration and activation (Bournazou et al, 2009). Lysophosphatidylcholine, which results from the hydrolysis of phosphatidylcholine by PLA2 in a caspase-3-dependent manner, is another ‘find-me’ signal that attracts monocytes and macrophages expressing the receptor for lysophosphatidylcholine, G2A, on their surface to apoptotic cells (Lauber et al, 2003). The chemokine CX3CL1 is released from apoptotic cells in a caspase-3 dependent manner and attracts macrophages which express the receptor for CX3CL1, CX3CR1, on their surface (Truman et al, 2008). Caspase-dependent release of nucleotides such as ATP and UTP from apoptotic cells has a chemoattractive effect on monocytes that express the receptor P2Y2 on their surface (Elliott et al, 2009).

Engulfment of apoptotic leukocytes by macrophages results in a reduction in the secretion of pro-inflammatory cytokines such as IL-1β, IL-12 and TNF-α and chemokines such as IL-8.
There is also an increase in secretion of IL-10 and TGF-β, both of which are anti-inflammatory cytokines (Voll et al., 1997; Fadok et al., 1998). TGF-β itself reduces secretion of IL-8 and the pro-inflammatory cytokines, and platelet-activating factor also achieves this effect. Phagocytosis of apoptotic cells increases levels of prostaglandin E2, which itself decreases levels of pro-inflammatory cytokines and IL-8 (Fadok et al., 1998). Apoptotic cell uptake additionally reduces the expression of iNOS in macrophages and the release of NO (Freire-de-Lima et al., 2006). Vascular endothelial growth factor is released following phagocytosis of apoptotic cells to initiate the process of tissue repair (Soehnlein and Lindbom, 2010).

Neutrophils have been demonstrated to display retrograde transmigration from inflammatory sites during the resolution phase as well as undergoing apoptosis and subsequently being removed by phagocytosis, as described previously (Matias et al., 2006). Inflammatory macrophages, however, do not undergo apoptosis and only emigrate from inflammatory sites to the lymphatic system (Bellingan et al., 1996). During the resolution of acute inflammation in the peritoneum, β1 integrins on macrophages interact with ICAM and VCAM-1 on mesothelium cells and this interaction is required for their exit (Bellingan et al., 2002). The β2 integrin, Mac-1, is also required for the efflux of macrophages from the peritoneum to the lymph nodes following inflammation and shedding of β2 integrins from macrophages in a metalloprotease-dependent manner is also necessary (Cao et al., 2005; Gomez et al., 2012). The trafficking of macrophages to lymph nodes may facilitate antigen presentation to lymphocytes and initiate the adaptive immune response (Cao et al., 2005). Undifferentiated monocytes also emigrate to the lymph nodes and differentiate into dendritic cells where they carry out antigen presentation functions too (Randolph et al., 1999). The resolution phase is characterised by an influx of lymphocytes; primarily B cells but also T cells and NK cells. These cells are not required to terminate the inflammatory response but are involved in protecting against secondary infection. The signal through which lymphocytes appear is unknown but is proposed to be COX-2 dependent (Rajakariar et al., 2008).
1.3 Chronic Inflammation Associated with Metabolic Diseases

1.3.1 Obesity

Obesity is a disease caused by excess accumulation of fat in the body either due to dietary intake of fat or from rare genetic disorders. Whilst very little is known about the acute inflammation associated with obesity, other than a transient influx of neutrophils to adipose tissue (AT) between 3 – 7 days following feeding on a high fat diet in mice, more is known about the chronic inflammation associated with this disease (Elgazar-Carmon \textit{et al}, 2008). \textit{In vivo} studies have shown that chronically obese mice, both those fed on a high fat diet for 16 weeks and those with genetic disorders fed a normal diet, display a significant upregulation of various inflammatory genes highly expressed on macrophages, including F4/80, ADAM8 and CD68 when compared to mice fed a standard chow diet (Xu \textit{et al}, 2003). This upregulation occurs only in white adipose tissue (WAT) and not in the liver, muscle or spleen. Furthermore, an infiltration of macrophages in WAT has been shown in these mice (Xu \textit{et al}, 2003).

Various \textit{in vitro} studies have been carried out to understand the mechanisms leading to the chronic inflammation associated with obesity. Such studies propose that TNF-α, produced by resident macrophages in AT, stimulates lipolysis in adipocytes through activation of ERK signalling (Souza \textit{et al}, 2003). This results in the release of free fatty acids (FFAs), which are proposed to activate signalling through MAPKs, leading to further production of TNF-α and thus enhancing lipolysis creating a vicious cycle (Suganami \textit{et al}, 2005). FFAs are also known to activate signalling through the TLR4 pathway in both adipocytes and macrophages resulting in activation of NF-κB (Suganami \textit{et al}, 2007). Production of TNF-α by resident macrophages leads to paracrine induction of MCP-1 expression in adipocytes resulting in the recruitment of macrophages to AT (Suganami \textit{et al}, 2005). The infiltrated macrophages in obese mice tend to be of a classically-activated (M1) pro-inflammatory phenotype, as opposed to the alternatively-activated (M2) anti-inflammatory phenotype which are abundant in the adipose tissue of lean mice (Lumeng \textit{et al}, 2007). This is shown by increased levels in expression of genes characteristic of M1 macrophages such as TNF-α and iNOS. In contrast, macrophages from the AT of lean individuals show high levels of genes characteristic of the
M2 phenotype, such as mannose receptor C type 2, arginase 1 and IL-10 (Lumeng et al, 2007). The scavenger receptor CD36, which is proposed to play a role in FA uptake, expressed on macrophages has been shown to be necessary for the M1 phenotype of macrophages in AT following feeding on a high fat diet in mice (Kennedy et al, 2011). Infiltrated macrophages contribute further to TNF-α production, exacerbating the inflammation even more. The cycle of inflammatory events occurring in adipose tissue during obesity is summarised in Fig 1.2:
1. Introduction

Figure 1.2: Cycle of Obesity-induced Inflammation in Adipose Tissue. Tumour necrosis factor alpha (TNF-α), produced by resident macrophages in adipose tissue, activates lipolysis in adipocytes leading to the release of free fatty acids (FFAs). Nuclear factor kappa B (NF-κB) signalling is also activated leading to the induction of MCP-1 expression, which recruits macrophages to the adipose tissue. FFAs activate TLR4 signalling in macrophages enhancing TNF-α production and creating a vicious cycle of inflammation (Figure adapted from Suganami et al, 2007).

Regulatory T (Treg) cells are a subpopulation of T cells which have immunosuppressive functions and are characterised by high expression levels of IL-10. They are enriched in the AT of lean mice where they maintain a non-inflammatory state by controlling the Th1 response. However, in the AT of mouse models of obesity, numbers of Treg cells are
reduced (Feuerer et al., 2009). The adipocyte-derived hormone, leptin, which controls nutritional status may be involved in the reduction in the Treg cell population in AT during obesity. It is proposed that during obesity, the increased secretion of leptin by adipocytes reduces the infiltration of Treg cells into AT by decreasing their proliferation. Increased leptin secretion also promotes infiltration of AT by Th1 cells, which leads to release of pro-inflammatory cytokines by these cells and establishment of a state of inflammation in AT (Matarese et al., 2010). Studies have demonstrated that leptin neutralisation with a blocking antibody stimulates Treg proliferation in mice, and that leptin deficient (ob/ob) and leptin receptor deficient (db/db) mice also show increased Treg cell proliferation (De Rosa et al., 2007). In vitro experiments have shown that the presence of inflammatory macrophages inhibits the differentiation of Treg cells (Deiuliis et al., 2011). Thus, in AT during obesity, it appears that there are changes in the profiles of both macrophages and T cells, which result in a pro-inflammatory state.

Liver X receptors (LXRs) are a family of nuclear receptors which have been demonstrated to reduce MCP-1 and IL-6 secretion in human adipocytes and thus antagonise the inflammation associated with obesity (Fernández-Veledo et al., 2009). LXRs are also expressed in macrophages, but their role in adipose tissue during the inflammation associated in obesity is unknown. The function of LXRs in macrophages appears to be species specific with an anti-inflammatory effect observed in mice and a pro-inflammatory effect in humans. In mice, activation of LXRs with synthetic agonists leads to a decrease in iNOS activity and expression as well as a reduction in expression of IL-6, IL-1β, MCP-1 and MMP-9 through interference with NF-κB signalling following LPS activation (Castrillo et al., 2003; Joseph et al., 2003). In contrast, in human macrophages, activation of LXRs with synthetic agonists increases expression of MCP-1 and TNF-α following LPS stimulation. Additionally, activation of LXRs enhances expression of NADPH oxidase subunits and increases activity of NADPH oxidase in human, but not murine, macrophages. LXR activation also results in phosphorylation of JNK, ERK and p38 and increases expression of TLR4 at the transcriptional level through binding to the TLR4 promoter, which does not occur in mouse macrophages (Fontaine et al., 2007). Given the conflicting activities of LXRs in human and murine macrophages, it will be interesting to see what role these nuclear receptors play in macrophages of mouse models of obesity compared to obese human patients.
1.3.2 Type II Diabetes

The inflammation associated with obesity can lead to the development of insulin resistance in adipose tissue, liver and skeletal muscle, resulting in type II diabetes (Zeyda and Stulnig, 2009). This occurs via the activation of JNK and NF-κB signalling (Shoelson et al, 2006). Lipid accumulation in adipose tissue in mouse models of obesity leads to both increased expression and activation of NADPH oxidase, resulting in elevated levels of ROS in adipocytes that activate JNK and NF-κB signalling (Furukawa et al, 2004; Shoelson et al, 2006). Furthermore, saturated fatty acids (SFAs) can form ceramides that interfere with insulin signalling possibly through activation of JNK and NF-κB pathways (Straczkowski et al, 2004; Shoelson et al, 2006). Ceramides also activate protein phosphatase 2A, which leads to inhibition of Akt/PKB, thus interfering with insulin signalling (Dobrowsky et al, 1993; Resjö et al, 2002). In vivo studies have shown that obesity generates stress in the ER that results in activation of JNKs (Özkan et al, 2004). ER stress also downregulates expression of the glucose transporter GLUT4 in adipocytes in vitro, which is required for glucose uptake in response to insulin signalling (Miller et al, 2007). JNK promotes insulin resistance by phosphorylating insulin receptor substrate (IRS)-1 (Lee Y et al, 2003). NF-κB, however, does so by inducing expression of target genes whose products promote insulin resistance (Shoelson et al, 2006). LXRα improve insulin sensitivity through preventing activation of JNK 1/2 and thus IRS-1 phosphorylation in human adipocytes. Additionally, activation of LXRα with agonists stimulates glucose uptake and promotes phosphorylation of Akt (Fernández-Veledo et al, 2008; Fernández-Veledo et al, 2009).

1.3.3 Atherosclerosis

Another inflammatory disease associated with accumulation of excess lipid in the body is atherosclerosis, which is characterised by the accumulation of fatty deposits in blood vessel walls. This results in narrowing of the lumen which reduces blood flow and can lead to cardiovascular complications. Low density lipoprotein (LDL) particles that have accumulated in the arterial intima become oxidised and the degree of oxidation affects the ability of these molecules to engage receptors (Glass and Witztum, 2001). Partially oxidised,
or minimally modified (mm), LDL is still able to bind to the LDL receptor (LDLR), whereas extensively oxidised LDL (oxLDL) is unable to bind to the LDLR, but can engage scavenger receptors on macrophages (Glass and Witztum, 2001). The oxidation of LDL is proposed to be due to ROS produced by ECs, macrophages and smooth muscle cells (SMCs). Oxidation of LDL molecules induces activation of ECs, resulting in upregulation of expression of CAMs on the surface of ECs, as well as release of chemoattractants (Rocha and Libby, 2009). E and P selectins have been shown to be required for the formation and progression of atherosclerotic lesions, indicating their importance as CAMs (Dong et al, 1998). Studies in mice have also demonstrated the requirement of both MCP-1 and its receptor CCR2 for the development of atherosclerosis (Boring et al, 1998; Gu et al, 1998). Monocytes are recruited which subsequently differentiate into macrophages as a result of macrophage colony stimulating factor release from ECs (Rajavashisth et al, 1990). Differentiated macrophages then bind to and internalise oxLDL, most likely through CD36 engagement (Febbraio et al, 2000). This generates lipid droplets in the macrophage cytoplasm, resulting in the macrophages becoming foam cells (Moore and Tabas, 2011). An aggregation of foam cells leads to the formation of an atherosclerotic plaque in the blood vessel wall (Rocha and Libby, 2009).
1.4 Zebrafish (Danio rerio) as Model Organisms in Immunological Research

1.4.1 Advantages and Limitations of Using Zebrafish to Understand Acute Diet-Associated Inflammation of Metabolic Diseases

At present, much is known about the chronic inflammation associated with diseases of the metabolic syndrome in terms of the cell populations involved, the signalling pathways activated and the localisation of this inflammation in sites such as AT and the vasculature. However, what is known about the signalling pathways involved derives largely from information gained from in vitro studies. Furthermore, there is a lack of real time in vivo information on the infiltration of immune cells at inflammatory sites. Despite these shortcomings, the acute inflammation that occurs following feeding on a high fat diet is much more poorly understood in terms of the localisation of the inflammation, and the cell types and signalling pathways involved. This is particularly the case for a feeding period in the timescale of a few hours.

The zebrafish (Danio rerio) may potentially shed some light on the mechanisms of acute inflammation following high fat feeding due to a number of advantages it has over other systems. This model organism could also provide further information on the chronic inflammation associated with the metabolic syndrome. Zebrafish larvae are optically transparent, permitting imaging at the organismal level and facilitating localisation of inflammation following acute feeding. There are a variety of transgenic strains available which express fluorescent proteins under promoters of immune cells. This allows the localisation and behaviour of these cells to be studied in vivo at the organismal level in real time after an acute feeding period which cannot be carried out in mice or other mammalian systems. Wholemount antibody staining can also be performed on zebrafish larvae in situations where there are no available transgenic strains. In mice, this technique can only be applied on tissue sections and is considerably more laborious, time consuming and does not provide information at the organismal level. The optical transparency of zebrafish larvae also allows the use of fluorogenic probes in vivo to investigate, for example, the activation of...
signalling pathways in acute diet-associated inflammation. In other systems, this technique can only be applied in vitro, limiting its applications. Similarly, the use of small molecule pharmacological inhibitors to investigate signalling pathways is largely restricted to in vitro use in mammalian systems. Zebrafish, however, are very amenable to this treatment as the inhibitors can be administered into the water. Thus, this allows the role of signalling pathways involved in acute inflammation associated with a high fat diet to be investigated in a high-throughput manner.

Morpholinos (MO) are oligonucleotides that block translation or splicing and can be used at the one-cell stage to transiently knock down genes in zebrafish embryos through microinjection (Nasevicius and Ekker, 2000). The effects of these knockdowns on immune signalling pathways involved in acute diet-associated inflammation can then be studied. Because zebrafish produce large numbers of offspring in a clutch through rapid external fertilisation, the developing embryos can be utilised without sacrificing the parent. Thus, a lot of embryos can be injected in a single experiment shortly after breeding the parents, which cannot be done in mammalian systems. Furthermore, in mice, the process of generating null mutants is considerably more time consuming than the transient gene knockdown that can be performed in zebrafish. The innate immune system in zebrafish develops well before full development of the adaptive immune system, which takes 4 – 6 weeks to fully develop (Lam et al, 2002; Sullivan and Kim, 2008). Therefore, the innate immune responses can be studied with or without involvement of the adaptive immune system (Sullivan and Kim, 2008).

As a model organism, the zebrafish itself has disadvantages. The entire zebrafish genome underwent a duplication event during evolution of teleost fish, which means that functional redundancy may occur with certain genes (Postlethwait et al, 2000). This may be beneficial when generating mutants which would otherwise be embryonic lethal but this functional redundancy hampers the ability to use morpholinos in gene knockdown. This is because it is possible that a paralogue of the gene being knocked down can exert a compensatory effect. A further limitation in the use of morpholinos is that if the gene of interest is involved in the early stages of development, it may result in an embryonic lethal phenotype preventing its effects at the desired stages from being studied. The innate immune system of zebrafish also differs in its ability to respond to external stimuli to that of mammals. This is due to differences in the function of accessory molecules between zebrafish and mammals or a lack
of certain accessory molecules in zebrafish that are present in mammalian systems (Iliev et al, 2005).

Zebrafish have been recently used to model the chronic basis of atherosclerosis. Feeding a high cholesterol diet to adult fish for 8 – 12 weeks results in hypercholesterolaemia, lipoprotein oxidation and the formation of fatty streaks in the arteries, consistent with the observations for mouse models of this disease (Stoletov et al, 2009). Furthermore, feeding zebrafish larvae a high cholesterol diet for 2 weeks leads to oxidation of lipoproteins containing apolipoprotein B (apoB), consistent with mmLDL observed in mammalian models (Fang et al, 2010). Larvae of transgenic strains fed a high cholesterol diet for 10 days show an accumulation of myeloid cells in the vascular wall (Stoletov et al, 2009). Thus, as a model organism, the zebrafish is capable of replicating at least some of the features associated with atherosclerosis observed in mammalian systems and could potentially provide insight into the acute basis of these metabolic diseases.

1.4.2 Development of the Innate Immune System in Zebrafish Through Haematopoiesis

The innate immune system is a first line of defence against infection and the cells involved develop through a process known as haematopoiesis. In vertebrates, haematopoiesis occurs in two successive waves, known as primitive (embryonic) and definitive (adult) (Warga et al, 2009). Primitive haematopoiesis in zebrafish occurs in two distinct sites of the lateral mesoderm: the development of erythroid and endothelial precursors occurs in the posterior lateral plate mesoderm (P-LPM), which forms the intermediate cell mass (ICM), whereas the development of myeloid cells occurs in the anterior lateral plate mesoderm (A-LPM), which forms the posterior blood island (PBI) (Dooley et al, 2005). Primitive haematopoiesis is controlled by the action of various transcription factors. One of these is Pu.1, which plays an important role in the development of myelomonocytes and granulocytes (Scott et al, 1994; McKercher et al, 1996). Pu.1 is known to function downstream of the early haematopoietic genes Cloche, Etsrp and Scl and is first expressed at around 12 hours post fertilisation (hpf) in the A-LPM, followed by expression in the ICM at around 22 hpf (Bennett et al, 2001; Ward et al, 2003; Rhodes et al, 2005; Bukrinsky et al, 2009). Pu.1 is initially expressed in erythro-myeloid progenitors and plays an important role in suppressing erythropoiesis in the
A-LPM by antagonising the function of Gata1, which is necessary for promoting erythropoiesis (Rhodes et al, 2005). At the same time, Pu.1 is involved in driving myelopoiesis through promoting the expression of markers of myeloid cells, such as MPO, L-plastin and Lysozyme C (LyzC). Studies have demonstrated that Pu.1 morphant embryos display a reduced expression of MPO, L-plastin and LyzC, and Gata1 morphants an enhanced expression of MPO and L-plastin compared to uninjected controls (Rhodes et al, 2005). MPO is expressed in granulocytes and is detected initially at 18 hpf within the ICM. MPO expressing cells spread over the yolk sac of the embryo and by 3 days post fertilisation (dpf) are present in the anterior yolk sac, posterior ICM and PBI (Bennett et al, 2001). L-plastin, an actin-binding protein expressed in both macrophages and neutrophils, is first detected at 18 hpf in the anterior yolk sac and by 28 hpf is expressed in the posterior ICM. Its expression however, is greatly reduced by 5dpf (Bennett et al, 2001, Matias et al, 2009). LyzC, like L-plastin, is expressed in both neutrophils and macrophages and first appears at approximately 20hpf in the anterior yolk sac. At around 30hpf, it is detected in the ICM (Berman et al, 2005). A schematic representation of haematopoiesis in zebrafish is shown in **Fig 1.3:**

![Schematic Representation of Haematopoiesis in Zebrafish](image)

**Figure 1.3: Schematic Representation of the Events of Primitive Haematopoiesis in Zebrafish Leading to Myeloid and Erythroid Differentiation.** Initial expression of genes indicated in hpf. (Figure adapted from Rhodes et al, 2005 and Bukrinsky et al, 2009)
1.4.3 *In Vivo* Real Time Imaging of Zebrafish Immune Cells in Response to Inflammatory Stimuli

The immune response following acute wounding injury has been investigated in detail in zebrafish and takes advantage of transgenic strains expressing fluorescent proteins under the promoters of immune cells. Studies have shown that up to a period of 6h following tailfin injury in larvae, there is an increase in the number of neutrophils, marked by green fluorescent protein (GFP) expressed under the MPO promoter, to the wounded tail. There is then a subsequent decrease such that by 24h, resolution of the inflammatory response has occurred through retrograde chemotaxis of neutrophils back to the vasculature (Matias *et al.*, 2006). Other groups have demonstrated that resolution of this neutrophil response can occur through apoptosis in a caspase-dependent manner, suggesting two mechanisms of resolution (Renshaw *et al.*, 2006; Loynes *et al.*, 2010). Myeloid cells marked by GFP expression under the LyzC promoter show a peak accumulation in a JNK-dependent manner at a wounded tail 4 – 6h post injury and retrograde resolution occurs 6 – 8h post injury (Zhang *et al.*, 2008). Studies simultaneously investigating the roles of macrophages and neutrophils have shown that neutrophils migrate more quickly than macrophages do to the injury site but macrophages remain at the site for longer and phagocytose apoptotic neutrophils. Such work has been carried out using two fluorescent proteins, one under a neutrophil promoter and the other under a macrophage promoter to track cells (Ellet *et al.*, 2011).

Real time imaging of fluorescent immune cells has also been used to examine chemically-induced inflammation. Treatment of larvae for 3 days with trinitrobenzene sulphonic acid (TNBS) results in an accumulation of neutrophils in the intestine which have migrated from the caudal haematopoietic tissue (CHT) (Oehlers *et al.*, 2011). It has additionally been shown that fluorescent LyzC and MPO cells migrate from the CHT and PBI to the midline of the tail and trunk in a ROS-dependent manner in response to copper sulphate treatment which damages neuromasts. The cells first appear 20 minutes after treatment and numbers begin to decrease approximately 2 – 3h post-treatment, such that by 6h resolution has occurred in a COX-dependent manner (d’Alençon *et al.*, 2010).

Thus, real time imaging of the behaviour of immune cells in several models of inflammation has been used to examine both the onset and resolution of inflammation *in vivo*, taking
advantage of the optical transparency of this model organism. As the signals that lead to macrophage and neutrophil emigration from inflammatory sites during the resolution phase of inflammation are currently not well understood, the ability to image these cell populations \textit{in vivo} may provide insight into this. Furthermore, neither the onset nor the resolution of acute inflammation associated with a high-fat diet is well understood and so this property of zebrafish can be exploited to understand these processes better. This may have potential therapeutic benefit in preventing the development of diseases such as obesity, diabetes and atherosclerosis, which result from an initial acute inflammation phase that fails to resolve.
1.5 Uptake and Processing of Dietary Fat

Dietary fat primarily takes the form of triacylglycerols (TAG) and is hydrolysed to fatty acids (FAs) and diacylglycerols (DAG) by lipases in the intestinal lumen (Tso et al, 2004; Niot et al, 2009). There are three proteins expressed on the apical surface of absorptive intestinal epithelial cells, also termed enterocytes, that are thought to be involved in the uptake of FAs, but with controversial involvement: plasma membrane fatty acid-binding protein (FABPpm), CD36 and fatty acid transport protein 4 (FATP4) (Masson et al, 2010). The role of FABPpm is questionable given that its amino acid sequence is identical to that of mitochondrial aspartate amino-transferase, a protein that itself is not involved in fatty acid uptake (Stump et al, 1993). There is conflicting evidence in the literature about the involvement of CD36 as studies investigating impaired function of CD36 on long chain FA uptake showed either no effect on uptake or a reduced uptake (Goudriaan et al, 2002; Nauli et al, 2006; Nassir et al, 2007). FATP4 was initially thought to be involved in long chain FA uptake as studies demonstrated that stable overexpression of FATP4 enhanced uptake of palmitic acid and reduced expression of FATP4 affected FA uptake by enterocytes (Stahl et al, 1999). However, more recent studies have indicated that FATP4 was localised to the ER membrane, thus bringing its proposed role as a FA uptake protein into question (Milger et al, 2006). FATP4 has also been shown to display acyl coA synthetase activity suggesting an alternative function of the protein. This may involve converting FAs to FA-CoAs, thereby reducing the intracellular FA concentration and promoting FA entry through a mechanism of mass entry if there are sufficient FAs in the lumen (Hall et al, 2005; Milger et al, 2006). Thus, it appears that CD36 is the only transport protein that may have a plausible potential role in FA uptake into enterocytes. It is possible that FAs initially bind to CD36 and then once in close proximity to the PM, enter through passive diffusion (Lynes and Widmaier, 2011).

Another major component of dietary fat is cholesterol which, like FAs, is also taken up by enterocytes and first incorporated into mixed micelles to enhance solubility in the intestinal lumen (Ros, 2000; Masson et al, 2010). The uptake protein Niemann-Pick C1-Like 1 (NPC1L1), which is expressed at the apical domain of enterocytes, is responsible for the uptake of cholesterol from the intestinal lumen into the enterocyte (Altmann et al, 2004). The localisation of NPC1L1 within the cell is dependent upon the cholesterol content of the
cell. When there is an abundance of cholesterol, NPC1L1 resides in the endocytic compartment, but upon cholesterol depletion, NPC1L1 is recycled to the PM (Yu et al., 2006). It is proposed that NPC1L1 binds to cholesterol using a predicted N-terminal sterol-sensing domain (Betters and Yu, 2010). The uptake of cholesterol by NPC1L1 is likely through clathrin-mediated endocytosis and it is thought that NPC1L1 dissociates from cholesterol during the endocytic stages (Ge et al., 2008; Betters and Yu, 2010). Two other proteins expressed at the apical domain of enterocytes are ATP-binding cassette G5 (ABCG5) and 8 (ABCG8), which are involved in the efflux of excess dietary cholesterol from the enterocyte back into the intestinal lumen (Masson et al., 2010). The expression of proteins involved in the uptake of cholesterol by enterocytes is controlled by the activity of LXR, which are activated by oxidised derivatives of cholesterol, known as oxysterols (Zhao and Dahlman-Wright, 2010). Activation of LXR decreases expression of NPC1L1, but increases expression of ABCG5, ABCG8 and ABCA1, thus reducing cholesterol uptake and promoting cholesterol efflux (Yu et al., 2003; Duval et al., 2006; Zhao and Dahlman-Wright, 2010).

FAs are then processed in the enterocytes to produce TAG, which are then combined with apoB48 through the action of microsomal transfer protein (MTP) (Wetterau et al., 1997; Niot et al., 2009). Following its uptake, cholesterol is esterified and the resulting cholesterol esters (CEs) are also combined with apoB lipoproteins through MTP (Lee et al., 2000; Iqbal et al., 2008). The association of TAG, CEs and apoB lipoproteins leads to the formation of chylomicrons (CMs) which are then subsequently transported in the blood to peripheral tissues. Additionally, a small proportion of FAs that are not processed into TAG are secreted from enterocytes as FFAs and some free cholesterol is combined with ApoA1 to form high density lipoproteins (HDLs) in a process mediated by ABCA1 (Masson et al., 2010). The FA content of CMs is released into AT and muscle, and the resulting CM remnants are taken up by the liver through endocytosis (Havel, 1998; Rader and Daugherty, 2008). The CEs are then combined with TAG, phospholipids and apoB100 to form very low density lipoproteins (VLDLs), which are subsequently processed to LDLs. LDLs are taken up by cells to release intracellular free cholesterol following hydrolysis in the lysosome (Rhainds and Brisette, 1999; Brewer, 2000; Daniels et al., 2009).

Zebrafish, like mammals, feed on a diet that contains lipids, express similar proteins involved in the transport of dietary fat, and process this fat in essentially the same way as mammals do. Dietary FAs are present in the plasma immediately after feeding and as TAG-enriched CMs...
following processing in the intestine. Dietary fat is transported from the intestine to the liver and in association with lipoproteins, as in mammals (Hölttä-Vuori et al., 2010). MTP has been characterised in zebrafish and is highly expressed in the anterior intestine, consistent with this location being the site of lipid uptake in zebrafish (Marza et al., 2005). The expression levels of MTP also increase in response to feeding on a fat-enriched diet, which is consistent with similar findings reported in other organisms (Marza et al., 2005; Schlegel and Stainier, 2006). NCP1L1 is conserved in zebrafish and has been shown to be involved in cholesterol uptake (Clifton et al., 2010). A zebrafish orthologue of mammalian Ang2, a protein involved in vesicular trafficking of lipids through the Golgi of enterocytes, termed fat free has been characterised (Ho et al., 2006). Lipoproteins such as ApoE, have also been characterised in zebrafish (Babin et al., 1997).

Thus, it appears that zebrafish are a useful model organism for the study of dietary fat uptake and processing. Therefore, in combination with their applications in immunological research, they can potentially be used to investigate the gastrointestinal inflammatory response associated with feeding on a fat-enriched diet. As zebrafish possess an intestine such as that in mammals, this allows the accumulation of cells in this region of the organism to be studied following fat feeding. Furthermore, the localised inflammation in the intestine following feeding on a fat-enriched diet has not been studied in detail before at either the acute or the chronic stage.
1.6 Aims

The aims of this thesis are:

- To establish and characterise zebrafish as a model for gastrointestinal inflammation following acute feeding on a fat-enriched diet.

- To investigate the role of the NLRP3 inflammasome in the inflammation associated with acute feeding on a fat-enriched diet.

- To attempt to generate a stable transgenic line of zebrafish that possesses an ablated population of immune cells.
CHAPTER 2: MATERIALS AND METHODS

2.1 Strains of Zebrafish (Danio rerio) Used and Their Maintenance

The strains of zebrafish used and their source are listed in Table 2.1:

<table>
<thead>
<tr>
<th>Name of Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type (WT) AB</td>
<td>Tübingen (Germany)</td>
</tr>
<tr>
<td>Pu.1 GFP</td>
<td>University of Sheffield (UK)</td>
</tr>
<tr>
<td>LyzC GFP</td>
<td>University of Bristol (UK)</td>
</tr>
<tr>
<td>LyzC dsRed</td>
<td>University of Bristol (UK)</td>
</tr>
<tr>
<td>Tra/Nac</td>
<td>Cancer Research Institute (UK)</td>
</tr>
</tbody>
</table>

Table 2.1: Strains of Zebrafish Used and Their Source.

Fish were maintained according to standard practices and all procedures conformed to UK Home Office requirements as per the Animals (Scientific Procedures) Act 1986. Prior to experimental procedures, larvae were initially reared in system water containing 3 x 10^{-5} \% methylene blue (Sigma) as an antifungal agent and 30mg/L 1-phenyl 2-thiourea (Sigma) to prevent melanisation.

2.2 Preparation of the Experimental Diets

1g of cholesterol (C75209; Sigma) or the SFAs, palmitic acid (P5585-10G; Sigma) and myristic acid (M3128-10G; Sigma), was dissolved in 10ml diethyl ether (Sigma) to create a 10\% solution. For preparing the unsaturated fatty acids (UFAs), linoleic acid (L1376-5G; Sigma) and oleic acid (01008-5G; Sigma), 1ml of the fatty acids was added to 9ml ether to create a 10\% solution. In all cases, 400\mu{l} was then added to 0.5g of standard zebrafish larval food (ZM; ZM Systems) to create an 8\% diet. 400\mu{l} of diethyl ether was added to 0.5g of ZM to serve as a control diet. The diets were then left overnight for the ether to evaporate and then the following day, were ground up into fine particles using a pestle and mortar (Adapted from Stoletov et al, 2009). All diets were stored at 4°C until required.
2.3 Feeding of Zebrafish Larvae

For studies on rates of ingestion and gastrointestinal transit, 6 dpf larvae were placed in a 3cm dish with system water containing 1.0µm polystyrene microspheres conjugated to YFP (excitation 505nm/emission 515nm; Invitrogen) at a dilution of 2 x 10⁷ microspheres/ml. Larvae were allowed to feed for 4h at 28°C. For fat feeding experiments, 6 dpf larvae were placed in system water containing clotted cream used at a 1:10 dilution (6.25% fat, 0.22% carbohydrate and 0.16% protein) and were fed for 6h at 28°C (Schlegel and Stainier, 2006; Kadereit et al, 2008). Larvae were also fed skimmed milk of an equivalent protein concentration to the cream, double cream containing 6.25% fat or were unfed for 6h. Additionally, 6dpf larvae were fed cholesterol, palmitic acid, myristic acid, linoleic acid and oleic acid (all at 8%) or a standard diet (ZM), prepared as described previously, for 6h at 28°C. Specific details of the timings of the feedings are described in the Results Sections.

2.4 Epifluorescent Microscopy

Larvae were anaesthetised in 0.016% MS222 (Sigma) and imaged using QCapture Pro software on an Olympus CKX41 epifluorescent microscope fitted with a QImaging QICAM camera, and FITC and TRITC filters. Specific details of the timings of imaging are described in the Results Sections.

2.5 Staining of Larvae

2.5.1 Fixation of Zebrafish Larvae

Larvae were fixed overnight in 4% paraformaldehyde (Polysciences Inc) at 4°C.

2.5.2 MPO Activity for Granulocyte Staining

Following fixation, the larvae were washed once in phosphate-buffered saline with 0.1 % Tween (Sigma) (PBST) and then once in wash buffer (1 × Trizmal buffer (Sigma), 0.01% Tween and H₂O) for 5 minutes each. The larvae were then stained for 30 minutes at 37°C in
stain solution (0.015% H_2O_2 AnalaR) and 1.5mg/ml dianinobenzidine (DAB) substrate (Sigma) in wash buffer), followed by two washes in PBST for 5 minutes (Matias et al, 2006). Stained larvae were imaged using Leica Application Suite software on a Leica MZ16F microscope. Cell numbers were counted in a blinded manner.

2.5.3 Whole Mount Immunofluorescent Staining of Myeloid Cells in Zebrafish Larvae with an Anti-L-plastin Antibody

Following fixation, larvae were washed twice for 5 minutes in PBS and then once for 5 minutes in de-mineralised (DM) H_2O. They were then permeabilised in acetone (Sigma) for 7 minutes at -20°C. Following this, they were rinsed in DM H_2O for 30 seconds and then incubated in blocking solution (5% donkey serum (Sigma), 1% dimethylsulphoxide (DMSO; Sigma) and 0.1% Tween in PBS) for 30 minutes. Larvae were subsequently incubated overnight at 4°C in rabbit anti-zebrafish L-plastin antibody (Gift from Paul Martin, University of Bristol, UK) diluted 1:500 in blocking solution. The following day, larvae were washed three times for 20 minutes in PBST and then twice for 20 minutes in blocking solution. They were then incubated for 4h at room temperature in secondary donkey anti-rabbit immunoglobulin G (IgG) conjugated to TRITC (Stratech) used at 15µg/ml in blocking solution. Following this, larvae were washed four times for 20 minutes each in PBST and then imaged using an epifluorescent microscope, as previously described. Cell numbers were counted in a blinded manner.

2.5.4 Whole Mount DAB Staining of Myeloid Cells in Zebrafish Larvae with an Anti-L-plastin Antibody

Larvae were stained as described above, but following washes in PBS and prior to permeabilisation in acetone, were incubated in peroxidase solution (0.5% H_2O_2 in PBS) for 20 minutes to block endogenous peroxidase activity. Additionally, larvae were incubated instead in a secondary goat anti-rabbit IgG conjugated to horseradish peroxidise (HRP) (Jackson Immunoresearch) used at 4µg/ml in block solution containing 5% goat serum (Sigma), 1% DMSO and 0.1% Tween in PBS. Following the final washes in PBST, larvae were incubated for 30 mins in 0.3mg/ml DAB in PBS, followed by incubation for 10 minutes in 0.3mg/ml DAB and 0.05% H_2O_2 in PBS. Larvae were then washed twice for 5 minutes in PBST and then stored at 4°C.
2.6 Paraffin Embedded Sectioning of Larvae

Larvae were embedded in a 3% agar block and then through wax processing, transferred to a paraffin block (performed by Lorraine Lawrence). 5µM transverse sections were cut using a rotary microtome and placed onto glass slides, which were left to dry overnight. The following day, sections were stained with haematoxylin and then mounted with coverslips (haematoxylin staining performed by Lorraine Lawrence). Stained sections were imaged using Axiovison Rel 4.7 software on a Leica MPS 60 microscope fitted with an Axiocam HRc camera.

2.7 Confocal Microscopy

Larvae were imaged on a Zeiss LSM-510 inverted confocal microscope fitted with an argon 488 laser and a helium/neon 543 (He/Ne 543) laser.

2.8 Small Molecule Inhibitor Treatment of Larvae

5 dpf WT larvae were treated with 12.5µg/ml or 25µg/ml ezetimibe (Sequoia Research Ltd) overnight, followed by feeding the next day. 6 dpf WT larvae were treated for 30 minutes with caspase-1 and 5 inhibitor N-Acetyl WEHD-al (Sigma), cathepsin B inhibitor Ca-074-Me (Calbiochem) and NADPH oxidase inhibitor VAS-2870 (Enzo Life Sciences), followed by feeding, as previously described. The inhibitors were administered into the water and were present for the duration of the experiment. Inhibitors were initially used at a range of concentrations to determine an optimum concentration, and then further experiments were carried out using the optimum concentration and with an appropriate vehicle control.

2.9 Treatment of Larvae with the Fluorogenic NADPH Oxidase Probe

6 dpf WT larvae were treated for 1 hour with 50µM fluorogenic NADPH oxidase probe Acetyl-pentafluorobenzenesulfonyl fluorescein (Calbiochem) or an equivalent volume of
DMSO (Niethammer et al, 2009). This was followed by a 10 minute feeding period after which larvae were anesthetised and imaged under an epifluorescent microscope, as previously described.

2.10 Treatment of Larvae with Fluorogenic Caspase-1 Substrate and Inhibitor

For experiments with the fluorogenic caspase-1 substrate Z-YVAD-AFC (Enzo Life Sciences), 6 dpf WT larvae were treated for 1 hour with the substrate used at concentrations of 10µM, 40µM and 100µM. Following this treatment period, larvae were anaesthetised and imaged on a Zeiss Axiovert 200 wide-field fluorescent microscope fitted with a CFP filter using Improvision Volocity Software.

For experiments with the fluorochrome inhibitor of caspase-1 (FLICA), FAM-YVAD-FMK (Immunochemistry Technologies, LLC), 6 dpf WT larvae were fed for 6h. After this feeding period, larvae were removed from the diet and at varying time points following removal, were incubated for 30 mins at 28°C in 1x FLICA. This was followed by a 30 minute wash in PBS, and after this larvae were anaesthetised and imaged under an epifluorescent microscope, as previously described (Adapted from Yeh et al, 2008).

2.11 Morpholino Treatment of Larvae

6 dpf WT larvae were treated with a splice-blocking MO that targets the exon 1 – intron 1 boundary sequence of zebrafish ASC, a translation blocking MO that blocks the translation initiation site of ASC, a specificity control MO containing mismatched base-pairs from the splice-blocking morpholino and a standard control MO (Gene-Tools). All MO were conjugated to carboxyfluorescein and administered into the water for 24h. After this period, the larvae were fed, as described previously in the presence of the MO. A range of concentrations of the splice-blocking morpholino were initially used, followed by further experiments using the optimum concentration and the other MO at this concentration also.
For MO delivery by micro-injection, WT embryos were injected at the single-cell stage into the yolk sac using a Narishige IM300 Microinjector (performed by Harriet Taylor). 0.1mM concentrations of splice-blocking morpholino, translation-blocking MO (both targeting zebrafish ASC) and standard fluorescent control MO were injected. 2h post injection, embryos were screened on the basis of the presence of carboxyfluorescein in the dividing cells. Embryos that displayed this fluorescence in the dividing cells only were retained and at 6 dpf, were fed, as described previously. A list of the MO used and their sequences is shown in Table 2.2:

<table>
<thead>
<tr>
<th>Name of Morpholino</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC Splice-blocking</td>
<td>CAATTGCACCTACATTGCCCTGTGT</td>
</tr>
<tr>
<td>ASC Translation-blocking</td>
<td>GGTGCTCCTTTGAAAGATTCCGCCAT</td>
</tr>
<tr>
<td>ASC Specificity Control for Splice-blocking</td>
<td>CAATTCCAGTTAGATTGCCGTGTCT</td>
</tr>
<tr>
<td>Standard Control</td>
<td>CCTCTTACCTCAGTTACAATTTATA</td>
</tr>
</tbody>
</table>

Table 2.2: Names and Sequences of Morpholinos Used and Their Sequences.

2.12 RNA Extraction from WT Larvae

Larvae were killed by anaesthetic overdose in 0.4% MS222 and then had their intestines dissected using forceps with the remaining carcases also being kept. 20 intestines were assimilated into a pool and the 20 remaining carcases were split into 4 pools of 5 carcases. Tissue was stored in lysis buffer containing β-mercaptoethanol and RNA was extracted using a MagMax 96 Total RNA Isolation Kit according to the manufacturer’s instructions (Ambion). Following this, cDNA was synthesised using the following reaction mixture (100µl): 85ng RNA, 1 x reaction buffer, 1 x random primers, 1 x dNTPs, reverse transcriptase (all available from Applied Biosystems) and H₂O. The reaction conditions were a 10 minute incubation at 25°C, followed by 2h at 37°C.
2.13 Quantitative Real Time PCR (q-RT PCR)

For TaqMan reactions, the mixture (10µl) was prepared using 1µl cDNA, 0.5µl probe (20x), 5µl mastermix (2x) (*Applied Biosystems*) and 3.5µl H$_2$O. For SYBR green reactions, the mixture was prepared using 1µl cDNA, 0.5µl forward (FWD) primer (0.5µM), 0.5µl reverse (REV) primer (0.5µM), 5µl mastermix (*Applied Biosystems*) and 3µl H$_2$O. Reactions were carried out in either duplicate or triplicate, depending on the gene and were run on an ABI 7500 Fast Real Time PCR System machine. The following cycling conditions were used: 1 cycle of 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. A list of genes and the type of reaction run is shown in Table 2.3:

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Type of Reaction</th>
<th>Taqman Assay Code / SYBR Green Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC</td>
<td>TaqMan</td>
<td>Dr03125114_m1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TaqMan</td>
<td>Dr03126850_m1</td>
</tr>
<tr>
<td>IL-17</td>
<td>TaqMan</td>
<td>Dr03141653_m1</td>
</tr>
<tr>
<td>IFN-1</td>
<td>TaqMan</td>
<td>Dr0310938_m1</td>
</tr>
<tr>
<td>m-17</td>
<td>TaqMan</td>
<td>Dr03098117_g1</td>
</tr>
<tr>
<td>Lta</td>
<td>TaqMan</td>
<td>Dr03109563_m1</td>
</tr>
<tr>
<td>IL-10</td>
<td>TaqMan</td>
<td>Dr03103209_m1</td>
</tr>
<tr>
<td>IL-12a</td>
<td>TaqMan</td>
<td>Dr03127924_m1</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>TaqMan</td>
<td>Dr03436643_m1</td>
</tr>
<tr>
<td>Cxcl12</td>
<td>TaqMan</td>
<td>Dr03119119_m1</td>
</tr>
<tr>
<td>IL-15</td>
<td>TaqMan</td>
<td>Dr03077605_m1</td>
</tr>
<tr>
<td>IL-8</td>
<td>SYBR Green</td>
<td><strong>FWD:</strong> TGTGTTATGGTTTCTCCTGGCATTTTC&lt;br&gt;<strong>REV:</strong> CTGTAGATCCACGCTGTCGC</td>
</tr>
<tr>
<td>EF1a (Housekeeping)</td>
<td>SYBR Green</td>
<td><strong>FWD:</strong> GGAGATTGCCGATTATGG&lt;br&gt;<strong>REV:</strong> CAGCAAGCGACCAAGAGGAG</td>
</tr>
<tr>
<td>Actin (Housekeeping)</td>
<td>TaqMan</td>
<td>Dr03432610_m1</td>
</tr>
<tr>
<td>18S (Housekeeping)</td>
<td>TaqMan</td>
<td>4308329</td>
</tr>
</tbody>
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Table 2.3: A List of Genes Analysed by qRT-PCR Along with the Type of Reaction Used.
2.14 Plasmid Vector Maps

Figure 2.1: Schematic Representation of the 14XUAS CFP-NTR Plasmid Vector. Sites for restriction enzymes that cut the vector once only are shown. Vector map provided by Jeff Mumm, Medical College of Georgia, USA.
2. Materials and Methods

Figure 2.2: Schematic Representation of the pTol(2.4kb LyzC)eGFP Plasmid Vector. Restriction sites are shown in bold and italic. Tol2L and Tol2R indicate minimal Tol2 transposable elements. PolyA indicates a polyadenylation sequence (Figure adapted from Kitaguchi et al., 2009).
Figure 2.3: Schematic Representation of the CS-TP Plasmid Vector. TPase indicates the Tol2 transposase cDNA sequence, P-CMV, the cytomegalovirus promoter and SV40 pA, a SV40 polyadenylation sequence. Restriction sites and their positions of cutting within the sequence are shown. Figure taken from the Kawakami Lab website http://kawakami.lab.nig.ac.jp/map2.html (Koichi Kawakami Laboratory, National Institute of Genetics, n.d.).
2. Materials and Methods

2.15 PCR Amplification of the CFP-NTR Ablation Cassette

The cyan fluorescent protein-nitroreductase (CFP-NTR) ablation cassette from the 14XUAS CFP-NTR plasmid vector (Gift from Jeff Mumm, Medical College of Georgia, USA) was amplified by PCR using the following reaction mixture: 1x reaction buffer (Promega), 200µM dNTPs (Promega), FWD primer (5’ – 3’; CAGTGATACACCATGGACGAATTCCGGTCGCCACCATGGT; 0.1µM, 0.5µM and 1µM; Sigma), REV primer (5’ – 3’; GTTCTAGAGGCTCGAGACCCGCTAAATCTTCAACCTGGGA; 0.1µM, 0.5µM and 1µM; Sigma), 3mM MgCl₂ (Promega), Pfu (Promega), H₂O and approximately 2ng DNA. The cycling conditions were: 1 cycle of 95°C for 90 seconds, 35 cycles of 95°C for 30 seconds, 63°C for 45 seconds and 72°C for 3 minutes, and 1 cycle of 72°C for 7 minutes. The reaction was run on a Programmable Thermal Controller – 100 PCR machine.

2.16 Restriction Digest of the pTol(2.4kbLyzC)eGFP Plasmid Vector

Approximately 3µg of pTol(2.4kb LyzC)eGFP (Gift from Atsuo Kawahara, National Cardiovascular Center Research Institute, Japan) was digested with NcoI in Reaction Buffer 3 (New England Biolabs (NEB)) to linearise the vector. The digested product was then column purified according to the manufacturer’s instructions (Qiagen). Following this, a second digest of the linearised vector was carried out using XhoI in reaction buffer 2 (NEB) to excise the GFP from the vector. The digested product was column purified. Restriction digests were carried out at 37°C for 4h and 100ng of DNA from the digestion reactions was run on a 1% agarose gel.

2.17 Recombination of the Digested Vector and PCR-amplified Insert Using the Infusion Kit

100ng of vector and 46.7ng of insert were used in the reaction according to the manufacturer’s instructions (Clontech). Following the reaction, 2.5ng and 10ng of DNA from the reaction mixture was added to 50µl of Library Efficiency DH5α cells (Invitrogen)
along with control reactions supplied with the kit. The cells were left on ice for 30 minutes and then heat shocked for 20 seconds at 42°C. They were then put back on ice for 2 minutes. Following this, 950µl of pre-warmed Luria-Bertani (LB) media (Sigma) was added to the cells and they were incubated at 37°C for 90 minutes. 10µl and 90µl volumes were then spread onto LB-agar (Sigma) plates containing 100µg/ml of ampicillin (Sigma).

2.18 Overnight Growth of Bacterial Colonies

Positive clones were picked and grown in 5ml LB media containing 100µg/ml ampicillin. The following day, DNA was miniprepped from the bacterial cultures according to the manufacturer’s instructions (Qiagen).

2.19 Restriction Digest of the Recombined Vector and Insert

To confirm the presence of the CFP-NTR ablation cassette in the pTol(2.4kb LyzC) vector, 250ng of miniprepped DNA was digested with EcoRV in Reaction Buffer 3 (NEB) along with 1x BSA (Promega) for 4h at 37°C. 100ng of the digestion reaction was run on a 1% agarose gel.

2.20 Sequencing of the pTol(2.4kbLyzC)CFP-NTR Plasmid Vector to Confirm the Presence of the CFP-NTR Ablation Cassette

The pTol(2.4kb LyzC)CFP-NTR plasmid vector was sent for sequencing at Beckman Coulter Genomics using four overlapping reverse primers (Sigma) along with an in-house M13R primer used to sequence the final 400bp of the CFP-NTR ablation cassette. Table 2.4 gives the sequences for the four overlapping primers and the position of the start site of the primer in the CFP-NTR sequence:
2. Materials and Methods

<table>
<thead>
<tr>
<th>Sequence of Primer (5’-3’)</th>
<th>Position of the Start Site in the Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAAGATGGTGCGCTCCTGGACGTA</td>
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</tr>
<tr>
<td>GCGGAGCTGACGCTGCCGTCCCTC</td>
<td>540</td>
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<tr>
<td>CTTAGTGAATGACGCTTTAAGGC</td>
<td>780</td>
</tr>
<tr>
<td>CCAGACATCCTCCATCGCGGTTTT</td>
<td>1020</td>
</tr>
</tbody>
</table>

Table 2.4: Sequences of the Overlapping Primers Used and the Position of Their Start Sites in the CFP-NTR Sequence.

2.21 Synthesis of mRNA from the Tol2 cDNA

The pCS-TP plasmid vector containing the Tol2 cDNA (*Gift from Koichi Kawakami, National Institute of Genetics, Japan*) was digested for 6h at 37°C with *NotI* in Reaction Buffer 3 (*NEB*) to linearise the plasmid vector. Following this, mRNA was synthesised from the Tol2 cDNA within the linearised vector using the SP6 mMessage mMachine kit according to the manufacturer’s instructions (*Ambion*).

2.22 Co-injection of pTol(2.4kb LyzC)CFP-NTR Plasmid Vector with Tol2 mRNA into Embryos at the Single-cell Stage

A DNA/RNA solution containing 25ng/µl of pTol(2.4kb LyzC)CFP-NTR plasmid vector and 25ng/µl of Tol2 mRNA was injected into WT and *Tra/Nac* embryos at the single-cell stage using a Narishige IM300 Microinjector (performed by Harriet Taylor). Larvae were screened at 3 dpf for the presence of CFP positive LyzC cells using Improvision Volocity software on a Zeiss Axiovert 200 wide-field fluorescent microscope fitted with a CFP filter.

2.23 Generation and Screening of F₁ Larvae

F₁ larvae were produced by crossing injected adult F₀ founder fish with age-matched WT or *Tra/Nac* fish. F₁ larvae were screened for the presence of CFP positive LyzC cells through a tail transection experiment, whereby 3 dpf F₁ larvae, LyzC GFP larvae and LyzC dsRed larvae were anaesthetised and had their tails transected. Larvae were then allowed to recover
through transfer to fresh water. 5h post transection, larvae were anaesthetised and imaged under a Zeiss Axiovert 200 wide-field fluorescent microscope fitted with CFP, GFP and rhodamine-TRITC filters for the presence of LyzC positive cells in the wounded tails.

2.24 Whole Kidney Marrow (WKM) Extraction from Adult Zebrafish

Adult fish were killed by anaesthetic overdose and then had their kidneys removed using forceps. The tissue was homogenised in PBS containing 10% foetal calf serum (Sigma) to obtain a single cell suspension. The resulting suspension was centrifuged at 1300 rpm for 4 minutes and the supernatant removed. The remaining pellet was then resuspended in PBS and kept on ice.

2.25 Analysis of WKM by Flow Cytometry

Tissue samples were analysed by flow cytometry on a BD LSR Fortessa machine using FACS DIVA software with a 405/450 excitation/emission channel and a 488/530 excitation/emission channel (performed by Fränze Progratzky).

2.26 Image Processing and Analysis

Acquired images were processed and analysed using ImageJ and Volocity software.

2.27 Statistical Analysis

Statistical analysis was performed for parametric data sets with two groups using a t-test or a paired t-test. For non-parametric data sets with two groups, a Mann-Whitney test or a Wilcoxon matched pairs test was used. For parametric data sets with more than two groups, a one-way analysis of variance (ANOVA) was used, followed by a Bonferroni post test. For non-parametric data sets with more than two groups, a Kruskall-Wallis test was used,
followed by a Dunn’s post test. P values of less than 0.05 were deemed statistically significant.
CHAPTER 3: ESTABLISHING AND CHARACTERISING ZEBRAFISH AS A MODEL FOR GASTROINTESTINAL INFLAMMATION FOLLOWING ACUTE FEEDING ON A FAT-ENRICHED DIET

3.1 Introduction

Whilst the process by which uptake of FAs into intestinal epithelial cells occurs is similar for all FAs, the inflammatory effects associated with different types of FAs vary. It is known that SFAs have pro-inflammatory properties in the context of the chronic inflammation associated with obesity and type II diabetes through activation of TLR4 and MAPK signalling (Suganami et al., 2005; Suganami et al., 2007). UFAs, however, are often non-inflammatory or anti-inflammatory and this has been demonstrated in a number of in vitro studies. Whilst SFAs such as palmitic acid or lauric acid have been shown to induce COX-2 expression in macrophages, UFAs such as linoleic acid or oleic acid, failed to do so. Additionally, lauric acid induced iNOS and IL-1α expression in an NF-κB-dependent manner, whereas UFAs inhibited NF-κB activation and also blocked LPS-induced expression of COX-2, iNOS and IL-1α (Lee et al., 2001). Palmitic acid and lauric acid increased expression of the monocyte chemokine, IP-10 in macrophages, but linoleic acid and oleic acid were unable to do so (Neville et al., 1997; Laine et al., 2007). Oleic acid and linoleic acid have also been shown to antagonise the effects of palmitic acid (Weigert et al., 2004; Kennedy et al., 2009). However, a study by Ciapaite et al. has shown that oleic acid can cause NF-κB activation just as palmitic acid can in human umbilical vein endothelial cells (Ciapaite et al., 2007). Thus, it appears that the effects of oleic acid may vary depending on cell or tissue type.

The ω-3 UFAs, DHA and EPA, in particular, have well characterised anti-inflammatory roles and have been shown to inhibit IL-1β and TNF-α production by monocytes, and IL-6 and IL-8 production by ECs (De Caterina et al., 1994; Khalfoun et al., 1997; Chu et al., 1999). DHA has also been demonstrated to decrease E-selectin, ICAM-1 and VCAM-1 expression on ECs,
and reduce the ability of monocytes to adhere to the endothelium (De Caterina et al, 1994; De Caterina and Libby, 1996). *In vivo* studies have shown that switching to a diet enriched in ω-3 UFAs for 5 weeks following a 15 week feeding period on a high fat diet in mice results in a phenotypic switch from M1 to M2 macrophages in AT compared to mice continually fed the high fat diet. The expression levels of M1 inflammatory genes such as IL-6, TNF-α, MCP-1, IL-1β and iNOS were also reduced upon switching diets. However, the expression levels of M2 anti-inflammatory genes such as arginase 1 and IL-10 were increased (Oh et al, 2010). Thus, the content of dietary fat affects its inflammatory properties. Western diets increasingly contain a high level of saturated fat and are therefore pro-inflammatory in comparison to healthier diets rich in ω-3 UFAs, which are anti-inflammatory. A large majority of the studies comparing the effects of SFAs and UFAs have been performed *in vitro* and would require *in vivo* verification. The zebrafish presents itself as a highly valuable system for examining this in more detail owing to its use for *in vivo* imaging at the organismal level.

As well as the similarity in the manner in which zebrafish process dietary fat to mammals, the zebrafish intestine is very similar to that of mammals in terms of both its structure and development. Both contain an epithelium layer that projects into the lumen, comprising the mucosa. Additionally, both the zebrafish and mammalian intestines are composed of a layer of inner circular and outer longitudinal smooth muscle, comprising the muscularis (Wallace et al, 2005). Cell renewal in the zebrafish intestine is also very similar to that in mammals and epithelial cell proliferation is regulated by the microbiota, as in mammals (Uribe et al, 1997; Rawls et al, 2004; Crosnier et al, 2005). However, whilst mammals acquire the microbiota in the womb prior to birth, zebrafish are colonised after hatching between 48 - 72 hpf, once they are no longer protected by their chorions (Bates et al, 2006). Despite the similarities between the zebrafish and mammalian digestive tracts, there are major differences. One of these is that the mammalian intestine is comprised of two distinct organs: a small intestine involved in nutrient absorption, and a large intestine involved in water and ion uptake. In contrast, the zebrafish intestine is a continuous structure extending from the mouth to the anus (cloaca) (Wallace et al, 2005). The zebrafish intestine also lacks a submucosa region, which is present in the mammalian intestine and contains blood vessels, lymphatics and enteric nerves. Another difference is that in mammals, the epithelium forms finger-like projections called villi that extend into the lumen, whereas in zebrafish these
projections are broad and irregular (Wallace et al, 2005). The differences in structure between the adult mammalian and zebrafish intestines are shown in Fig 3.1:
3. Establishing and Characterising the Model

Figure 3.1: Differences in the Structure and Organisation of the Mammalian (A) and Zebrafish (B) Intestines. The mammalian intestine contains a submucosa layer (yellow) that is absent in that of the zebrafish. Additionally, the epithelial projections in the mammalian intestine are finger-like, whereas they are more broad and irregular in the zebrafish intestine (Figure adapted from Wallace et al., 2005).
The zebrafish intestine is comprised of two regions: the anterior intestine, or intestinal bulb (IB), and the posterior intestine (PI). The two regions carry out distinct roles in the processing and absorption of ingested food. The IB is involved in lipid absorption and this is highlighted by the enriched expression of intestinal fatty acid binding protein (I-FABP) in this region of 5 dpf, 7 dpf and 9 dpf larvae (Her et al, 2004; Crosnier et al, 2005). The anterior portion of the PI is involved in protein absorption and the posterior portion of the PI near the cloaca in water absorption and ion uptake (Crosnier et al, 2005). Essentially, the roles of the IB and the anterior portion of the PI are similar to that of the mammalian small intestine, and the role of the posterior portion of the PI is similar to that of the mammalian large intestine.

The primitive zebrafish intestine initially arises at 26 – 30 hpf from endodermic tissue running from what will form the mouth to the site of where the cloaca will develop. From 30 – 52 hpf, formation of the intestinal lumen occurs, which begins in the IB and progresses caudally to the PI (Ng et al, 2005). Following this, the intestine then develops in two stages: an expansion stage characterised by rapid cell proliferation and a differentiation stage (Wallace et al, 2005). The epithelium of the intestine undergoes a phase of rapid proliferation until approximately 74 hpf, whereby there is a decline in proliferation uniformly in all regions of the intestine and junctional complexes have now fully formed between epithelial cells (Wallace et al, 2005). After 74 hpf, epithelial cells adopt a columnar shape and undergo polarisation, with the nuclei being localised at the base of the cell (Ng et al, 2005). Enteric neurones and smooth muscle progenitor cells both first appear at approximately 50 hpf and follow a coincidental differentiation program from about 74 hpf (Wallace et al, 2005). Hormone-producing enteroendocrine cells first appear at approximately 52 hpf in the PI and then appear later in the IB (Ng et al, 2005). At 74 hpf, mucus-secreting goblet cells first start to appear primarily in the PI, before differentiating at around 100 hpf (Crosnier et al, 2005; Ng et al, 2005; Wallace et al, 2005). The cloaca opens at around 98 hpf and the lumen in the IB enlarges at this time too. Epithelial folds begin to develop in the IB at around 96 hpf and continue until approximately 120 hpf, whereby a fully functional intestine has developed (Ng et al, 2005). Between 6 dpf and 8 dpf, there is an increase in the number of neutrophils in the intestine as a result of microbial colonisation during this period. This is necessary to establish a homeostatic population of neutrophils in the intestine (Bates et al, 2007).
3.2 Aim

The aim of this Chapter was to establish and characterise zebrafish as a model for acute gastrointestinal inflammation following feeding on a fat-enriched diet. This was achieved in the following ways:

- Feeding zebrafish larvae a fat-enriched diet and observing the recruitment of various populations of immune cells to the intestine.
- Observing the localisation of immune cells within different regions of the intestine following feeding on a fat-enriched diet and examining this in more detail using histological sectioning.
- Detecting changes in expression of pro-inflammatory cytokines and chemokines following feeding on a fat-enriched diet.
- Investigating the effect of switching from a fat-enriched diet to a standard diet on the accumulation of immune cells in the intestine.
3.3 Results

3.3.1 Structure and Histology of the Zebrafish Larval Intestine

Figure 3.2: Anatomy of the Zebrafish Larval Intestine at 7 dpf. (A) Low magnification image of a live 7 dpf zebrafish larva. (B) Intestinal bulb (IB) and (C) posterior intestine (PI) of a fixed 7 dpf zebrafish larva. Areas of the IB and PI outlined by a black box are enlarged in (D) and (E), respectively. The intestine is outlined in red, (A), (B) and (C), the swim bladder depicted by a red arrow and the eye by a green arrow, (A) and (B). Larvae are oriented sagitally from anterior (left) to posterior (right). Scale bar (white) represents 1mm, black represents 0.4mm and red represents 0.1mm. Data shown for n = 1 experiments (number of times experiment was performed).
The zebrafish larval intestine (Fig 3.2, outlined in red) at 7 dpf is composed of two parts: the anterior (IB) (Fig 3.2B and D) and the posterior intestine (PI) (Fig 3.2C and E). The entire area outlined in red is the region in which the immune cells are counted in subsequent experiments.

In order to further examine the detailed histological structure of the zebrafish larval intestine, transverse sections were taken of both the IB and PI in paraffin-embedded 7 dpf larvae.
Figure 3.3: Morphology of the Zebrafish Larval Intestine at 7 dpf. 7 dpf WT larvae were fixed in 4% paraformaldehyde overnight at 4°C and then one group was not subjected to immunohistochemistry (no Ab) (A) and (B). Another was stained with a secondary IgG antibody conjugated to HRP raised in goat, followed by staining with DAB to check for non-specific staining of the secondary antibody (C) and (D). A final group was stained with a primary antibody against zebrafish L-plastin raised in rabbit, followed by staining with the secondary antibody, and then with DAB (E) and (F). Larvae were then embedded in a paraffin block and 5µM sections were cut using a rotary microtome, followed by staining with haematoxylin. Panels (A), (C) and (E) represent sections taken from the IB and panels (B), (D) and (F) represent sections taken from the PI. Stained L-plastin positive cells are indicated with an arrow (E) and (F). l denotes lumen, g goblet cells, e epithelial layer and m muscularis. Scale bar (black) represents 20µM and red represents 10µM. Data shown for n = 1 experiments (number of times experiment was performed).
There are three identifiable regions in both the IB and PI: the innermost lumen (l), the epithelial layer (e) and the outermost muscularis (m) (Fig 3.3). Additionally, goblet cells (g) are absent in the IB (Fig 3.3A, C and E) but present in the PI (Fig. 3.3B), although not across the entire length of the PI. This is shown by some sections in this region lacking goblet cells (Fig 3.3D and F). Staining larvae with a primary antibody against zebrafish L-plastin in combination with the secondary antibody and DAB gave positive staining of myeloid cells (Fig 3.3E and F, arrows). Staining zebrafish larvae with the secondary antibody followed by DAB, gave no positive staining indicating absence of both endogenous peroxidase activity in the larvae and non-specific staining of the secondary antibody (Fig 3.3C and D).

3.3.2 Accumulation of Immune Cells in the Zebrafish Larval Intestine in Response to an Enriched Fat Diet

* Differences in Rates of Ingestion and Transit of Fluorescent Microspheres by Zebrafish Larvae

As zebrafish larvae are capable of feeding from 5 dpf onwards, it was important to verify that they were able to swallow and ingested material would pass down the digestive tract. Fluorescent microspheres were thus used as a marker for following feeding. 6 dpf embryos were fed fluorescent microspheres for 4h. After this feeding period (0h post feeding), larvae were selected on the basis of whether or not they had ingested a sufficient number of microspheres to fill the IB. Those with sufficient numbers of microspheres were washed, anaesthetised and imaged. Larvae were also imaged at 4h and 20h post feeding.
3. Establishing and Characterising the Model

![Image of Figure 3.4: Accumulation and Localisation of Ingested Fluorescent Microspheres by Zebrafish Larvae.](image)

**Figure 3.4: Accumulation and Localisation of Ingested Fluorescent Microspheres by Zebrafish Larvae.** 6 dpf larvae were fed fluorescent microspheres for 4h. Following this, (0h post feeding), a selection of larvae were washed, anaesthetised and imaged. Larvae were also similarly imaged at 4h and 20h post feeding. (A) Accumulation of green microspheres in a larva after feeding for 4h (0h post feeding). (B) Localisation of microspheres in a larva 4h post feeding. (C) Clearance of microspheres from the intestine in a larva 20h post feeding. The swim bladder is depicted by a red arrow. Larvae are oriented sagitally from anterior (left) to posterior (right). Representative images are shown. Scale bar represents 0.5 mm. Data shown for n = 1 experiments (number of times experiment was performed).

There was sufficient accumulation of fluorescent microspheres to fill the IB of approximately 75% of 6 dpf larvae 0h post feeding (**Fig 3.4A**). At 4h post feeding, the microspheres were still present in the intestines of the larvae, but there were variations in the localisation within the intestine. Approximately 50% of the larvae showed a localisation pattern as in **Fig 3.4B**, with the microspheres having progressed along the length of the IB. The remaining 50% showed a localisation pattern as in **Fig 3.4A**, with the microspheres displaying the same localisation at 0h, indicating no progress along the IB. At 20h post feeding, approximately 90% of the larvae had passed the microspheres out of their systems (**Fig 3.4C**). Collectively, these results demonstrate that 6dpf zebrafish larvae are capable of swallowing, but show variations in the transit of the microspheres along their intestines.

**Accumulation of Granulocytes in the Intestine of Zebrafish Larvae in Response to an Enriched Fat Diet**

To study the effect of a fat-enriched diet on the number of granulocytes found in the intestine, larvae were fed for 6h either clotted cream, milk of an equivalent protein concentration or were unfed. Larvae were then stained for MPO activity, a marker of these immune cells, and numbers of cells were counted in the intestine.
3. Establishing and Characterising the Model

Figure 3.5: Accumulation of Granulocytes in the Intestines of Zebrafish Larvae Fed a Fat-enriched Diet. 6 dpf WT larvae were fed either clotted cream containing 6.25% fat, milk of an equivalent protein concentration to the cream or were unfed for 6h. Then they were fixed overnight at 4°C and then stained for MPO activity of granulocytes. (A) Unfed larva, (B) larva fed cream and (C) larva fed milk. The red box highlights the area of interest, with the intestine outlined in white and the swim bladder depicted by an arrow. MPO positive granulocytes are stained black and examples are highlighted with a black open arrowhead. Larvae are oriented sagittally from anterior (left) to posterior (right). Scale bar represents 0.5 mm. Representative images are shown. Comparison of the numbers of granulocytes in the intestines of individual larvae (D) and mean numbers of granulocytes (E) in the intestines under the various feeding conditions. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. *** denotes p < 0.001 and ** p < 0.01. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (number of times experiment was performed).

Successful staining in each experiment of the cell type was verified by presence in the blood island, the principal region for haematopoiesis in the larvae. The number of granulocytes in the intestine showed a statistically significant increase of ~ 1.5 fold in larvae fed cream compared to unfed larvae (Fig 3.5D and E). As larvae fed milk did not show this significant
accumulation, the protein component alone of the cream is not responsible for this effect (Fig 3.5D and E). Taken together, the results show that feeding zebrafish larvae an enriched fat diet results in a significant accumulation of granulocytes in the intestine.

**Accumulation of Pu.1 Expressing Cells Peaks at 12h After a 6h Feed on a Fat Enriched Diet and Subsides by 27h After Feeding**

To examine the effects of acute fat feeding on the accumulation of immune cells in the intestines of larvae over a period of time after feeding for 6h, larvae were anaesthetised at 3h intervals and imaged over a 27h period after feeding and removal from the cream (Fig 3.6). This experiment took advantage of transgenic zebrafish, which express GFP under the influence of the Pu.1 promoter. Pu.1 is a transcription factor that drives myelopoiesis and is an early marker of myeloid cells (Scott *et al*, 1994; McKercher *et al*, 1996).

**Figure 3.6: Design of the Acute Fat Feeding Time Course of 6 dpf Pu.1 GFP Larvae.** Larvae were either fed double cream or were unfed for 6h. At the end of this period (6h + 0h), larvae were removed from the cream and placed in system water. Numbers of Pu.1 expressing cells accumulating in the intestines of cream fed and unfed larvae were counted at 3h intervals after removal from the cream.
3. Establishing and Characterising the Model

Figure 3.7: Accumulation of Pu.1 Expressing Cells Peaks at 12h After a 6h Feed on a Fat Enriched Diet and Subsides by 27h After Feeding. 6 dpf Pu.1 GFP transgenic larvae were either fed double cream containing 6.25% fat or were unfed for 6h. Double cream was found to disperse into the water more uniformly than clotted cream following its administration. After this feeding period, larvae were removed from the diet and at 3h intervals post removal, numbers of GFP positive cells were counted in anaesthetised larvae. Mean numbers of GFP positive cells in the intestines of larvae at 3h intervals from the diet. Statistical significance was determined using a t-test or a Mann-Whitney test comparing between unfed and cream fed larvae at each time point. *** denotes p < 0.001, ** p < 0.01 and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (number of times experiment was performed).

During the time course there was a slight increase in the number of GFP positive cells in the intestines of unfed larvae, suggesting an increase in cell numbers with increasing age. Immediately after removal (0h), there was approximately twice the number of cells in the intestines of larvae fed cream compared to the unfed control. Cell numbers increased in cream-fed larvae until a peak was reached 12h after removal from the cream (~ 9 cells). After this peak, there was a decrease in the number of cells until they reached the level of the unfed control 27h after removal from the cream (~ 4 cells). There was a statistically significant difference in the cell numbers in larvae fed cream compared to unfed controls.
from 0h to 15h after removal from the cream, at which point the significance was lost (Fig 3.7). These results show that the number of GFP positive cells in the intestines of larvae fed cream increases until a peak is reached 12h after removing the larvae from the cream and this is followed by a subsequent decline. 27h after removing the larvae from the cream, the inflammatory effect of feeding an enriched fat diet appears to subside.

Accumulation of Myeloid Cells in the Intestines of Larvae fed a Fat-enriched Diet for 6h Peaks at 18h Following Removal from the Diet

Having seen an accumulation of Pu.1 expressing cells in the intestines of larvae fed a fat-enriched diet, the behaviour of other myeloid cell populations was then investigated. Two such markers of myeloid cells were LyzC and L-plastin, which are expressed in both neutrophils and macrophages but are expressed later in development than Pu.1 (Bennett et al, 2001; Berman et al, 2005). A transgenic line of zebrafish expressing dsRed under the influence of the LyzC promoter was used along with WT larvae that were stained with an antibody against L-plastin. Larvae were fed for 6h on either a fat-enriched diet or were unfed and at various time points following removal from the diet, numbers of dsRed positive cells were counted in the intestine. At these same time points, WT larvae were fixed, stained and of L-plastin positive cells were enumerated.
3. Establishing and Characterising the Model

Feeding a fat-enriched diet in the form of cream resulted in an increase in the number of dsRed positive cells and L-plastin positive cells until 18h following removal from the diet, when a peak was reached (~19 and ~26 cells, respectively). At 24h following removal from the diet, there was a decrease in the number of cells to a level approximately equal to that of the unfed control. The differences in the numbers of both dsRed and L-plastin positive cells at 0h, 6h, 12h and 18h after removal from the diet were statistically significant compared to the unfed groups at these time points. The fold changes between the cream-fed and unfed larvae at these time points were also approximately the same for L-plastin and dsRed positive cells. However, there were slightly higher numbers of L-plastin positive cells compared to the numbers of dsRed positive cells for the unfed group as a result of the resident population of L-plastin positive cells in the intestine being higher than that of the dsRed positive cells. Consequently, L-plastin positive cells accumulated in greater numbers than dsRed positive cells following feeding on a fat-enriched diet (Fig 3.8). Collectively, these results demonstrate that the number of myeloid cells accumulating in the intestine following feeding on a fat-enriched diet increases until a peak is reached at 18h, after which there is a decline and the fold change between cream-fed and unfed larvae is the same for both L-plastin and

Figure 3.8: Accumulation of Myeloid Cells in the Intestines of Larvae fed a Fat-enriched Diet for 6h Peaks at 18h Following Removal from the Diet. 6 dpf LyzC dsRed and WT larvae were fed double cream or were unfed for 6h. At various time points following removal from the diet, numbers of dsRed positive cells were counted in the intestines of LyzC dsRed larvae. At the same time points following removal from the diet, WT larvae were fixed, stained with an antibody against zebrafish L-plastin and numbers of L-plastin positive cells were counted in the intestine. Mean numbers of dsRed positive cells under the LyzC promoter (A) and L-plastin positive cells (B) in the intestines of larvae fed either cream or unfed over the time course. Statistical significance was determined using a t-test or a Mann-Whitney test comparing between cream-fed larvae and unfed larvae at each time point. *** denotes p < 0.001, ** p < 0.01 and * p < 0.05. Error bars represent 95% confidence intervals. Data for n = 1 experiments (number of times experiment was performed).
dsRed positive cells at the respective time points. Additionally, there are a slightly higher number of resident L-plastin positive cells present in the intestine compared to dsRed positive cells and these cells consequently accumulate in slightly greater numbers following feeding on a fat-enriched diet.

3.3.3 Accumulation of Immune Cells in the Zebrafish Larval Intestine in Response to a Cholesterol-enriched Diet

*Feeding a Cholesterol-enriched Diet has a Similar Effect to Feeding Cream in both the Kinetics of the Accumulation of Pu.1 Positive Cells in the Intestine and Numbers of Cells*

After establishing that feeding 6 dpf larvae an enriched fat diet in the form of cream results in a localised accumulation of immune cells to the intestines of larvae, the next step was to use a more refined diet enriched in single components which were present in cream. Due to the crude nature of the cream, in that the fat element was very heterogeneous in terms of its composition, it was necessary to see whether or not such individual components were capable of achieving the same inflammatory effect as the cream. Thus, such components may potentially be responsible for the effect observed with the cream. Pu.1 GFP larvae were fed either cream, a standard diet (ZM), ZM enriched in cholesterol, palmitic acid, or myrsitic acid, or were unfed for 6h. At various time points following feeding, numbers of GFP positive cells were counted in the intestines of the larvae.
3. Establishing and Characterising the Model

Figure 3.9: Effect of Feeding Various Components of Cream on the Accumulation of GFP Positive Cells in the Intestines of Larvae. 6 dpf Pu.1 GFP larvae were fed double cream, standard diets (ZM) enriched in palmitic acid, myristic acid, or cholesterol, ZM or were unfed for 6h (Cholesterol and fatty acids used at a concentration of 8%). At various time points following removal from the diet, the larvae were anaesthetised and numbers of GFP positive cells were counted in the intestine. The graph shows the comparison of the numbers of GFP positive cells in the intestines of the larvae fed the various diets at several time points following removal from the diets. Error bars represent 95% confidence intervals. Data for n = 1 experiments (number of times experiment was performed).

Feeding larvae a diet enriched in cholesterol resulted in a significant accumulation of GFP positive cells in the intestines of these larvae compared to unfed larvae. The kinetics of the cell recruitment and the numbers of cells recruited was similar between larvae fed a cholesterol-enriched diet and those fed cream with both effects peaking at 12h following removal from the diets. At this time point, there were approximately 2.5x the number of cells in the intestines of larvae fed these two diets compared to unfed larvae. Larvae fed a palmitic acid-enriched diet also showed a significant accumulation of cells. However, this peaked at
18h following removal from the diet, with the numbers at 12h being lower than both cream and cholesterol. Feeding larvae a myristic acid-enriched diet resulted in a peak recruitment of cells at 18h following removal from the diet, but unlike the effect of a palmitic acid-enriched diet, this was not significantly different to unfed larvae. Larvae that were fed ZM showed a very similar accumulation of cells to that of unfed larvae, indicating that the standard diet alone has no inflammatory effect and that any effect seen is due to cholesterol or palmitic acid (Fig 3.9). These results show that cholesterol has a similar effect to cream in terms of both the kinetics and the numbers of accumulated cells in the intestine.

*L-Plastin Positive Cells Show the Greatest Accumulation in the Intestine Following Feeding on a Cholesterol-enriched Diet*

After observing that feeding larvae on a cholesterol-enriched diet resulted in an accumulation of Pu.1 expressing cells in the intestine, the effect of feeding such a diet on other immune cell populations was studied. WT and transgenic strains of larvae expressing GFP under the Pu.1 or LyzC promoter were fed and at various time points post-feeding, numbers of immune cells were quantified in the intestines of transgenic larvae and WT larvae fixed and stained for MPO and L-plastin.
3. Establishing and Characterising the Model

Figure 3.10: Comparison of the Kinetics of the Accumulation of Different Immune Cell Populations in the Intestine Following Feeding on a Cholesterol-enriched Diet. 6 dpf WT, Pu.1 GFP or LyzC GFP larvae were fed either 8% cholesterol enriched standard diet (ZM) or ZM for 6h. At various time points following removal from the diet, the numbers of MPO positive cells (A), Pu.1 GFP cells (B), L-Plastin positive cells (C) and LyzC GFP cells (D) were counted in the intestine. MPO positive cells were counted in WT larvae that had been fixed and stained for MPO activity in granulocytes. L-Plastin positive cells were counted in WT larvae that had been fixed and antibody stained. Statistical significance was determined using a t-test or a Mann-Whitney test comparing between cholesterol-fed and ZM-fed groups at each time point. ** denotes p < 0.01 and * denotes p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (number of times experiment was perfomed).

Feeding larvae a cholesterol-enriched diet resulted in an accumulation of all four populations of immune cells in the intestine compared to larvae fed a standard diet. There was also an increase in the number of immune cells in the ZM-fed group for all of the populations.
through the time course, suggesting an age-related increase. However, the rate of increase of immune cells for each successive time point in the cholesterol-fed groups was greater than the rate of increase in the ZM-fed groups. This indicates that the accumulation over time following feeding on a cholesterol-enriched diet was due to a recruitment of immune cells over time and not an initial recruitment at 0h post removal, followed by an age-related increase (**Fig 3.10**). The peak accumulation of MPO positive cells and cells expressing GFP under the Pu.1 promoter (Pu.1 GFP cells) occurred at 12h post-removal (~ 9 cells and ~ 8 cells, respectively; **Fig 3.10A** and **B**), whereas the peak accumulation of L-plastin positive cells and cells expressing GFP under the LyzC promoter (LyzC GFP cells) occurred at 18h post-removal (~ 28 cells and ~ 15 cells, respectively; **Fig 3.10C** and **D**). Additionally, the numbers of recruited L-plastin positive cells following cholesterol feeding were the highest of all of the immune cell populations throughout the time course and showed the greatest level of statistical significance across the time course compared to the numbers of the ZM-fed larvae (**Fig 3.10C**).

After 12h, there was a reduction in the numbers of MPO positive and Pu.1 GFP cells in cholesterol-fed larvae, such that by 24h, the numbers were approximately the same as that of the ZM-fed control (~ 5 cells and ~ 4 cells, respectively, **Fig 3.10A** and **B**). After 18h, there was a reduction in the numbers of L-plastin positive and LyzC GFP cells in cholesterol-fed larvae, such that by 24h post-removal, the numbers of LyzC GFP cells were approximately equal to that of ZM-fed larvae (~ 10 cells; **Fig 3.10C** and **D**). However, although the number of L-plastin positive cells was reduced at 24h following removal from the diet, they still appeared to be higher than that of the ZM-fed larvae (~ 22 cells compared to ~ 17; **Fig 3.10D**). Across the ZM-fed groups, L-plastin positive cells also showed the greatest numbers indicating a higher resident population of L-plastin positive cells in the intestine compared to the other immune cell populations (**Fig 3.10C**). These results demonstrate that L-plastin positive cells show the greatest accumulation of the four immune cell populations following feeding on a cholesterol-enriched diet and cell numbers peak at 18h post-removal.
A Diet Enriched in 8% Cholesterol Gives the Greatest Accumulation of L-Plastin Positive Cells in the Intestine

Given that an 8% cholesterol diet resulted in a similar response to cream in terms of the accumulation of Pu.1 positive cells in the intestine, it was then necessary to determine whether this was the optimum concentration of cholesterol to use as an enriched diet. In order to do this, 6 dpf larvae were fed a range of concentrations of cholesterol or ZM made up both in the presence and absence of ether, the solvent in which the diets are prepared, for 6h. This was to exclude the possibility of an inflammatory effect associated with ether in the diets, although it evaporates from the diets prior to their use. 18h after removal from the diet, the larvae were fixed and stained and L-plastin positive cells were enumerated in the intestine.

Figure 3.11: A Diet Enriched in 8% Cholesterol Gives the Greatest Accumulation of L-Plastin Positive Cells in the Intestine. 6 dpf WT larvae were fed either a standard diet (ZM) prepared with or without ether or a cholesterol-enriched diet containing various concentrations of cholesterol from 2% - 10% for 6h. 18h following removal from the diet, larvae were fixed, stained and numbers of L-plastin positive cells were counted in the intestine. Comparison of cell numbers in individual larvae (A) and mean numbers of cells (B) in larvae fed either ZM or cholesterol. Statistical significance was determined using a one-way ANOVA followed by a Bonferroni post test. *** denotes p < 0.001 and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (number of times experiment was performed).

Feeding larvae a diet enriched in a range of concentrations of cholesterol resulted in a dose-dependent increase in the number of L-plastin positive cells accumulating in the intestine up
to an 8% concentration. A 10% diet gave a similar number of cells (~ 19) as an 8% diet, indicating that a peak is reached at an 8% concentration. Feeding larvae a diet enriched in 4%, 8% or a 10% concentration of cholesterol resulted in a statistically significant accumulation of cells compared to ZM-fed larvae, with 8% and 10% giving the greatest level of significance (p < 0.001) (Fig 3.11B). Furthermore, feeding larvae ZM prepared either in the presence or absence of ether gave approximately the same number of cells (~ 11), indicating that the ether has no inflammatory effect, as expected. These results show that a diet enriched in 8% cholesterol is sufficient to give the peak accumulation of L-plastin positive cells in the intestine.

Treating Cholesterol-Fed Larvae with Ezetimibe Reduces the Number of L-Plastin Positive Cells Accumulating in the Intestine

Whether the uptake of cholesterol was required for the accumulation of immune cells in the intestine to occur was then investigated. This involved the use of a drug, ezetimibe, which is an inhibitor of the cholesterol transport protein NPC1L1, thus preventing cholesterol uptake by intestinal epithelial cells (Betters and Yu, 2010). Larvae were pre-treated with ezetimibe or DMSO and then fed a cholesterol-enriched or palmitic acid-enriched diet for 6h. Numbers of L-plastin positive cells were counted in larvae fixed and stained 18h after removal from the diet.
3. Establishing and Characterising the Model

Figure 3.12: Treating Cholesterol-Fed Larvae with Ezetimibe Prevents L-Plastin Positive Cells from Accumulating in the Intestine. 5 dpf WT larvae were pre-treated overnight with either ezetimibe, or an equivalent volume of DMSO, or untreated. The following day, one group of untreated larvae were fed a standard diet (ZM) and an untreated group and the treated groups were fed a cholesterol-enriched diet or palmitic acid-enriched diet for 6h, whilst in the presence of the drug. 18h following removal from the diet, but still in the presence of ezetimibe, larvae were fixed, stained and numbers of L-plastin positive cells were counted in the intestines. Comparison of cell numbers in individual larvae (A) and mean numbers of cells (B) in larvae treated with 12.5µM or 25µM ezetimibe, or an equivalent volume of DMSO to the concentrations of ezetimibe used (low or high being equivalent to 12.5µM or 25µM, respectively), or untreated under the different feeding conditions. Comparison of cell numbers in individual larvae (C) and mean numbers of cells (D) in larvae treated with 25µM ezetimibe or DMSO or untreated under the different feeding conditions. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. *** denotes p < 0.001 and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (A) and (B), and n = 1 experiments (C) and (D) (numbers of times experiments were performed).
Treating larvae with 25µM ezetimibe, followed by feeding a cholesterol-enriched diet resulted in a reduction in the number of L-plastin positive cells accumulating in the intestine compared to cholesterol-fed larvae exposed to an equivalent volume of DMSO to that of 25µM ezetimibe (~ 7 - 8 compared to ~ 13 - 14). However treatment with 12.5µM ezetimibe had no effect (Fig 3.12A and B). Larvae fed a palmitic acid-enriched diet exposed to 25µM ezetimibe showed no reduction in cell numbers compared to larvae pre-treated with DMSO, as expected. This indicates that palmitic acid is taken up independently of NPC1L1 and validates the specificity of ezetimibe in blocking cholesterol uptake only (Fig 3.12C and D). Taken together, these results suggest that the uptake of cholesterol may be required for the recruitment of L-plastin positive cells to the intestine following feeding on a cholesterol-enriched diet.

3.3.4 Localisation of L-Plastin Positive Cells Within the Zebrafish Larval Intestine

*L-plastin Positive Cells Tend to Accumulate in the Posterior Intestine of Larvae Fed a Cholesterol-enriched Diet*

Establishing that acute feeding of a cholesterol-enriched diet to 6dpf larvae resulted in an accumulation of a variety of immune cell populations in the intestines, of which L-plastin positive cells showed the greatest response, the finer localisation of this population of cells within the intestine was examined. Larvae were fed a cholesterol-enriched diet or ZM and numbers of L-plastin positive cells were quantified in the IB and PI of the larvae.
3. Establishing and Characterising the Model

Figure 3.13: L-plastin Positive Cells Tend to Accumulate in the Posterior Intestine of Larvae Fed a Cholesterol-enriched Diet. 6 dpf WT larvae were fed either a standard diet (ZM), or a cholesterol-enriched diet for 6h. 18h following removal from the diet, larvae were fixed, stained and numbers of L-plastin positive cells in the intestine were counted. Examples of L-plastin positive cells are depicted with a white arrow and the intestine is outlined in white. IB denotes the intestinal bulb (A) and (C), and PI posterior intestine (B) and (D). Larvae are oriented sagitally from anterior (left) to posterior (right). Scale bar represents 0.4mm. Comparison of cell numbers in the entire intestines of individual larvae (E) and mean numbers of cells (G) in the entire intestines of larvae fed either ZM or cholesterol. Comparison of cell numbers in the IB and PI of individual larvae (F) and mean numbers of cells (H) in the IB and PI of larvae fed either ZM or cholesterol. Statistical significance was determined using a Wilcoxon matched pairs test (E) and (G) or a Kruskall-Wallis test followed by a Dunn’s post test (F) and (H). *** denotes p < 0.001. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (number of times experiment was performed).
3. Establishing and Characterising the Model

The majority of L-plastin positive cells accumulating in the intestines of larvae fed a cholesterol-enriched diet tended to do so in the PI with only a relatively small number in the IB. Numbers in the IB appeared to be similar between ZM-fed and cholesterol-fed larvae but there were considerably more cells accumulating in the PI of cholesterol-fed larvae compared to ZM-fed larvae. (Fig 3.13A – D). When quantifying the numbers of L-plastin positive cells in the entire intestines of cholesterol-fed larvae compared to ZM-fed larvae, there were approximately twice the number of cells in the intestines of larvae fed a cholesterol-enriched diet compared to larvae fed ZM (Fig 3.13E and G). When quantifying the number of cells in the IB and PI of cholesterol and ZM-fed larvae, there were no significant differences in cell numbers in the IB between the two diets. However, there was a significant difference of approximately three-fold in the PI of larvae fed the two diets (Fig 3.13F and H). These results demonstrate that the majority of L-plastin positive cells accumulating in the intestines of larvae fed a cholesterol-enriched diet do so in the PI and there is relatively little accumulation in the IB.

*L-plastin Positive Cells are Localised in the Muscularis of the Intestine for Both Cholesterol and ZM-fed Larvae*

Given that the majority of cells were localised in the PI, the next step was to determine the localisation of these cells within the histology of the intestine. Fixed and stained larvae were embedded in paraffin and 5µM sections were cut.
3. Establishing and Characterising the Model

**Figure 3.14: L-plastin Positive Cells are Localised in the Muscularis of the Intestine for Both Cholesterol and ZM-fed Larvae.** 6 dpf WT larvae were either fed a standard diet (ZM) or a cholesterol-enriched diet for 6h. 18h after removal from the diet, larvae were fixed and antibody stained followed by DAB staining. Stained larvae were then embedded in a paraffin block and 5µM sections were cut using a rotary microtome, followed by staining with haematoxylin. Panels (A) and (C) represent sections taken from the intestinal bulb (IB) and panels (B) and (D) represent sections taken from the posterior intestine (PI). Stained L-plastin positive cells (brown) are indicated with an arrow. l denotes lumen, g goblet cells, e epithelial layer and m muscularis. Scale bar (black) represents 20µM and red represents 10µM. Data shown for n = 1 experiments (number of times experiment was performed).

L-plastin positive cells (arrows) in the intestines of larvae fed ZM were localised in the muscularis layer of the gut for both the IB and PI (**Fig 3.14A and B**). In the intestines of larvae fed a cholesterol-enriched diet, the cells were also distributed in the muscularis for both the IB and PI (**Fig 3.14C and D**). However, there were approximately two cells per section in the PI for cholesterol-fed larvae compared to one cell for ZM-fed larvae, consistent with the significant difference in this region (**Fig 3.14B and D**). Additionally, a greater number of PI sections from cholesterol-fed larvae contained cells than those from ZM-fed
larvae, as expected. The morphology of the guts between ZM-fed and cholesterol-fed larvae was the same for both the IB and PI. These results show that there is no change in localisation of recruited L-plastin positive cells in the intestine after feeding a cholesterol-enriched diet compared to the resident population in the ZM-fed group. The localised inflammation associated with feeding on a cholesterol-enriched diet also does not affect the morphology of the intestine compared to feeding on ZM.

3.3.5 Analysis of Cytokine and Chemokine Transcripts Following Feeding on a Cholesterol-enriched Diet

To further study the pro-inflammatory effect of feeding a cholesterol-enriched diet to zebrafish larvae, changes in expression of cytokines and chemokines in response to a cholesterol-enriched diet were analysed. Larvae were fed for 4h and immediately after feeding, had their intestines dissected and the remaining carcasses also kept. RNA was extracted and cDNA synthesised and analysed by qRT-PCR for expression of a variety of cytokines and chemokines (Table 3.1).
### Table 3.1: Analysis of Transcripts for Various Cytokines and Chemokines Following Feeding a Cholesterol-enriched Diet

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Undetected</td>
</tr>
<tr>
<td>IL-17</td>
<td>Undetected</td>
</tr>
<tr>
<td>IFN-1</td>
<td>Undetected</td>
</tr>
<tr>
<td>Lta</td>
<td>Undetected</td>
</tr>
<tr>
<td>M-17</td>
<td>Undetected</td>
</tr>
<tr>
<td>IL-10</td>
<td>Undetected</td>
</tr>
<tr>
<td>IL-12a</td>
<td>Undetected</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>Detectable but no difference</td>
</tr>
<tr>
<td>Cxcl12</td>
<td>Detectable but no difference</td>
</tr>
<tr>
<td>IL-15</td>
<td>Detectable but no difference</td>
</tr>
<tr>
<td>IL-8</td>
<td>Detectable but no difference</td>
</tr>
</tbody>
</table>

6 dpf larvae were fed either a cholesterol-enriched diet or standard diet (ZM) for 4h and following this, had their intestines dissected with the remaining carcasses also kept. RNA was extracted from the tissue and cDNA synthesized and analysed by qRT-PCR for cytokine and chemokine expression. Undetected refers to a gene that gave a C_{T} of above 35 or was stated as beyond the level of detection. Detectable but no difference refers to a gene that gave a detectable C_{T} but there was no difference in expression between the cholesterol and ZM-fed larvae in both the intestines and remaining carcasses. Data shown for n = 1 experiments (number of times experiment was performed).

11 cytokines and chemokines were analysed for expression changes by qRT-PCR, but 7 out of the 11 were beyond the level of detection. Of the remaining four that were detectable, there was no difference in expression levels in any of these between the cholesterol-fed and ZM-fed groups in both the intestines and remaining carcasses (Table 3.1).

Changes in IL-8 expression between the two feeding conditions over a time course were then measured.
3. Establishing and Characterising the Model

**Figure 3.15: Analysis of IL-8 Expression Following Feeding for a Variety of Time Periods.** 6 dpf WT larvae were fed either a cholesterol-enriched diet or a standard diet (ZM) for 1h, 2h, 4h or 6h. At the end of these time points, larvae had their intestines dissected and the remaining carcasses also kept. RNA was extracted from pooled intestines (20 intestines/pool) and from one pool of carcasses (5 carcasses/pool) for each condition, and cDNA was synthesised. Expression levels of IL-8 were determined by qRT-PCR, normalised to EF1α and readings were calibrated to a group of larvae that were killed immediately prior to the start of feeding (Unfed 0h). Comparison of changes in IL-8 expression in the intestine (A) and remaining carcasses (rest) (B) during feeding on either ZM or a cholesterol-enriched diet. C_T values for IL-8 in both the intestines and remaining carcasses ranged between 29-30. C_T values for EF1α both in the intestines and remaining carcasses ranged between 18-19. Data shown for n = 1 experiments (number of times experiment was performed).
There appeared to be a reduction in IL-8 expression in cholesterol-fed larvae compared to unfed larvae at 0h in both the intestines and remaining carcasses after feeding for 1h. The level of expression then seemed to slightly decrease in the intestines and very slightly increase in the remaining carcasses after feeding for 2h. After a 4h feed, there was an increase in expression in both the intestines and remaining carcasses. After a 6h feed, the level of expression decreased slightly in the intestines and remained the same in the remaining carcasses. The trend observed for changes in IL-8 expression throughout the time course is very similar in both the intestines and remaining carcasses of ZM-fed larvae with only small fluctuations in expression between time points (Fig 3.15). There were also differences in the level of expression in the intestine between cholesterol and ZM-fed larvae with expression levels being ~1.3 fold lower in cholesterol-fed larvae compared to ZM-fed larvae, except after a 4h feed where levels of expression were ~1.5 fold higher in cholesterol-fed larvae. In the remaining carcasses, expression levels were lower in cholesterol-fed larvae compared to ZM-fed larvae (~2 fold at 1h and ~1.2 fold at 2h and 6h), except after 4h, where levels were ~1.3 fold higher. However, these differences are not biologically relevant. IL-8 expression levels were also normalised to other housekeeping genes, but also showed no meaningful differences between cholesterol-fed and ZM-fed larvae.

3.3.6 Unsaturated Fatty Acids do not Cause a Significant Accumulation of Immune Cells in the Intestines of Larvae unlike a Saturated Fatty Acid

The data so far has shown that feeding larvae on a palmitic acid-enriched diet resulted in an accumulation of immune cells in the intestine compared to ZM-fed larvae (Fig 3.9). Given that palmitic acid is SFA, the effect of feeding a diet enriched in unsaturated fatty acids was studied. Larvae were fed diets enriched in palmitic acid or the UFA, linoleic acid and oleic acid, or ZM for 6h. 18h after removal from the diet, larvae were fixed and stained for L-plastin positive cells.
Figure 3.16: Unsaturated Fatty Acids do not Cause a Significant Accumulation of L-plastin Positive Cells in the Intestines of Larvae Unlike Palmitic Acid. 6 dpf WT larvae were fed either a standard diet (ZM), or diets enriched in either palmitic acid, linoleic acid or oleic acid for 6h. 18h following removal from the diet, larvae were fixed and stained, and numbers of L-plastin positive cells were counted in the intestines. Comparison of cell numbers in individual larvae (A) and mean numbers (B) in larvae fed either ZM or varying concentrations of palmitic acid. Comparison of cell numbers in individual larvae (C) and mean numbers (D) in larvae fed either ZM or the FA-enriched diets (8%). Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test (A) and (B) or a one-way ANOVA followed by a Bonferroni post test (C) and (D). *** denotes p < 0.001, ** p < 0.01 and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (number of times experiment was performed).

Feeding larvae a palmitic acid-enriched diet resulted in a dose-dependent increase in the number of L-plastin positive cells in the intestine compared to ZM-fed larvae up to an 8% concentration, as was the case with cholesterol. Beyond 8%, the response formed a plateau with 10% giving a similar number of cells to 8% (~ 17 - 18 cells). Feeding larvae a diet
enriched in 4%, 8% or a 10% concentration of palmitic acid resulted in a statistically significant accumulation of cells compared to ZM-fed larvae, with 8% giving the greatest level of significance (p < 0.001). Feeding larvae ZM prepared either in the presence or absence of ether gave approximately the same number of cells in the intestine (~12), indicating that the ether had no inflammatory effect, as expected. In contrast to the inflammatory effect observed with palmitic acid, the unsaturated fatty acids, linoleic acid and oleic acid, failed to show a significant increase in the number of L-plastin positive cells in the intestine compared to ZM (Fig 3.16C and D). These results demonstrate that a SFA, such as palmitic acid, can induce a localised accumulation of L-plastin positive cells in the intestine, but UFAs are unable to do so.

As UFAs did not cause a statistically significant increase in the number of L-plastin positive cells in the intestine, unlike palmitic acid, the behaviour of another cell population over a time course was investigated. Larvae were fed the same diets but at various time points post-feeding, GFP cells under the LyzC promoter were enumerated in the intestines of anaesthetised larvae.
3. Establishing and Characterising the Model

Figure 3.17: Unsaturated Fatty Acids do not Cause a Significant Accumulation of LyzC GFP Cells in the Intestines of Larvae Unlike Palmitic Acid Over a Time Course. 6 dpf LyzC GFP larvae were fed diets enriched in palmitic acid, linoleic acid, oleic acid or a standard diet (ZM) for 6h. At various time points following removal from the diet, the larvae were anaesthetised and numbers of GFP positive cells were counted in the intestines. The graph shows the comparison of the mean numbers of GFP positive cells in the intestines of the larvae fed the various diets at several time points following removal from the diets. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. *** denotes p < 0.001, ** p < 0.01 and * p < 0.05 and indicate statistical significance between palmitic acid and ZM. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (number of times experiment was performed).

Feeding larvae a palmitic acid-enriched diet resulted in an increase in the accumulation of GFP positive cells in the intestine up to 18h post removal (~ 21 cells), after which the numbers of cells decreased. There was a statistically significant difference in cell numbers between palmitic acid and ZM at 0h, 12h and 18h post-removal, with the greatest difference at 18h post removal (p < 0.001). In contrast, both linoleic acid and oleic acid fail to show a significant difference in the numbers of cells compared to ZM at any point in the time course.
3. Establishing and Characterising the Model

(Fig 3.17). These results thus show that UFAs do not cause a significant accumulation of GFP positive cells in the intestine, at least at the concentrations used, and this is consistent with the result from the previous experiment with the effect on L-plastin cell accumulation in the intestine.

3.3.7 Reversion to a Standard Diet Following Enriched Fat Feeding Leads to Accelerated Resolution of Inflammation

*Switching to a Standard Diet Following High Fat Feeding Results in a Reduction in the Number of Pu.1 GFP Cells Accumulating in the Intestine*

The results have demonstrated that feeding a diet enriched in either saturated fat or cholesterol results in a localised accumulation in the number of immune cells in the intestine compared to either unfed larvae or larvae fed a standard diet. The number of immune cells also peaks at a specific time point following removal from the diet after which there is a decline in the number of cells. However, the effect of switching to a standard diet following acute feeding of a fat enriched or cholesterol-enriched diet had not been been examined. 6 dpf Pu.1 GFP larvae were fed either cream or were unfed for 6h. Following removal from the diet, one group of cream-fed and unfed larvae were fed a standard diet whereas another group were unfed. 0h, 12h and 18h after switching diets, numbers of GFP positive cells were counted in the intestine.
Figure 3.18: Switching to a Standard Diet Following Feeding on a Fat-enriched Diet Results in a Reduction in the Number of Pu.1 GFP Cells Accumulating in the Intestine. 6dpf Pu.1 GFP larvae were either fed double cream for 6h or were unfed. Immediately after this feeding and upon removal, one group of unfed and one group of cream fed larvae were given ZM (Unfed ZM and Cream ZM, respectively), whilst another group of unfed and cream fed larvae were not (Unfed Unfed and Cream Unfed, respectively). Numbers of GFP positive cells were counted in the intestines of larvae at 0h, 12h and 18h after switching the diets. Comparison of the cells numbers in individual larvae under different feeding conditions at 0h (A), 12h (B) and 18h (C) after switching the diets. Comparison of the mean number of cells in larvae under the different feeding conditions at 0h (D), 12h (E) and 18h (F) after switching the diets. Statistical significance was determined using a Wilcoxon matched pairs test (A) and (D) or a Kruskall-Wallis test followed by a Dunn’s post test (B) and (E), and (C) and (F). *** denotes p < 0.001 and ** denotes p < 0.01. Error bars represent standard 95% confidence intervals. Data shown for n = 4 experiments for 0h and 18h, and n = 2 experiments for 12h (number of times experiments were performed).

There were approximately twice the number of GFP positive cells accumulating in the intestines of larvae fed cream compared to those unfed immediately after feeding, confirming the result shown in Fig 3.7 (Fig 3.18A and D). 12h after removing the larvae from the cream, there was an increase in the number of GFP positive cells compared to the numbers observed immediately removing larvae from the cream (Fig 3.18B and E). However, there was no significant effect of switching to a standard diet following an acute fat diet at this stage (Fig
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18h after removing the larvae from the cream and switching to a standard diet, there was a significant reduction in the number of cells compared to larvae fed an enriched fat diet and then starved (~ 5 cells compared to ~ 8; **Fig 3.18F**). This reduction resulted in a similar number of cells present in the intestine to that of the unfed control. These results show that feeding a standard diet following a high fat diet causes a more rapid reduction in the number of cells than observed with no refeeding.

*Switching to a Standard Diet Following Feeding on a Cholesterol-enriched Diet Results in a Reduction in the Number of Pu.1 GFP Cells Accumulating in the Intestine*

After observing that switching to a standard diet following feeding on a fat-enriched diet resulted in an accelerated reduction in the number of Pu.1 GFP positive cells in the intestine compared to no refeeding, the next step was to see if this were the case with feeding on a cholesterol-enriched diet. Pu.1 GFP larvae were fed as in the previous experiment, except cholesterol was used instead of cream.
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Figure 3.19: Switching to a Standard Diet Following Feeding on a Cholesterol-enriched Diet Results in a Reduction in the Number of Pu.1 GFP Cells Accumulating in the Intestine. 6 dpf Pu.1 larvae were fed either a cholesterol-enriched diet or were unfed for 6h. After this feeding period, one group of unfed larvae were fed a standard diet (ZM) whilst another group were unfed. Additionally, one group of cholesterol-fed larvae were left unfed and another group were fed ZM. 12h and 18h after switching diets, numbers of GFP positive cells were counted in the intestines of anaesthetised larvae. Comparison of the cell numbers in individual larvae (A) and (B), and mean numbers (C) and (D) at 12h and 18h after switching diets under the different feeding conditions. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. ** denotes p < 0.01. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (number of times experiment was performed).

Cholesterol-fed larvae that were not fed a standard diet after the 6h feeding period showed approximately twice the number of GFP positive cells in the intestine compared to unfed
larvae 12h after switching diets. There was a trend towards a reduction in the number of cells following switching to ZM after feeding on a cholesterol-enriched diet compared to larvae that were fed a cholesterol-enriched diet and starved afterwards, but it was not statistically significant (Fig 3.19A and C). 18h following switching diets, there was a stronger trend towards a reduction in the number of cells in larvae fed ZM compared to those that were starved after a cholesterol feed in comparison to 12h, but it was also not statistically significant (Fig 3.19B and D). Taken together, these results indicate that switching to a standard diet following feeding on a cholesterol-enriched diet shows a trend towards a reduction at both 12h and 18h post-switching.

_Switching to a Standard Diet Following Feeding on a Cholesterol-enriched Diet Results in a Reduction in the Number of L-Plastin Positive Cells Accumulating in the Intestine_

The effect of switching diets on another population of immune cells was thus investigated in order to establish if there was a similar effect to that on Pu.1 GFP cells and whether this effect was stronger with potentially higher numbers of immune cells in the cholesterol-fed groups that were starved after the initial feed. Therefore, L-plastin expressing cells were chosen as the immune cell population to study given that such cells show the greatest accumulation in response to feeding on a cholesterol-enriched diet. Larvae were fed either a cholesterol-enriched diet or were unfed for 6h. After this feeding period, cholesterol-fed larvae were either fed a further cholesterol-enriched diet or ZM or unfed for 12h or 18h. Unfed larvae were either fed ZM or unfed for 12h or 18h. Larvae were fixed and stained at 12h and 18h post-switching.
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Figure 3.20: Switching to a Standard Diet Following Feeding on a Cholesterol-enriched Diet Results in a Reduction in the Number of L-Plastin Positive Cells Accumulating in the Intestine. 6 dpf WT larvae were fed either a cholesterol-enriched diet or were unfed for 6h. After this feeding period, one group of unfed larvae were fed a standard diet (ZM) whilst another group were unfed. Additionally, one group of cholesterol-fed larvae were left unfed, another group were fed a cholesterol-enriched diet and a final group were fed ZM. 12h and 18h after switching diets, larvae were fixed, stained and numbers of L-plastin positive cells were counted in the intestines. Comparison of the cell numbers in individual larvae at 12h and 18h after switching diets (A) and (B), and mean numbers of cells in larvae at 12h and 18h after switching diets (C) and (D). Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. ** denotes p < 0.01. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (number of times experiment was performed).

There was an increase in the number of L-plastin positive cells accumulating in the intestines of cholesterol-fed larvae that are either left unfed or fed a further cholesterol-enriched diet
12h after switching diets compared to unfed larvae. There was also no difference in the cell numbers of larvae that were fed a further diet enriched in cholesterol compared to those that were left unfed after the initial cholesterol-enriched feed (~ 20 cells). This shows that a prolonged cholesterol feed does not induce an enhanced effect than the initial 6h feed. However, switching to ZM following a cholesterol-enriched feed resulted in a trend towards a decrease in the number of cells compared to cholesterol-fed larvae that were left unfed or fed a further diet enriched in cholesterol 12h after switching diets (~ 17 – 18 cells; Fig 3.20A and C). 18h after switching diets, there was an increase in cell numbers in larvae that were switched to a second cholesterol-enriched diet or were left unfed compared to 12h after switching diets. There was also no difference in the number of cells accumulating in the intestines of larvae that were switched to a second cholesterol-enriched diet compared to those that were left unfed 18h after switching diets (~ 22 – 23 cells). However, there was a statistically significant decrease in cell numbers in larvae switched to ZM after the initial cholesterol feed compared to those that were fed a further cholesterol-enriched diet or were left unfed (~ 16 cells; Fig 3.20B and D). Collectively, these results demonstrate that switching from a cholesterol-enriched diet to a standard diet results in a reduction in the number of L-plastin positive cells accumulating in the intestine. Additionally, feeding a prolonged diet enriched in cholesterol does not result in an increase in the number of cells accumulating in the intestine compared to an initial 6h feed.
3.4 Discussion

In brief, the results have demonstrated that acute feeding of zebrafish larvae a diet enriched in either cholesterol or saturated fat results in a significant accumulation of myeloid cells in the intestines compared to control larvae. However, a diet enriched in UFAs does not have this effect. The results also show that of the cell populations examined, L-plastin expressing cells display the greatest level of accumulation in the intestine and peak 18h after removal from the diet. These cells accumulate primarily in the PI but show the same localisation in the histology of the intestine in both cholesterol-fed and ZM-fed larvae. Treatment with ezetimibe reduces the recruitment of L-plastin positive cells to the intestine following feeding on a cholesterol-enriched diet. Finally, switching from a fat-enriched diet to a standard diet results in an accelerated resolution of the inflammatory effect.

3.4.1 Differences in Rates of Ingestion and Transit of Fluorescent Microspheres by Zebrafish Larvae.

The variations in the passage of ingested material along the digestive tract are consistent with observations reported by Field et al, who also showed that there are variations in transit times across the intestine of 7dpf larvae (Fig 3.4). In this study, by 24h post feeding, the majority of larvae have cleared a fluorescent tracer consisting of powdered larval feed and yellow-green fluorescent 2.0μm polystyrene microspheres from their intestine, but a small number still contain this tracer. Field et al also show that approximately 50% of the larvae that were fed the tracer had actually ingested it after 2h of feeding (Field et al, 2009). This indicates that whilst larvae are capable of feeding at this age, not all of them ingest material at the same rate nor does the ingested material progress along the digestive tract at the same rate in those that have fed. This could account for the variability observed in numbers of immune cells recruited to the intestines of larvae within the same treatment group as it is possible that larvae which have ingested a greater amount of a fat-enriched diet display a higher number of recruited cells than those that have ingested less.
3.4.2 A Fat-Enriched Diet Results in the Localised Recruitment of Leukocytes to the Intestine

The observed recruitment of leukocytes to the intestines of larvae following feeding on a fat-enriched diet has never, to our knowledge, been observed before. It also represents a process of inflammation associated with enriched-fat feeding that has not, to our knowledge, been previously investigated. The ability of the SFA, palmitic acid, to cause a significant accumulation of myeloid cells in the intestines of larvae is consistent with its well-established pro-inflammatory effects. In contrast, the inability of the UFAs, oleic acid and linoleic acid, to do so is also supported by their anti-inflammatory properties in the literature (Fig 3.16 – 3.17). Palmitic acid impairs insulin signalling and induces production of ceramide and synthesis of DAG in C2C12 myotubes. It has also been demonstrated to activate NF-κB and MAPK signalling pathways in adipocytes leading to induction of MCP-1 expression, resulting in the recruitment of monocytes to adipose tissue (Ajuwon and Spurlock, 2005; Takahashi et al, 2008). Palmitic acid causes inflammation in WAT through oxidative stress, ROS production and protein kinase C signalling, whereas oleic acid antagonises the inflammatory effects of palmitic acid (Kennedy et al, 2009). Murine 3T3-L1 adipocytes treated with palmitic acid display increased TNF-α expression and secretion, and reduced expression and secretion of the anti-inflammatory cytokine IL-10. Oleic acid, however, fails to increase TNF-α expression or secretion (Bradley et al, 2008). Linoleic acid has been shown to antagonise the activation of NF-κB and IL-6 expression caused by palmitic acid in human myotubes (Weigert et al, 2004). Additionally, treatment of macrophages in vitro with linoleic acid results in decreased levels of IL-6 and MCP-1 compared to palmitic acid (Wang et al, 2009). Mice that are deficient in TLR4 are protected from the inflammatory effects of feeding on a high palmitic acid diet, indicating that palmitic acid exerts its effects through TLR4 signalling (Davis et al, 2008). However, the mechanism by which palmitic acid achieves this inflammatory effect in the larval intestine may be different. This is especially given that zebrafish lack CD14 and MD-2 and thus are unable to signal through TLR4 (Iliev et al, 2005).

The accumulation of cells expressing dsRed or GFP under the LyzC promoter and L-plastin expressing cells in the intestines of larvae fed a fat-enriched diet confirmed a possible involvement of monocytes and macrophages. This is due to these two populations of cells showing a greater difference in numbers between fat fed and control diet fed larvae than...
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MPO positive cells. If the response to feeding on a fat-enriched diet was solely neutrophil-driven, then the difference in numbers of L-plastin positive and LyzC dsRed or GFP positive cells between between the two diets would be similar to that of the MPO positive cells. L-plastin marks both macrophages and neutrophils as shown by studies demonstrating the presence of both L-plastin positive cells with MPO expression and those lacking MPO expression, the former being neutrophils and the latter being macrophages (Meijer et al., 2008; Matias et al., 2009). An explanation for the differences in numbers of L-plastin positive cells and dsRed or GFP positive cells in the intestine is that whilst L-plastin and LyzC are both markers of macrophages and neutrophils, L-plastin may encompass a greater population of cells. This is supported by findings by made by Hall et al who demonstrated that whereas all LyzC expressing cells also express L-plastin, not all L-plastin positive cells express LyzC (Hall et al., 2007). Thus, L-plastin encompasses both LyzC expressing cells (L-plastin* LyzC*) and other non-LyzC expressing cells (L-plastin* LyzC*). Given that both L-plastin and LyzC encompass the majority of neutrophils, as shown by their colocalisation with MPO expression, the L-plastin* LyzC* cells are most likely macrophages. Therefore, L-plastin is a more selective marker of macrophages than LyzC (Hall et al., 2007; Meijer et al., 2008; Matias et al., 2009).

A study of acute inflammation in response to tailfin injury in zebrafish larvae by Loynes et al revealed that the peak accumulation of macrophages follows that of neutrophils and that macrophages remained at the injury site for longer (Loynes et al., 2010). This is consistent with the observed peak accumulation of L-plastin positive cells occurring after that of MPO positive cells and taking longer to resolve following feeding on a cholesterol-enriched diet (Fig 3.10A and C). Although LyzC GFP cells also show a peak accumulation after MPO positive cells do, the resolution of this accumulation occurs more rapidly than that of L-plastin cell accumulation and is consistent with LyzC not being as selective a marker of macrophages as L-plastin (Fig 3.10A, C and D). Loynes et al also found that macrophages phagocytose neutrophils that have undergone apoptosis at the site of injury (Loynes et al., 2010). It is possible that the macrophages being recruited to the intestine following feeding on a cholesterol-enriched diet may be carrying out the same role. At 18h post-removal, there is a reduction in the number of MPO positive cells, suggesting a resolution of the neutrophil response and a coincidental peak accumulation of L-plastin positive cells. Thus, the L-plastin positive cells accumulating at this time point may be anti-inflammatory M2 macrophages, in addition to pro-inflammatory M1 macrophages that may have accumulated earlier. Studies in
zebrafish have identified markers of M1 and M2 macrophages, TNF-α and arginase, respectively, which colocalise with L-plastin indicating the possibility of pro-inflammatory and anti-inflammatory macrophages in this model organism (Feng et al, 2010).

The involvement of macrophages in chronic inflammation associated with metabolic disorders is well characterised. In atherosclerosis, the immune cells that are recruited to the sites of vascular lesions are primarily macrophages (Glass and Witztum, 2001). In mouse models of chronic obesity, there is an infiltration of macrophages in the adipose tissue of mice fed a high fat diet compared to mice fed a standard diet. There is also an upregulation of macrophage-enriched genes upon feeding a high fat diet (Xu et al, 2003). However, very little is known about the acute inflammation associated with feeding on a fat-enriched diet. Neutrophil recruitment to AT has been observed as early as 3 days of feeding on a high fat diet and persists until 7 days. By 14 days of feeding, neutrophils are no longer detected, suggesting a transient infiltration. Macrophages, though, are not observed in great numbers until several weeks of feeding (Xu et al, 2003; Elgazar-Carmon et al, 2008). Thus, the involvement of macrophages in the acute stages of feeding on a fat-enriched diet has not been previously documented. These results, however, do suggest a potential involvement of macrophages in the localised inflammatory response in the intestine to acute feeding on a fat-enriched diet after a period of a few hours.

3.4.3 Cholesterol Uptake into Intestinal Epithelial Cells May be Required for Localised Inflammation to Occur

The results suggest that the uptake of cholesterol by intestinal epithelial cells may be required for the inflammatory effect observed upon feeding on a cholesterol-enriched diet (Fig 3.12). NPC1L1 is expressed predominantly in intestinal epithelial cells and hepatocytes, but is also expressed in macrophages, albeit at a much lower level (Seedorf et al, 2004). In human macrophages, expression is approximately 0.3 – 0.5% of that in the liver and intestine. It has also been demonstrated that ezetimibe blocks oxLDL uptake by macrophages, presumably through inhibition of Class II scavenger receptors (Seedorf et al, 2004). This is consistent with macrophages not having a characterised role in cholesterol uptake and any effect of ezetimibe in macrophages would likely be on another target than NPC1L1. However, in the context of acute fat feeding, it is unlikely that the observed effect of ezetimibe is on blocking
oxLDL uptake by macrophages due to the relatively short timescale of the feeding period within which formation of oxLDL would be not expected. This is consistent with observations that vascular lesions resulting from oxLDL uptake by macrophages take a period of weeks to develop in zebrafish (Stoletov et al., 2009). Given that in order for cholesterol to form LDLs, it needs to first be taken up by intestinal epithelial cells and processed by the body, it is more likely that ezetimibe is acting on NPC1L1 on intestinal epithelial cells than on macrophage-expressed scavenger receptors in the context of an acute fat feeding period.

It has been proposed that intracellular accumulation of cholesterol in the liver through uptake by NPC1L1 results in ER stress which leads to activation of p38. This accumulation also results in production of ROS by mitochondria, leading to activation of JNK and can progress to insulin resistance through downregulation of Akt activity (Nomura et al., 2009). Ezetimibe treatment, however, has been shown to decrease expression of NADPH oxidases, IL-6, TNF-α and IL-1β in the livers of rats fed a high fat diet in comparison to untreated animals fed on such a diet. In hepatocytes in vitro stimulated with free cholesterol, ezetimibe treatment decreases ROS production and reduces activation of JNK and p38 compared to free cholesterol-stimulated hepatocytes in the absence of ezetimibe (Nomura et al., 2009). It is possible that ezetimibe treatment of larvae may reduce the accumulation of L-plastin positive cells in the intestine compared to untreated larvae by a decrease in expression levels of IL-1β and NADPH oxidase in intestinal epithelial cells. This is supported by intestinal epithelial cells being known to express both IL-1β and NADPH oxidase and both of whose expression levels are affected by ezetimibe treatment in hepatocytes (Waterhouse and Stadnyk, 1999; Flores et al., 2010).

In rabbits fed a hyperlipidaemic diet, ezetimibe treatment results in a reduced expression of MCP-1 and reduction in activation of NF-κB (Gómez-Garre et al., 2009). Atherosclerotic plaques are smaller in size and have a lower macrophage content in ezetimibe-treated animals than untreated ones. Ezetimibe treatment has also been shown to decrease monocyte transmigration in vitro. (Gómez-Garre et al., 2009). The observation of a reduced transmigration of monocytes following ezetimibe treatment is consistent with lower numbers of L-plastin positive cells in the intestines of ezetimibe-treated larvae. This is because the accumulation of L-plastin positive cells in the intestine is likely due to transmigration of cells from outside the intestine. It is possible that MCP-1 may be involved in the recruitment of L-
plastin positive cells to the intestine due to its expression being reduced following ezetimibe treatment in vivo in rabbit models of atherosclerosis, suggesting cholesterol induces MCP-1 expression. MCP-1 has been also shown to be expressed in intestinal epithelial cells (Reinecker et al, 1995).

3.4.4 Pro-inflammatory Cytokines and Chemokines do not Show Detectable Differences in Changes in Expression Between Cholesterol-Fed and ZM-fed Larvae

IL-8 functions as a potent chemoattractant primarily for neutrophils, but also to a lesser extent for monocytes (Oppenheim et al, 1991; Gerszten et al, 1999). It is expressed in the zebrafish intestine along with its receptors, CXR1 and CXR2 (Oehlers et al, 2010). IL-8 transcript levels have been demonstrated to be upregulated as measured by qRT-PCR following treatment with a variety of inflammatory stimuli. Bacterial infection of 4 dpf zebrafish larvae with Listonella anguillarum for 6h resulted in an approximate 2.5 fold increase in IL-8 expression levels in the whole body compared to untreated controls. LPS treatment increased expression levels in the intestine by 2.5 fold and treatment of 3 dpf larvae with TNBS for 3 days resulted in an increase in expression levels by 4 fold in the whole body compared to untreated controls (Oehlers et al, 2010). In a further study by Oehlers et al, a significant upregulation of IL-8 expression was observed in the intestines of larvae but not in the remaining carcasses following 3 days of treatment of 3 dpf larvae with TNBS (Oehlers et al, 2011). Thus, it appears that changes in expression levels of IL-8 in response to inflammatory stimuli are detectable by qRT-PCR both in the intestine and remaining carcasses.

It appears odd that the results in Fig 3.15 do not show biologically relevant increases in IL-8 expression levels in at least the intestines of cholesterol-fed larvae compared to ZM-fed larvae. This is especially unusual given that even immediately after a 6h feeding period, there is an accumulation of immune cells in the intestines of larvae fed either cream or a cholesterol-enriched diet, suggesting that migration of immune cells to the intestine has begun. Thus, it would be expected that there should be an increase in expression levels of IL-8 prior to the accumulation of myeloid cells. IL-8 has been shown to be upregulated in obesity and its production in human adipocytes is induced by TNF-α (Kobashi et al, 2009). Circulating levels of IL-8 have also been shown to be elevated in obese human patients (Kim
et al, 2006). Thus, IL-8 has been demonstrated to be involved in the chronic inflammation associated with a high fat diet and both neutrophils and macrophages are involved in the inflammation associated with a high fat diet, albeit with different kinetics (Xu et al, 2003; Elgazar-Carmon et al, 2008). Taken together, this evidence supports a potential involvement of IL-8 in the acute inflammation associated with feeding on a fat-enriched or cholesterol-enriched diet that is not observed in the data. It is, however, possible that the localised inflammation in the intestine is occurring independently of IL-8 and that another chemokine may be involved in immune cell accumulation, despite IL-8 having a well-established role in chronic diet-associated inflammation.

Several other cytokines and chemokines that were tested for expression changes by qRT-PCR upon feeding a cholesterol-enriched diet were undetected or showed no difference in expression levels between the intestines and remaining carcasses of cholesterol-fed or ZM-fed larvae (Table 3.1). A number of these tested cytokines have been shown to be involved in the chronic inflammation associated with obesity. TNF-α, which was not detected, plays an important role in stimulating lipolysis in AT and activating NF-κB signalling leading to production of MCP-1. TNF-α is also an activator of JNK signalling, which results in insulin resistance (Souza et al, 2003; Suganami et al, 2005; Shoelson et al, 2006). M-17, which was also not detected, is a member of the IL-6 family and elevated levels of IL-6 expression are observed in obese mice (Harkins et al, 2004). Furthermore, co-culture of pre-adipocytes with monocytes in vitro leads to an upregulation in IL-6 expression in pre-adipocytes, suggesting a paracrine effect of monocytes on IL-6 expression in pre-adipocytes (Couturier et al, 2012). Another undetected cytokine was the anti-inflammatory IL-10, which is a characteristic marker of M2 macrophages and is downregulated in adipose tissue during obesity as the proportion of M1 macrophages increases (Lumeng et al, 2007). Thus, it is possible that this cytokine would show a decreased expression in cholesterol-fed larvae compared to ZM-fed larvae.

CXCL1 is an example of a chemokine that was detected but showed no difference in expression levels between cholesterol-fed or ZM-fed larvae. Like IL-6, CXCL1 expression has been shown to be upregulated in pre-adipocytes following co-culture with monocytes (Couturier et al, 2012). CXCL1 is both a neutrophil and macrophage chemoattractant that is expressed in intestinal epithelial cells (Masumoto et al, 2006). Given that there is an observed accumulation of myeloid cells to the intestines of cholesterol-fed larvae, the
inability to detect TNF-α, and CXCL1 showing no difference between the two feeding conditions is not consistent. TNF-α promotes macrophage chemotaxis by activating MCP-1 expression and CXCL1 is itself a chemokine for both neutrophils and macrophages. Thus, both of these two cytokines would be expected to be involved in the inflammation observed with feeding a cholesterol-enriched diet. However, as is the case with IL-8, the localised immune cell accumulation in the intestine may be occurring independently of TNF-α and CXCL1, despite their established roles in chronic inflammation associated with obesity.

Oehlers *et al* also investigated changes in expression of TNF-α in the same study as IL-8 expression. They found that bacterial infection resulted in a 3 fold increase in expression levels in whole larvae compared to untreated controls. LPS treatment resulted in a 2 fold increase in TNF-α expression levels in the intestine and TNBS exposure in a 7 fold increase in expression levels in the whole body compared to untreated controls (Oehlers *et al*, 2010). In a subsequent study investigating the effects of TNBS exposure, Oehlers *et al* found that 3 days of treatment of 3dpf larvae with TNBS resulted in a significant increase in TNF-α levels in the remaining carcasses but no change in the intestine (Oehlers *et al*, 2011). These studies, together with the measurements of IL-8 expression, provide further evidence that it is possible to detect cytokine expression in both the bodies and intestines of larvae.

A potential explanation for the lack of observed detection of cytokines in the intestines of larvae may be due to the intestine being a very heterogeneous population of cells. This means that the amount detected in certain cell populations could potentially be diluted out by a non-detectable signal in other cell populations. This may then result in an overall non-detectable signal. The same may be true for expression in the remaining carcasses with only a small proportion of the carcass giving a detectable signal. This explanation can also account for cytokines that were detectable but showed no difference in expression levels in the intestine between cholesterol-fed and ZM-fed larvae. There may have been an increased expression in certain populations of cells in the intestine that was diluted out by an unchanged expression in other cell populations in cholesterol-fed larvae. Thus, the overall expression levels would be the same for both the cholesterol-fed and ZM-fed larvae. A possible explanation for no change in expression levels in the remaining carcasses may be due to potential changes in expression levels of the cytokines being limited only to the intestine, consistent with a localised inflammation in this region. Thus, expression levels in the remaining carcasses would be unaffected. Given the inability to detect certain cytokines
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and chemokines in zebrafish or to detect differences in expression, wholemount *in situ* hybridisation (WISH) can be used as an alternative method, as discussed further in Chapter 6.

### 3.4.5 Reversion to a Standard Diet Following Enriched Fat Feeding Leads to Accelerated Resolution of Inflammation Possibly Due to the Anti-Inflammatory Effects of Omega-3 Fatty Acids

ZM is enriched in ω-3 FAs and these may be a component of the standard diet that is responsible for the observed reduction in immune cell numbers in the intestine following switching to such a diet. ω-3 FAs are converted to protectins and resolvins, which block neutrophil transmigration and promote their phagocytosis, thus enabling the resolution of inflammation (Schwab *et al.*, 2007; Serhan *et al.*, 2008; Soehnlein and Lindbom, 2010). It is possible that protectins and resolvins may cause an initial reduction in numbers of neutrophils accumulating in the intestine following a switch to ZM. It has also been demonstrated *in vitro* that ω-3 FAs are converted to oxo-derivatives by COX-2 in activated macrophages and these derivatives are capable of suppressing IL-6 and MCP-1 expression. Additionally, the oxo-derivatives suppressed expression of iNOS and functioned as agonists of peroxisome proliferator receptor-γ (PPARγ) (Groeger *et al.*, 2010). Thus, oxo-derivatives of ω-3 FAs have anti-inflammatory properties and may be involved in the accelerated resolution of inflammation upon switching to a ZM diet following feeding on a fat-enriched or cholesterol-enriched diet. In support of this, it is likely that there is a presence of activated macrophages in the intestines of cholesterol-fed larvae which can convert the ω-3 FAs present in ZM into oxo-derivatives and thus resolve the inflammation caused by the feeding of a cholesterol-enriched diet.

Feeding mice a diet enriched in ω-3 FAs for 5 weeks results in an increase in the expression levels of PPARγ, GLUT4 and IRS-1, thus leading to improved insulin sensitivity. Intraperitoneal injection of resolvins into mice also results in enhanced expression of PPARγ, GLUT4 and IRS-1 (González-Périz *et al.*, 2009). Thus, both ω-3 FAs and their metabolites are capable of improving insulin sensitivity, highlighting their anti-inflammatory properties. Studies by Oh *et al* have identified GPR120 as the receptor through which ω-3 FAs exert their anti-inflammatory effects, which is highly expressed on M1 macrophages and adipocytes (Oh *et al.*, 2010). *In vitro*, ω-3 FAs have been shown to inhibit phosphorylation of
JNK and IKKB in both macrophages and adipocytes. It is likely that this occurs through GPR120 signalling inhibiting TAB1 and preventing its interaction with TAK1, which is necessary for activation of both JNK and NF-κB signalling (Oh et al., 2010).

Feeding mice a high fat diet for 15 weeks and then switching one group of mice to an ω-3 FA-enriched diet for 5 weeks, whilst continuing another group on a high fat diet for 5 weeks results in improved insulin sensitivity and lipid metabolism in the ω-3 FA-fed group compared to the high fat diet-fed group. Mice fed the diet enriched in ω-3 FAs also displayed a decrease in levels of F4/80 expression and reduced macrophage chemotaxis (Oh et al., 2010). Expression levels of IL-6, CD11c and MCP-1 were all reduced in the ω-3 FA-fed group compared to the high fat diet-fed group. Furthermore, there was a reduction in expression of genes characteristic of M1 macrophages and an increase in expression levels of genes characteristic of M2 macrophages upon feeding a diet enriched in ω-3 FAs (Oh et al., 2010). This indicates that ω-3 FAs are able to induce a switch in macrophage phenotype from pro-inflammatory to anti-inflammatory. Thus, perhaps it is possible that the ω-3 FAs in ZM are capable of exerting their anti-inflammatory properties in the intestine and accelerating resolution of the inflammatory effect of a fat enriched or cholesterol-enriched diet without themselves being converted into oxo-derivatives, or protectins and resolvins. This is supported by the observed potential involvement of macrophages in the acute response in the intestine, which may express GPR120 and thus allow signalling through the receptor through binding of ω-3 FAs. Therefore, the observed reduction in immune cell numbers in the intestines of larvae that are fed a standard diet following feeding on a fat-enriched or cholesterol-enriched diet may be mediated through the action of ω-3 FAs on M1 macrophages or products of ω-3 FAs metabolism being processed by activated macrophages.

3.4.6 Conclusion

In conclusion, feeding zebrafish larvae a fat-enriched diet results in a localised recruitment of both neutrophils and potentially macrophages to the intestine. It also appears that the peak accumulation of neutrophils precedes that of macrophages following feeding on a cholesterol-enriched diet. The results suggest that the uptake of cholesterol into intestinal epithelial cells may be required for acute inflammation in the intestine to occur. This could possibly be due to cholesterol uptake increasing expression of NADPH oxidase or IL-1β,
both of which are expressed in intestinal epithelial cells and whose expression levels are reduced by blocking cholesterol uptake in other studies. A long chain SFA such as palmitic acid is capable of causing a localised accumulation of myeloid cells in the intestine, whereas UFAs are unable to likely due to their anti-inflammatory nature. Finally, switching to a standard diet following feeding on a fat-enriched diet results in an accelerated resolution of the inflammatory effect. This is possibly due to the anti-inflammatory effects of ω-3 FAs in the standard diet on macrophages, or processing of ω-3 FAs to anti-inflammatory derivatives by macrophages.
CHAPTER 4: INVESTIGATING THE ROLE OF THE NLRP3 INFLAMMASOME IN THE INFLAMMATION ASSOCIATED WITH FEEDING ON A FAT-ENRICHED DIET

4.1 Introduction

As described in Chapter 1, the NLRP3 inflammasome is activated by a variety of danger signals, including components of dietary fat. Recently, it has been shown that crystalline particles of cholesterol are capable of activating the NLRP3 inflammasome in vitro. When LPS-primed mouse macrophages are treated with cholesterol crystals, the crystals are taken up through phagocytosis and activate the NLRP3 inflammasome in a cathepsin B-dependent manner (Duewell et al., 2010; Rajamäki et al., 2010). Incubation of unprimed macrophages with oxLDL results in internalisation of this material and crystalline particles forming in the phagolysosomal compartment, ultimately leading to the release of mature IL-1β. It is possible that the crystals form from the action of cholesterol ester hydrolases converting cholesteryl esters from the oxLDL to free cholesterol (Duewell et al., 2010). As oxLDL is capable of priming cells for NLRP3 inflammasome activation through binding to the TLR4/6 heterodimer and co-receptor CD36, and is capable of being converted into crystalline particles of cholesterol, there is no requirement for priming with LPS. This is because oxLDL alone is capable of providing both signals required for full NLRP3 inflammasome activation (Stewart et al., 2010). The SFA, palmitate, has also recently been shown to induce IL-1β secretion from bone marrow-derived macrophages in the presence of LPS in an NLRP3 inflammasome-dependent manner. The mechanism by which this occurs is independent of the lysosome and therefore cathepsin B, but requires the production of ROS. The unsaturated fatty acid oleate is unable to do so, however (Wen et al., 2011).

The NLRP3 inflammasome is implicated in a variety of metabolic diseases with activation of caspase-1 and release of IL-1β being contributing factors underlying the development and progression of these diseases. Obesity is associated with an increase in ceramide, SFAs, ROS and ATP, all of which are activators of the inflammasome (Lukens et al., 2011). Caspase-1
activity has been shown to be increased in both diet-induced and genetic mouse models of obesity, with the resulting increase in activity in AT and liver leading to impaired insulin signalling and promotion of β cell apoptosis in the pancreas (Stienstra et al., 2010; Vandanmagsar et al., 2011). This leads to insulin resistance and development of type II diabetes. During type II diabetes, islet amyloid polypeptide (IAPP) is secreted by β cells and deposited in the pancreas. IAPP activates caspase-1 in an NLRP3 and lysosome dependent manner further exacerbating defective insulin signalling and enhanced β cell apoptosis. The priming of the NLRP3 inflammasome is due to partially oxidised, or mmLDL, and glucose metabolism. This indicates a role of dietary intake in providing the first signal for induction of IL-1β expression (Masters et al., 2010). In atherosclerosis, crystalline particles of cholesterol are deposited in the lesions relatively early during the development of the disease and their localisation correlates with that of macrophages as disease development progresses. Additionally, LDLR KO mice reconstituted with bone marrow deficient in components of the NLRP3 inflammasome showed decreased lesion size (Duewell et al., 2010). Given that these particles are capable of activating the NLRP3 inflammasome in vitro, the inflammasome may play a role in development and progression of atherogenesis in vivo (Duewell et al., 2010; Rajamäki et al., 2010).

In zebrafish, an orthologue of mammalian caspase-1, termed Caspy, has been characterised, which displays 54% similarity to its mammalian counterpart. As in mammals, Caspy interacts with the zebrafish orthologue of ASC (zASC) and this interaction is required for activity of Caspy (Masumoto et al., 2003). Caspy has been demonstrated to cleave IL-1β in zebrafish primary leukocytes in vitro following Francisella noatunensis infection (Vojtech et al., 2012). Cathepsin B has also been characterised in zebrafish (Zhang T et al., 2008). Furthermore, members of the NADPH oxidase family have been identified, including phagocyte NADPH oxidase, Nox2, expressed in macrophages and neutrophils, and Duox, expressed in intestinal epithelial cells (Flores et al., 2010; Brothers et al., 2011). Thus, it appears that zebrafish possess many of the components required for signalling through the NLRP3 inflammasome and this pathway may play a role in the accumulation of immune cells in the gut following acute feeding on an enriched fat diet.
4.2 Aim

The aim of this Chapter was to investigate the role of the NLRP3 inflammasome in the inflammation associated with feeding on a fat-enriched diet. This was approached in the following ways:

Using (1) pharmacological inhibitors, (2) fluorogenic tools such as a H$_2$O$_2$ probe or a caspase-1 substrate and (3) splice-blocking morpholinos to target various components involved in signalling through the NLRP3 inflammasome, the effect on acute inflammation in the intestine following feeding on a fat-enriched diet was investigated. Fig 4.1 indicates the targets (red) of pharmacological inhibition and MO knockdown:
Figure 4.1: Components of the NLRP3 Inflammasome that were Targeted by Pharmacological Inhibition and MO Knockdown. Names of inhibitors are shown in bold and specificities in italic (Figure adapted from Tschopp and Schroder, 2010).
4.3 Results

4.3.1 Pharmacological Inhibition of Cathepsin B Activity Reduces the Accumulation of L-Plastin Positive Cells in the Intestines of Cholesterol-fed, but not Palmitic Acid-fed Larvae

In order to study the role of cathepsin B induced by a cholesterol-enriched diet, a pharmacological inhibitor of cathepsin B, Ca-074-Me, was used. Ca-074 has been shown to bind to Cys 29 in the active site of bovine cathepsin B and irreversibly inhibits its activity (Yamamoto et al, 1997). Larvae were fed cholesterol-enriched or palmitic acid-enriched diets in the presence of the drug and following this, L-plastin positive cells in the intestine were enumerated in fixed and stained larvae.
Figure 4.2: Pharmacological Inhibition of Cathepsin B Activity Reduces the Accumulation of L-Plastin Positive Cells in the Intestines of Cholesterol-fed, but not Palmitic Acid-fed, Larvae. 6 dpf WT larvae were pre-treated with the cathepsin B inhibitor, Ca-074-Me, or DMSO of an equivalent volume, or were untreated for 30 mins. Following this, they were then fed for 6h whilst in the presence of the drug or DMSO. 18h after removal from the diets but in the presence of the drug, larvae were fixed, stained, and numbers of L-plastin positive cells were counted in the intestines. Comparison of the numbers of cells in individual larvae (A) and mean numbers of cells in larvae (B) fed either a standard diet (ZM) or a cholesterol-enriched diet and treated with varying concentrations of Ca-074-Me. Comparison of the numbers of cells in individual larvae (C) and mean numbers of cells in larvae (D) fed either ZM or a cholesterol-enriched diet and treated with 100µM Ca-074-Me or DMSO, or untreated. Comparison of the numbers of cells in individual larvae (E) and mean numbers of cells in larvae fed either ZM, or diets enriched in either cholesterol or palmitic acid (PA) and treated with 100µM Ca-074-Me, or DMSO or untreated (F). Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. *** denotes p < 0.001, and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (A) and (B), (E) and (F), and n = 2 experiments (C) and (D) (numbers of times experiments were performed).
Treating larvae with a range of concentrations of Ca-074-Me resulted in a dose-dependent reduction in the accumulation of L-plastin positive cells in the intestines of larvae fed a cholesterol-enriched diet. 100µM was statistically significant (p < 0.05) whereas 10µM and 25µM were not. Additionally, exposure to an equivalent volume of DMSO to 100µM did not reduce the inflammatory infiltrate compared to untreated larvae (Fig 4.2A and B). ZM-fed larvae exposed to 100µM showed no reduction in cell numbers compared to ZM-fed larvae that were treated with DMSO. However, cholesterol-fed larvae showed a statistically significant reduction between these two groups, validating the effect observed in the previous titration experiment (Fig 4.2A – D). In contrast, this concentration had no inhibitory effect on the numbers of cells in larvae on a palmitic acid-enriched diet as compared to DMSO-treated larvae (Fig 4.2E and F). Taken together, these results indicate that pharmacological inhibition of cathepsin B activity results in a reduction in the number of L-plastin positive cells accumulating in the intestines of larvae fed a cholesterol-enriched diet, but not a palmitic acid-enriched diet. This suggests that the manner in which palmitic acid induces an accumulation of L-plastin positive cells in the intestine occurs independently of cathepsin B activity.

4.3.2 Pharmacological Inhibition of NADPH Oxidase Activity Reduces the Accumulation of L-Plastin Positive Cells in the Intestines of Cholesterol-fed Larvae

Since NADPH oxidases are expressed in zebrafish intestinal epithelial cells and are a potential source of ROS capable of activating NLRP3 (Dostert et al, 2008; Dostert et al, 2009; Flores et al, 2010), larvae were pre-treated with a range of concentrations of the NADPH oxidase inhibitor, VAS-2870, or the appropriate controls and fed with cholesterol in the presence of the drug. The numbers of L-plastin positive cells were evaluated as described previously.
A dose-dependent reduction in the number of L-plastin positive cells recruited to the intestines of larvae fed a cholesterol-enriched diet was observed following treatment with VAS-2870, with the greatest level of statistical significance seen with 1µM VAS-2870 (p < 0.001). However, such a reduction was not observed upon exposure to DMSO of an equivalent volume to 1µM (~ 17 cells). No reduction in cell numbers occurred following treatment with all concentrations of inhibitor in larvae fed ZM compared to untreated or DMSO-treated larvae (all ~ 8 – 9 cells; Fig 4.3). These results thus indicate that pharmacological inhibition of NADPH oxidase activity reduces the number of L-plastin positive cells accumulating in the intestines of larvae fed a cholesterol-enriched diet.

Given that NADPH oxidase activity appears to be involved in the inflammation, the next step was to attempt to visualise this activity through the use of a fluorogenic H$_2$O$_2$ probe. H$_2$O$_2$ is
4. NLRP3 Inflammasome

A ROS that is produced by NADPH oxidases and larvae were pre-treated with this probe, fed and then imaged (Nathan, 2006). Larvae pre-treated with DMSO showed no detectable fluorescence in the intestine (Fig 7.1A; Appendix). However, in all of the feeding conditions, the level of fluorescence intensity of the probe was the same indicating no difference in H2O2 production upon feeding a cholesterol-enriched diet compared to feeding ZM or unfed larvae (Fig 7.1B – D; Appendix). As DMSO treatment displayed no fluorescence, this indicates that the fluorescence observed in the intestines of larvae pre-treated with the probe was due to the effect of the probe and not autofluorescence associated with DMSO or autofluorescence in the intestine itself. These results thus suggest that it is not possible to distinguish between the fluorescence intensity of the probe in larvae fed ZM, a cholesterol-enriched diet or unfed larvae.

4.3.3 Pharmacological Inhibition of Caspase-1 Activity Reduces the Accumulation of L-Plastin Positive Cells in the Intestines of Cholesterol-fed and Palmitic Acid-fed Larvae

The role of caspase-1, a component of the inflammasome itself downstream of cathepsin B and NADPH oxidase, was then studied. Caspase-1 is involved in the processing of IL-1β to its mature, cleaved form and an orthologue of which has been characterised in zebrafish (Masumoto et al, 2003; Schroder et al, 2010; Tschopp and Schroder, 2010). Experimental groups were set up as described for experiments using the cathepsin B inhibitor, but in this case using a reversible, competitive caspase-1 and 5 inhibitor, N-Acetyl WEHD-al (Rano et al, 1997).
Figure 4.4: Pharmacological Inhibition of Caspase-1 Activity Reduces the Accumulation of L-Plastin Positive Cells in the Intestines of Cholesterol-fed and Palmitic Acid-Fed Larvae. 6 dpf WT larvae were pre-treated with the caspase-1 and 5 inhibitor, N-Acetyl WEHD-al, or were untreated for 30 mins. Following this, they were then fed for 6h whilst in the presence of the drug. 18h following removal from the diet but in the presence of the drug, larvae were fixed, stained, and numbers of L-plastin positive cells were counted in the intestines. Comparison of the numbers of cells in individual larvae (A) and mean numbers of cells in larvae (B) fed either a standard diet (ZM) or a cholesterol-enriched diet and treated with varying concentrations of N-Acetyl WEHD-al. Comparison of the numbers of cells in individual larvae (C) and mean numbers of cells in larvae (D) fed either ZM or a cholesterol-enriched diet and treated with 100µM N-Acetyl WEHD-al or untreated. Comparison of the numbers of cells in individual larvae (E) and mean numbers of cells in larvae (F) fed either ZM, a cholesterol-enriched diet or a palmitic acid (PA)-enriched diet and treated with 100µM N-Acetyl WEHD-al or untreated. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. *** denotes p < 0.001, ** p < 0.01 and * p < 0.05. Error bars represent 95% confidence intervals (CI). Data shown for n = 1 experiments (A) and (B), and n = 2 experiments (C) – (F) (number of times experiments were performed).
As was observed with Ca-074-Me treatment (Fig 4.4A and B), a dose-dependent reduction in numbers of L-plastin positive cells was seen upon treatment of cholesterol-fed larvae with N-Acetyl WEHD-al. Similarly, only the highest concentration tested (100µM) gave a statistically significant difference compared to untreated larvae (~ 11 cells compared to ~ 20; Fig 4.4A and B). Exposure of ZM-fed larvae to this concentration showed no difference in cell numbers to that of untreated ZM-fed larvae (~ 10 – 11 cells; Fig 4.4A and B) and repeating the experiment with only 100µM of inhibitor validated the observed result in the titration experiment (Fig 4.4A – D). Larvae fed a palmitic acid-enriched diet that were treated with 100µM also showed a statistically significant reduction in the number of cells, though the level of this reduction was not quite as strong as that observed with exposure of larvae fed a cholesterol-enriched diet (Fig 4.4E and F). Collectively, these results indicate that pharmacological inhibition of caspase-1 or caspase-5 activity results in a reduction in the number of L-plastin positive cells accumulating in the intestines of larvae fed diets enriched in either cholesterol or palmitic acid.

As N-Acetyl WEHD-al primarily inhibits caspase-1 activity but also targets caspase-5 with much lower affinity, the use of a fluorogenic probe that is specific to caspase-1 would therefore allow the activity of this protease to be studied in the context of feeding on a cholesterol enriched-diet without the potential involvement of caspase-5. Two such probes were available to do this. One was a fluorogenic substrate of caspase-1, Z-YVAD-AFC, and the other a FLICA, FAM-YVAD-FMK, that binds to activated caspase-1. Larvae were treated with Z-YVAD-AFC, DMSO or were untreated, and then imaged. In order to visualise caspase-1 activity with FAM-YVAD-FMK, larvae were fed for 6h and then immediately following this, were treated with FAM-YVAD-FMK for 30 mins, followed by a 30 min wash in PBS. Larvae were then imaged.

The levels of fluorescence in the intestines of larvae treated with all concentrations of Z-YVAD-AFC were equivalent to those of DMSO treated larvae and untreated larvae, indicating that it was not possible to distinguish between fluorescence associated with potential caspase-1 activity and autofluorescence in the intestine (Fig 7.2Ai – iii; Appendix). In contrast, when using FAM-YVAD-FMK, untreated larvae showed no detectable fluorescence in the intestine, indicating absence of autofluorescence in this region (Fig 7.2Bi; Appendix). Approximately 50% of larvae fed either ZM or a cholesterol-enriched diet and treated with FAM-YVAD-FMK showed very little or no detectable fluorescence in the
intestine (Fig 7.2Bii and Biv; Appendix). The remaining 50% showed a stronger green fluorescence in the intestine that was easily detectable (Fig 7.2Biii and Bv; Appendix). In all cases, there appeared to be detectable fluorescence in the gallbladder of the larvae. Given that the untreated larvae showed no detectable autofluorescence, the fluorescence observed in the treated larvae was due to FAM-YVAD-FMK. These results demonstrate that it was not possible to distinguish between fluorescence observed with Z-YVAD-AFC treatment and autofluorescence in the intestine. The results also show that whilst the fluorescence observed with FAM-YVAD-FMK treatment is detectable, there are variations in the intensity of this fluorescence in treated larvae fed either ZM or a cholesterol-enriched diet.

To further investigate the differences in fluorescence intensity within ZM-fed and cholesterol-fed larvae treated with FAM-YVAD-FMK, larvae were segregated into those that had bright intestines and those with dim intestines after feeding and treatment with FAM-YVAD-FMK 3h after removal from the diet. Following this, numbers of L-plastin positive cells were counted in the intestines of larvae. However, there were no detectable differences in numbers of cells in larva that had bright intestines or those with dim intestines for both feeding conditions (Fig 7.3; Appendix). These results demonstrate that there is no correlation between the numbers of L-plastin positive cells accumulating in the intestines of larvae and the brightness of the intestines.

To validate whether the fluorescence observed in the intestines of larvae treated with FAM-YVAD-FMK was associated with caspase-1 activity, inhibitors of caspase-1 and cathepsin B, N-Acetyl-WEHD-al and Ca-074-Me, respectively, were used to block caspase-1 activity either directly or through inhibition of an upstream target. Larvae were treated with inhibitors and fed as previously described. 3h following removal, they were treated with FAM-YVAD-FMK and levels of fluorescence intensity in the intestines were then quantified. The fluorescence intensity observed in the intestines of untreated larvae was higher in ZM-fed larvae compared to cholesterol-fed larvae in one experiment but approximately the same in another. Furthermore, DMSO treatment resulted in an increase in fluorescence intensity compared to untreated larvae and there was no meaningful decrease following treatment with either N-Acetyl-WEHD-al or Ca-074-Me (Fig 7.4; Appendix).
4.3.4 The Effect of Treating Larvae with Morpholinos against ASC on the Accumulation of L-Plastin Positive Cells in the Intestines of Cholesterol-fed Larvae

*Localisation of FITC Fluorescence in the Inner Region of the Intestine of a 7 dpf Larva Following Administration of a Fluorescently Conjugated Morpholino into the Water.*

In order to validate the potential role of the NLRP3 inflammasome in the inflammation associated with feeding on a cholesterol-enriched diet, the outcome of reduced expression of ASC on the inflammatory cell infiltrate was investigated. ASC associates with NLRP3 and recruits the pro-form of caspase-1 prior to its activation (Masumoto et al., 2003; Schroder et al., 2010; Tschopp and Schroder, 2010). Given that ASC is not activated in the signalling transduced through the NLRP3 inflammasome, but functions as an adaptor bridging between NLRP3 and caspase-1, a pharmacological inhibitor-based approach to study the role of ASC was not feasible. An alternative approach was to utilise a MO to transiently knock down gene expression of ASC and observe the effect of this knockdown on the inflammation associated with feeding on a cholesterol-enriched diet. A splice-blocking MO that targets the first exon and intron boundary sequence of ASC was chosen, as the effect of the knockdown could be determined by qRT-PCR. The effect of this MO is that the first intron is retained following splicing, instead of being removed. A translation-blocking MO was also used that targets the translation initiation site. Firstly, the ability for zebrafish larvae to take up a MO into the intestine needed to be examined. To achieve this, larvae were treated for 24h with a standard control MO conjugated to FITC or untreated and imaged by confocal microscopy.
Figure 4.5: Localisation of FITC Fluorescence in the Inner Region of the Intestine of a 7 dpf Larva Following Administration of a Fluorescently Conjugated Morpholino into the Water. 6 dpf WT larvae were treated with either 20µM FITC-conjugated control MO or were untreated for 24h. Following this, larvae were anaesthetised and then imaged on a confocal microscope for presence of green (FITC) fluorescence in the intestine. Images shown are individual slices taken from a Z-stack. Inner slices represent those taken from the interior of the intestine, near the lumen. Outer slices represent those taken near the exterior of the intestine, where the muscularis is located. (A) Inner slice taken from a MO-treated larva at 10x magnification and 63x magnification (B). Outer slice taken from a MO-treated larva at 10x magnification (C) and 63x magnification (D). (E) Inner slice and (F) outer slice taken from an untreated larva at 10x magnification. (G) Schematic representation of a transverse section of the intestine indicating the relative positions in the intestine, where inner and outer slices are taken. l denotes lumen, e epithelial layer and m muscularis. Larvae are oriented sagitally from anterior (left) to posterior (right). Slices taken at intervals of 1.6µm at 10x magnification and at intervals of 0.9µm at 63x magnification. Scale bar (black) represents 0.4mm and red represents 0.1mm. Data shown for n = 1 experiments (number of times experiment was performed).
Administration of a fluorescently conjugated MO led to an accumulation of FITC fluorescence in the inner, but not in the outer layer of the intestine (Fig 4.5A – D). In the absence of a MO, there was no detectable fluorescent signal in the intestines of larvae in either the outer layer of the intestine, near the muscularis, or the inner layer, near the lumen (Fig 4.5E and F). Given that there was no fluorescent signal observed in the absence of a MO, the fluorescent signal seen in the MO treated larvae is due to the FITC of the MO and not autofluorescence in the intestine. These results show that administration of a fluorescently conjugated MO into the water leads to an accumulation of fluorescence in the inner region of the intestine but not the outer region. However, the fluorescence may correspond to just the FITC and not the MO as the two may no longer be conjugated following the uptake of the MO into the intestine.

Prior Administration of a Splice-Blocking or Translation Blocking Morpholino Against ASC into the Water Reduces the Accumulation of L-Plastin Positive Cells in the Intestine Following Feeding on a Cholesterol-enriched Diet

The next step was to use a MO against ASC and determine its effect on cholesterol-induced cell recruitment in the intestine. Larvae were firstly pre-treated for 24h with MO or were untreated and following this treatment period, were fed for 6h whilst in the presence of the MO. 18h following removal from the diet, larvae were fixed, stained and L-plastin positive cells enumerated.
Figure 4.6: Prior Administration of a Splice-Blocking or Translation Blocking Morpholino Against ASC into the Water Reduces the Recruitment of L-Plastin Positive Cells to the Intestine Following Feeding on a Cholesterol-enriched Diet. 6 dpf WT larvae were pre-treated with MO, or were untreated for 24h. Following this, larvae were either fed a cholesterol-enriched diet or a standard diet (ZM) for 6h, whilst in the presence of the MO. 18h following removal from the diet, larvae were fixed, stained and numbers of L-plastin positive cells were counted in the intestines. Comparison of the numbers of cells in the intestines of individual larvae (A) and mean numbers of cells in larvae (B) treated with varying concentrations of the splice-blocking and control MO. Comparison of the numbers of cells in individual larvae (C) and mean numbers of cells in larvae (D) treated with 20µM splice-blocking MO, or control MO or untreated. Comparison of the numbers of cells in individual larvae (E) and mean numbers of cells in larvae (F) treated with 20µM splice-blocking MO, translation-blocking MO, specificity control MO, control MO or untreated. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. ** denotes p < 0.01 and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (A) and (B), (E) and (F), and n = 2 experiments (C) and (D) (number of times experiments were performed).
Treating larvae with a splice-blocking MO against ASC led to a dose-dependent reduction in the number of L-plastin positive cells in the intestine. A concentration of 20µM gave a statistically significant reduction in the cell numbers to an equivalent level to that of ZM-fed larvae, whereas lower concentrations showed a non-significant trend towards a reduction. The standard control MO, however, had no effect at any concentration, as expected (Fig 4.6A and B). ZM-fed larvae exposed to 20µM of the splice-blocking MO showed no reduction in cell numbers, unlike cholesterol-fed larvae, validating the result in the previous experiment (Fig 4.6 A – D). Treatment with either a splice-blocking or a translation blocking MO against ASC resulted in a statistically significant reduction in the number of cells in cholesterol-fed larvae to a level similar to that of ZM-fed larvae. However, a standard control or a specificity control did not have this effect, as expected (Fig 4.6E and F). Taken together, these results indicate that prior administration of a splice-blocking or translation blocking MO against ASC reduces the recruitment of L-plastin positive cells to the intestine following feeding on a cholesterol-enriched diet.

Microinjection of a Splice-Blocking or a Translation-Blocking Morpholino into Single-Cell Zebrafish Embryos Does Not Affect L-plastin Positive Cell Accumulation in the Intestine Following Feeding on a Cholesterol-enriched Diet

In an attempt to confirm the effects of feeding the ASC-specific MO, it was administered by microinjection. Embryos were microinjected at the single-cell stage with either a splice-blocking MO against ASC, a standard control MO, or were non-injected. At 6 dpf, larvae were fed either a cholesterol-enriched diet or ZM for 6h. In a separate experiment, embryos were microinjected with either translation-blocking MO against ASC, standard control MO, or were non-injected and fed as in the previous experiment. In both experiments, 18h following removal from the diet, larvae were fixed, stained and numbers of L-plastin positive cells were counted in the intestines.
Figure 4.7: Microinjection of a Splice-Blocking or a Translation-Blocking Morpholino into Single-Cell Zebrafish Embryos Does Not Affect L-plastin Positive Cell Recruitment to the Intestine Following Feeding on a Cholesterol-enriched Diet. WT embryos at the single-cell stage were micro-injected with either a splice-blocking MO against ASC, control MO (both at 0.1mM), or were not injected. In a separate experiment, single-cell stage WT embryos were micro-injected with either a translation-blocking MO against ASC, control MO (both at 0.1mM) or were not injected. In both experiments, at 6 dpf, the larvae were fed either a standard diet (ZM) or a cholesterol-enriched diet for 6h. 18h following removal from the diet, larvae were fixed, stained and numbers of L-plastin positive cells were counted in the intestines. Comparison of the numbers of cells in individual larvae (A) and mean numbers of cells in larvae (B) injected with either the splice-blocking morpholino, control morpholino or non-injected. Comparison of the numbers of cells in individual larvae (C) and mean numbers of cells in larvae (D) injected with either the translation-blocking morpholino, control morpholino or non-injected. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test, (A) and (B), or a one way ANOVA followed by a Bonferroni post test, (C) and (D). *** denotes p < 0.001 and ** p < 0.01. Error bars represent 95% confidence intervals. Data shown for n = 2 experiments (number of times experiment was performed).
Feeding larvae a cholesterol-enriched diet following microinjection with a splice-blocking MO against ASC at the single-cell stage did not result in a reduction in the number of L-plastin positive cells accumulating in the intestine compared to larvae that were injected with a standard control MO or were non-injected. Such larvae still showed a statistically significant difference in the number of cells compared to ZM-fed larvae injected with the same MO (~19 – 20 cells compared to ~12 – 13 cells; Fig 4.7A and B). Feeding larvae a cholesterol-enriched diet following microinjection with a translation-blocking MO against ASC also did not reduce the inflammatory infiltrate compared to controls and such larvae also showed a statistically significant difference (p < 0.001) compared to ZM-fed larvae injected with the same MO (~14 cells compared to ~7 – 8 cells; Fig 4.7C and D). Collectively, these results demonstrate that feeding on a cholesterol-enriched diet following microinjection of either a splice-blocking or a translation-blocking MO against ASC at the single-cell stage does not reduce the number of L-plastin positive cells accumulating in the intestine. This is not consistent with the observed reduction in the previous experiments when these MO were administered into the water.

4.3.5 The Effect of Treating Larvae with a Splice-Blocking Morpholino Against ASC on the Expression of ASC in the Intestine

To try to resolve the different outcomes of MO feeding to that of microinjection, ASC expression was measured by qRT-PCR to assess whether or not both routes of delivery had indeed knocked down gene expression.

Administration of a Splice-Blocking MO Against ASC into the Water Does Not Result in a Detectable Knockdown of ASC Expression

6 dpf larvae were treated with a splice-blocking MO against ASC, a standard control MO or were untreated for 30h. Following this, larvae had their intestines dissected and the remaining carcasses were also kept. RNA was extracted from the intestines and carcasses, cDNA was generated and changes in ASC expression were measured by qRT-PCR. A probe against ASC that targets the boundary sequence between exon 1 and 2 was used. Expression
changes were normalized against the housekeeping genes EF1a (Fig 4.8), actin (Fig 4.9) and 18S (Fig 4.10).
Figure 4.8: Administration of a Splice-Blocking Morpholino Against ASC into the Water Does Not Result in a Detectable Knockdown of ASC Expression. 6 dpf WT larvae were treated with either 20µM splice-blocking MO against ASC, 20µM control MO, or were untreated for 30h. Following this treatment, larvae were killed and the intestines dissected from the larvae (20 intestines/condition) with the remaining carcasses also being kept. RNA was extracted from the intestines and carcasses, and cDNA generated. Changes in ASC expression was measured by qRT-PCR and calibrated against the non-injected intestine readings. Fold changes in expression of ASC in both pooled larval intestines (20 intestines/pool) and 4 pooled carcasses (Rest, 5 carcasses/pool) for each of the treatment conditions for two separate experiments (A and B). Mean fold changes in expression of ASC in pooled larval intestines and carcasses for the different treatment conditions for two separate experiments (C and D). ASC expression normalised to EF1a. Error bars represent standard error of the mean. C_T values for ASC in Experiment 1 ((A) and (C)) ranged from 27.5 – 28.5 (Intestine) and 28 – 30 (Rest), and in Experiment 2 ((B) and (D)) from 27.5 – 28 for both intestines and remaining carcasses. C_T values for EF1a in Experiment 1 ranged from 19 – 20 (Gut) and 20.5 – 22 (Rest), and in Experiment 2 from 18.5 – 19.5 (Intestine) and 19 – 20 (Rest). Data shown for n =1 experiments (number of times experiment was performed).
Administering a splice-blocking MO against ASC into the water did not result in a reduction in gene expression of ASC in either the intestines or the remaining carcasses of larvae from Experiment 1 (Fig 4.8A and C). In Experiment 2, treatment with both the ASC and control MO decreased ASC expression levels in the intestines and the remaining carcasses, indicating no reduction in expression solely due to the effect of the ASC MO (Fig 4.8B and D). Taken together, these results suggest that there is no detectable decrease in ASC expression levels in either the intestines or the remaining carcasses of larvae pre-treated with a MO against ASC administered into the water.
Figure 4.9: Administration of a Splice-Blocking Morpholino Against ASC into the Water Does Not Result in a Detectable Knockdown of ASC Expression. 6 dpf WT larvae were treated with either 20µM splice-blocking MO against ASC, 20µM control MO, or were untreated for 30h. Following this treatment, larvae were killed and the intestines dissected from the larvae (20 intestines/condition) with the remaining carcasses also being kept. RNA was extracted from the intestines and carcasses, and cDNA generated. Changes in ASC expression was measured by qRT-PCR and calibrated against the non-injected intestine readings. Fold changes in expression of ASC in both pooled larval intestines (20 intestines/pool) and 4 pooled carcasses (Rest, 5 carcasses/pool) for each of the treatment conditions for two separate experiments (A) and (B). Mean fold changes in expression of ASC in pooled larval intestines and carcasses for the different treatment conditions for two separate experiments (C) and (D). ASC expression normalised to actin. Error bars represent standard error of the mean. \( C_T \) values for ASC in Experiment 1 ((A) and (C)) ranged from 27.5 – 28.5 (Intestine) and 28 – 30 (Rest), and in Experiment 2 ((B) and (D)) from 27.5 – 28 for both intestines and remaining carcasses. \( C_T \) values for actin in Experiment 1 ranged from 24.5 – 25.5 (Intestine) and 26.5 – 27.5 (Rest), and in Experiment 2 from 25 – 25.5 (Intestine) and 25.5 – 27 (Rest). Data shown for \( n = 1 \) experiements (number of times experiment was performed).
Administering a splice-blocking MO against ASC into the water failed to decrease ASC expression levels in both larval intestines and remaining carcasses compared to untreated larvae in Experiment 1 as judged by qRT-PCR (Fig 4.9A and C). In Experiment 2, there appeared to be no reduction in ASC expression levels in the intestines of larvae treated with the ASC MO compared to untreated but a 20% decrease in expression levels of ASC in larvae treated with the control MO relative to untreated larvae. In contrast, in the remaining carcasses, there appeared to be ~2 fold increase in ASC expression levels in larvae treated with either the ASC or control MO compared to untreated larvae (Fig 4.9B and D). Collectively, these results also suggest that there is no detectable decrease in ASC expression levels in either the intestines or the remaining carcasses of larvae pre-treated with a MO against ASC administered into the water.
Figure 4.10: Administration of a Splice-Blocking Morpholino Against ASC into the Water Does Not Result in a Detectable Knockdown of ASC Expression. 6 dpf WT larvae were treated with either 20µM splice-blocking MO against ASC, 20µM control MO, or were untreated for 30h. Following this treatment, larvae were killed and the intestines dissected from the larvae (20 intestines/condition) with the remaining carcasses also being kept. RNA was extracted from the intestines and carcasses, and cDNA generated. Changes in ASC expression were measured by qRT-PCR and calibrated against the non-injected intestine readings. Fold changes in expression of ASC in both pooled larval intestines (20 intestines/pool) and 4 pooled carcasses (Rest, 5 carcasses/pool) for each of the treatment conditions for two separate experiments (A) and (B). Mean fold changes in expression of ASC in pooled larval intestines and carcasses for the different treatment conditions for two separate experiments (C) and (D). ASC expression normalised to 18S. Error bars represent standard error of the mean. C_T values for ASC in Experiment 1 ((A) and (C)) ranged from 27.5 – 28.5 (Intestine) and 28 – 30 (Rest), and in Experiment 2 ((B) and (D)) from 27.5 – 28 for both intestines and remaining carcasses. C_T values for 18S in Experiment 1 ranged from 11.5 – 12.5 (Intestine) and 12.5 – 13.5 (Rest), and in Experiment 2 from 11 – 12 for both intestines and remaining carcasses. Data shown for n =1 experiments (number of times experiment was performed).
Administering a splice-blocking MO against ASC into the water resulted in an approximate 25% decrease in ASC expression in the intestines of larvae compared to untreated larvae in Experiment 1. However, since there was also a 50% reduction in expression levels in the larvae treated with the control MO, this reveals that the ASC MO does not specifically reduce expression. Similarly, both the ASC and the control MO reduced expression in the remaining carcasses (Fig 4.10A and C). The changes in ASC expression in the intestines of larvae in Experiment 2 are very similar to that of Experiment 1. However, there is no change in ASC expression levels in the remaining carcasses of larvae treated with the ASC MO compared to untreated larvae (Fig 4.10B and D). Collectively, these results confirm that there is no marked or specific decrease in ASC expression levels in larvae pre-treated with an ASC-specific MO administered into the water.

**Microinjection of a Splice-Blocking MO Against ASC into Embryos Results in an Observed Reduction of ASC Expression**

The results from the previous experiments failed to demonstrate mechanistically that the reduction in inflammatory cells following cholesterol feeding correlated with reduced gene expression. Therefore, the biological activity of the ASC MO by microinjection was examined. Embryos were microinjected with either a splice-blocking MO against ASC, a standard control MO or were non-injected. At 7dpf, larvae were processed for cDNA and ASC expression levels analysed by qRT-PCR as for the previous experiments.
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Figure 4.11: Microinjection of a Splice-Blocking Morpholino Against ASC into Embryos Results in an Observed Reduction of ASC Expression. WT larvae at the single-cell stage were micro-injected with either 0.1mM splice-blocking MO against ASC, 0.1µM control MO, or were non-injected. At 7 dpf, larvae were killed and the intestines dissected from the larvae (20 intestines/condition) with the remaining carcasses also being kept. RNA was extracted from the intestines and carcasses, and cDNA generated. Changes in ASC expression was measured by qRT-PCR and calibrated against the non-injected intestine or carcass readings. Fold changes in expression of ASC in both pooled larval intestines (20 intestines/pool) (A) and 4 pooled carcasses (Rest, 5 carcasses/pool) (B) for each of the treatment conditions. Mean fold changes in expression of ASC in pooled larval intestines (C) and carcasses (D) for the different treatment conditions. Numbers in (D) represent fold changes relative to mean of non-injected carcasses. ASC expression normalised to 18S. Error bars represent standard error of the mean. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. ** denotes p < 0.01. C<sub>T</sub> values for ASC ranged from 27 – 28 (Intestine) and 27 - 29 (Rest). C<sub>T</sub> values for 18S ranged from 11.5 - 12 for both intestine and remaining carcasses. Data shown for n = 1 experiments (number of times experiment was performed).
Microinjection of the control MO led to an approximate 25% reduction in expression levels of ASC in the intestines of larvae compared to non-injected larvae. However, microinjection of a splice-blocking MO against ASC resulted in a 60% reduction in expression levels compared to non-injected larvae and a 45% reduction compared to control MO injected larvae. Thus, there is a decrease in ASC expression levels following injection of the ASC MO confirming its activity, but there was also a reduction in ASC expression levels following injection of the control MO (Fig 4.11A and C). In the remaining carcasses, microinjection of a control MO led to a 35% reduction in expression levels of ASC compared to non-injected larvae whereas injection of the ASC MO resulted in a 65% reduction. The trend was similar to that observed in the intestine, although the effect of the control MO was more marked. Overall, the ASC MO had greater effects than the control on gene expression in the remaining carcasses (Fig 4.11B and D). These results indicate that microinjection of a splice-blocking MO against ASC leads to a reduction in ASC expression levels in both the intestines and remaining carcasses.

To validate the effect of the MO further, 5 dpf and 7 dpf larvae that had either been microinjected with the splice-blocking MO, control MO or non-injected and processed as previously described, except RNA was extracted from whole larvae.
Figure 4.12: Microinjection of a Splice-Blocking Morpholino Against ASC into Embryos Results in an Observed Reduction of ASC Expression. WT larvae at the single-cell stage were microinjected with either 0.1mM splice-blocking MO against ASC, 0.1µM control MO, or were non-injected. At 5 dpf and 7 dpf, larvae were killed and grouped into pools (5 larvae/pool). RNA was extracted from whole larvae and cDNA generated. Changes in ASC expression was measured by qRT-PCR and calibrated against the non-injected readings. Fold changes in expression of ASC in 5 dpf larvae (A) and 7 dpf larvae (B) for each of the treatment conditions. Mean fold changes in expression of ASC in 5 dpf larvae (C) and 7 dpf larvae (D) for the different treatment conditions. Numbers in (C) and (D) represent fold changes relative to mean of non-injected carcasses. ASC expression normalised to EF1a. Error bars represent standard error of the mean. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. * denotes p < 0.05. 

$C_T$ values for ASC ranged from 28 – 30 for both 5dpf and 7dpf larvae. $C_T$ values for EF1a ranged from 20 - 21 for 5 dpf larvae and from 19.5 – 21 for 7 dpf larvae. Data shown for n = 1 experiments (number of times experiment was performed).
Microinjection of a splice-blocking ASC MO reduced gene expression by ~ 70% compared to non-injected larvae at both 5dpf and 7dpf (p < 0.05). However, at 5dpf and 7dpf, the control MO gave a 10% and 25% reduction in ASC expression, respectively (Fig 4.12). These results validate the findings of the previous experiment and confirm that microinjection of a splice-blocking MO against ASC leads to a reduction in ASC expression levels in whole larvae.
4.4 Discussion

In brief, inhibition of cathepsin B, NADPH oxidase and caspase-1 activity reduces the number of L-plastin positive cells in the intestines of larvae fed a cholesterol-enriched diet. The caspase-1, but not the cathepsin B, inhibitor also reduced the inflammatory response to a palmitic acid diet. Furthermore, administration of a fluorescently conjugated MO into the water results in an accumulation of fluorescence in the inner layer of the intestine near the lumen, but not the outer layer. Feeding of a splice-blocking ASC-specific MO leads to a decrease in the number of L-plastin positive cells in the intestines of cholesterol-fed larvae but has no detectable effect on ASC expression. In contrast, following microinjection of this MO, ASC expression was reduced but there was no detectable change in the inflammatory cell infiltrate.

4.4.1 The Accumulation of L-Plastin Positive Cells in the Intestine Following Feeding on a Cholesterol-enriched Diet Appears to be Dependent on the Activities of Cathepsin B, NADPH Oxidase and Caspase-1

One potential explanation for the difference in effects of cathepsin B inhibition observed following feeding on a cholesterol-enriched diet compared to a diet enriched in palmitic acid may be due to the manner in which cholesterol and palmitic acid are taken up by intestinal epithelial cells (Fig 4.2). Cholesterol is taken up by clathrin-mediated endocytosis following its interaction with NPC1L1 and as such is present in the lysosome. Thus, cholesterol could activate cathepsin B if the lysosomal membrane ruptures (Ge et al., 2008; Betters and Yu, 2010; Tschopp and Schroder, 2010). Palmitic acid however, enters intestinal epithelial cells either through means facilitated by fatty acid transport proteins at the cell surface or through passive diffusion, or a combination of both (Masson et al., 2010; Lynes and Widmaier, 2011). So, since palmitic acid enters the cell independently of endocytosis it is unlikely to cause activation of cathepsin B. Support of this comes from in vitro studies which have shown that palmitic acid can induce secretion of IL-1β from bone marrow derived macrophages, but is independent of the lysosome (Wen et al., 2011). It is known that not all danger signals that activate the NLRP3 inflammasome do so by activation of cathepsin B, nevertheless they all
appear to result in ROS production, including palmitic acid (Hornung et al, 2008; Schroder et al, 2010; Tschopp and Schroder, 2010; Wen et al, 2011). Crystal particles of cholesterol have been demonstrated to activate the NLRP3 inflammasome in a cathepsin B-dependent manner, consistent with a similar activation by other crystalline particles, such as silica or alum (Hornung et al, 2008; Duewell et al, 2010; Rajamäki et al, 2010). It is however, unlikely in a 6h feeding period that cholesterol crystals will have formed as such particles are first observed in apoE/− mice two weeks after initiating feeding on a cholesterol-enriched diet (Duewell et al, 2010). Nevertheless, given that dietary cholesterol itself is taken up in an endocytic manner, it is possible that the potential cathepsin B activation observed in this data occurs in the lysosome following internalisation of this dietary cholesterol into the intestinal epithelial cells.

The results also suggest that the inflammation associated with feeding on a cholesterol-enriched diet is dependent upon NADPH oxidase activity (Fig 4.3). It has been demonstrated in vivo that blocking cholesterol uptake with ezetimibe decreases expression of NADPH oxidases and in vitro that ezetimibe treatment reduces ROS production (Nomura et al, 2009). Therefore, cholesterol is likely to be involved in enhancing expression of NADPH oxidases and possibly stimulating ROS production by NADPH oxidases. Crystal particles of cholesterol can activate the NLRP3 inflammasome and all known activators of the inflammasome appear to induce ROS production (Duewell et al, 2010; Rajamäki et al, 2010; Schroder et al, 2010; Tschopp and Schroder, 2010). Thus, it is possible that the potential activation of NADPH oxidases by cholesterol may trigger NLRP3 signalling. This suggests that the acute inflammatory response that is observed following cholesterol feeding may involve the NLRP3 inflammasome. In support of this is the finding that a cathepsin B inhibitor reduces the inflammatory cellular infiltrate, since cathepsin B is an upstream activator of the NLRP3 inflammasome. However, there is currently no evidence in the literature that supports a role for dietary cholesterol, other than in the form of crystals, in activating the NLRP3 inflammasome.

The source of ROS involved in the activation of the NLRP3 inflammasome is currently a matter of controversy with findings from some groups indicating that NADPH oxidases are the source and not mitochondria (Dostert et al, 2008; Dostert et al, 2009). However, findings from other groups indicate that ROS production occurs independently of NADPH oxidases and mitochondria are the potential source (van Bruggen et al, 2010; Zhou et al, 2011). The
data in this thesis indicates a role of NADPH oxidases as a source of ROS production following feeding on a cholesterol-enriched diet. This is in agreement with two studies by Dostert et al but a mitochondrial-derived source of ROS may also be involved (Dostert et al, 2008; Dostert et al, 2009). Whilst the results suggest a potential role for NADPH oxidase activity in the acute inflammatory response induced by a cholesterol-enriched diet, no detectable differences were observed in levels of H$_2$O$_2$ indicative of NADPH oxidase activity in the intestine following feeding on a cholesterol-enriched diet compared to a ZM-fed diet or unfed larvae (Fig 7.1; Appendix). It is possible that the fluorescence observed with the probe is an artefact and thus not a measure of the levels of H$_2$O$_2$ produced.

The recruitment of L-plastin positive cells to the intestines of larvae fed either a cholesterol or a palmitic acid-enriched diet appears to be dependent upon caspase-1 activity (Fig 4.4). This is in agreement with published reports that palmitic acid and crystal particles of cholesterol activate NLRP3 in a caspase-1-dependent manner (Duewell et al, 2010; Rajamäki et al, 2010; Wen et al, 2011). Despite the findings obtained with N-Acetyl WEHD-al, no meaningful changes in caspase-1 activity could be detected when using a fluorogenic inhibitor, FAM YVAD-FMK, of active caspase-1. An explanation for this is that the fluorescence observed with FAM-YVAD-FMK is an artefact and not a measure of caspase-1 activity. It is possible that with N-Acetyl WEHD-al treatment, there is a smaller level of inhibition of caspase-5 given that the inhibitor has a lower level of affinity for caspase-5 in addition to its primary target, caspase-1. However, the predominant effect of inhibition is likely to be on caspase-1. Studies have demonstrated that the $K_i$ of N-Acetyl WEHD-al for caspase-1 is approximately 750 times lower than that of caspase-5 (0.056nM compared to 43nM, respectively) confirming the much higher affinity of the inhibitor for caspase-1 over caspase-5 (Rano et al, 1997; Garcia-Calvo et al, 1998). This is consistent with caspase-5 having a $K_{cat}/K_M$ of ~ 100 times lower than caspase-1 for substrates containing the WEHD recognition motif (Garcia-Calvo et al, 1998). Caspase-5 is an inflammatory caspase and forms part of the NLRP1 inflammasome, which also involves caspase-1 and ASC (Martinon et al, 2002). However, the NLRP1 and NLRP3 inflammasomes differ in that activation of caspase-1 in NLRP1 is not dependent upon ASC; ASC only enhances caspase-1 activation (Faustin et al, 2007). Additionally, activation of the NLRP1 inflammasome is not known to be dependent upon NADPH oxidase activity which appears to be involved in the inflammatory response to a cholesterol-enriched diet. NLRP1 is activated by microbial
4. NLRP3 Inflammasome

products and as yet appears to have no role in the response to dietary fat (Boyden and Dietrich, 2006; Faustin et al, 2007).

As well as the NLRP3 inflammasome, components of dietary fat have been shown to activate TLR signalling. Palmitic acid activates TLR4 in a MyD88-dependent manner and is likely to activate p38 and JNK signalling through upstream TLR4 activation (Shi et al, 2006; Kim et al, 2007; Håversen et al, 2009; Maloney et al, 2009). Palmitic acid also forms ceramides, which directly activate p38 and JNK, although given that ceramides take a period of hours or days to form, it is unlikely that they are responsible for any effect observed in these results (Perry and Hannun, 1998; Schmitz-Peiffer et al, 1999; Håversen et al, 2009). OxLDL has been demonstrated to activate a TLR4/6 heterodimer in association with CD36 and independently of CD14 (Stewart et al, 2010). Similarly, mmLDL has been shown to activate TLR4 signalling both in a MyD88 dependent and independent manner (Cushing et al, 1990; Bae et al, 2009; Chávez-Sánchez et al, 2010). Oxidised derivatives of cholesterols, known as oxysterols have been demonstrated to enhance IL-8 and MCP-1 in an ERK-dependent manner in human retinal cells (Dugas et al, 2010). In human macrophages, oxysterols induce NF-κB activation and increase phosphorylation of ERK1/2 and JNK (Palozza et al, 2010). Nevertheless, although there is no evidence that a cholesterol-enriched diet induces inflammation via NLRP1, it cannot be excluded due its dependence upon caspase-1. Collectively, however, the reduction in intestinal inflammation that is observed with the cathepsin B, NADPH oxidase and caspase-1 inhibitors all point to NLRP3 playing a role in the acute response to a cholesterol-enriched diet.

There is cross-talk between TLR signalling and the NLRP3 inflammasome in that NF-κB activation results in expression of the pro-form of IL-1β. TLR4 or TLR2 signalling following LPS or peptidoglycan stimulation respectively, has also been demonstrated to induce expression of NLRP3 itself, which is necessary for its activation possibly due to its constitutive low level of expression (Bauernfeind et al, 2009; Qiao et al, 2012). Given that components of dietary fat are known to activate TLRs, it is possible that the priming signal required for generation of pro-IL-1β may occur in a TLR-dependent manner in response to cholesterol or palmitic acid and that induction of expression of NLRP3 may also. However, whether or not this is the case in zebrafish remains to be examined in light of zebrafish lacking CD14 and MD-2 which are required for signalling through TLR4 (Iliev et al, 2005). Alternatively, it is possible that the microbiota in the intestine may provide the priming signal.
as there is a low level of inflammation occurring in the intestine during colonisation with microbiota, leading to neutrophil infiltration and establishment of a resident immune cell population (Bates et al., 2007). However, in order to prevent an excessive inflammatory response against the microbiota, the induction of expression of other cytokines and chemokines would need to be relatively low. Another possibility is that the priming signal may occur as a result of the microbiota in combination with cholesterol or palmitic acid feeding. These possibilities are discussed in greater detail in Chapter 6.

4.4.2 Administration of a Splice-Blocking Morpholino Against ASC into the Water May Result in a Reduction in Expression Levels of ASC in Intestinal Epithelial Cells that Cannot be Detected by qRT-PCR in the Entire Intestine

The results suggest that the recruitment of L-plastin positive cells to the intestines of larvae fed a cholesterol-enriched diet appears to be dependent upon ASC expression. Despite this, however, there was no detectable reduction in expression levels of ASC by qRT-PCR neither in the intestines nor the remaining carcasses in MO treated larvae (Figs 4.8 – 4.10). This may be due to the MO localising to the inner region of the intestine where the fluorescent signal was detected near the lumen and where epithelial cells are present since no fluorescent signal was detected in the outermost region of the intestine near the muscularis (Fig 4.5). However, this relies on the fluorescent signal being a measure of the MO localisation and thus the MO being conjugated to FITC. If this is the case, ASC knockdown may occur in only a proportion of the cells in or associated with the intestinal tract. It is unlikely that the ASC-specific splice-blocking MO had an off target effect that prevented cell recruitment to the intestine, given that the specificity control MO which has only 5 mismatched bases from the splice-blocking MO had no effect on the numbers of cells and the ASC-specific translation-blocking MO had the same effect as the splice-blocking MO. Thus, it is likely that the reduction in cell numbers following ASC-specific MO treatment is due to an effect on ASC expression. It is known that intestinal epithelial cells express the NLRP3 inflammasome and therefore ASC, and additionally that these cells are responsible for taking up cholesterol in the intestine (Altmann et al., 2004; Kummer et al., 2007; Cario, 2010). Thus, perhaps it is possible that cholesterol may activate the NLRP3 inflammasome within these cells leading to an accumulation of L-plastin positive cells in the intestine. Treatment of larvae with a MO against ASC by administration into the water could potentially decrease expression levels of
ASC in the intestinal epithelial cells in the inner region of the intestine and so prevent NLRP3 inflammasome-dependent accumulation of L-plastin positive cells following cholesterol-feeding.

**4.4.3 Microinjection of an ASC-specific Splice-Blocking MO May Reduce Levels of ASC More Efficiently in Cells Whose ASC Expression is Not Necessary in the Response to Feeding on a Cholesterol-enriched Diet**

Microinjection of a splice-blocking ASC-specific MO reduces expression of ASC in both the intestines and remaining carcasses of larvae compared to control larvae (Fig 4.11). ASC has been shown to be expressed in mature, differentiated colonic epithelial cells near the lumen but is absent from immature epithelial cells near the crypts that are further from the lumen (Masumoto et al, 2001; Yokoyama et al, 2003). This pattern of ASC expression in the mucosal epithelium is consistent with its expression in highly differentiated epithelium of both skin and tonsils in humans (Masumoto et al, 2001). The full development of the zebrafish larval intestine occurs by ~ 120 hpf from its onset at ~ 26 – 30 hpf (Ng et al, 2005). During this period, the epithelium undergoes a phase of rapid proliferation until ~ 74hpf, followed by a differentiation phase and ASC may possibly be expressed in these cells at the latter stage (Wallace et al, 2005). As this phase commences around 3 days after the MO was microinjected into the embryos, the effect of knockdown of gene expression in mature, differentiated epithelial cells may be reduced compared to a potential knockdown in these cells following feeding of the MO. In this context, the MO is instantly taken up by mature ASC-expressing intestinal epithelial cells from the lumen in 6 dpf larvae with a fully developed intestine.

ASC is also expressed in SMCs of the human colon and in macrophages and neutrophils (Masumoto et al, 2001; Kolly et al, 2010). Macrophages and neutrophils first appear at 15.5 and 18 hpf, respectively, and SMC progenitors at 50 hpf which is relatively early compared to the commencement of the differentiation program of epithelial cells (Herbomel et al, 1999; Bennett et al, 2001; Wallace et al, 2005). Therefore, the effect of ASC knockdown in these cells in the intestine may be stronger than that occurring in the mature epithelial cells which might express ASC later. Thus, despite the potential reduced knockdown in the epithelial cells, the possible stronger level of knockdown of expression additionally in macrophages,
neutrophils and SMCs would result in an overall reduction in expression in the intestine, which is observed in the data (Fig 4.11).

However, despite the observed decrease in ASC expression, there is no reduction in the numbers of L-plastin positive cells recruited to the intestines of larvae fed a cholesterol-enriched diet following microinjection of the splice-blocking MO compared to the controls (Fig 4.7). An explanation for this is that ASC expression in cells that are potentially more greatly affected by the microinjected MO, such as SMCs, macrophages and neutrophils, may not be necessary for the recruitment of L-plastin positive cells to the intestine in response to a cholesterol-enriched diet. Microinjection of the ASC-specific splice-blocking MO may not reduce ASC expression in the lumenal epithelial cells as greatly and as a result of this, there is no reduction in the immune cell infiltrate. This is supported by the observation that a potential knockdown of ASC expression in the epithelial cells in the inner region of the intestine following MO feeding is sufficient to significantly reduce the numbers of L-plastin positive cells without any further knockdown in expression of cells in the outer region (Fig 4.6). SMCs form the muscularis of the intestine and L-plastin positive cells, encompassing both neutrophils and macrophages, are also localised in the muscularis (Fig 3.14). As this is in the outermost region of the intestine, such ASC-expressing cells may not be affected by the MO. This suggests that it may be the expression of ASC in the intestinal epithelial cells that drives the inflammatory response to feeding on a cholesterol-enriched diet. However, in order to confirm the localisation of cellular ASC expression in the intestine, WISH can be used as discussed further in Chapter 6.

4.4.4 Conclusion

The recruitment of L-plastin positive cells to the intestines of larvae in response to a cholesterol-enriched diet appears to be dependent upon the activities of cathepsin B, NADPH oxidase and caspase-1. The cell recruitment may also be dependent upon the expression of ASC in intestinal epithelial cells. Taken together, these results suggest that the inflammation associated with feeding on a cholesterol-enriched diet is at least in part dependent on the NLRP3 inflammasome.
CHAPTER 5: ATTEMPTING TO GENERATE A NOVEL STABLE TRANSGENIC LINE OF ZEBRAFISH WITH ABLATED LYZC EXPRESSING CELLS

5.1 Introduction

The optical transparency of zebrafish larvae permits live imaging at the whole organism level and is an advantage of this model system over mammalian ones. This property has been capitalised on by a large number of research groups and led to the development of various transgenic strains of zebrafish that express fluorescent reporters under the influence of cell or tissue specific promoters, allowing temporal and spatial control of expression. The principles of zebrafish transgenesis date back almost 25 years and since then, several developments have been made enabling this technique to become widely used today.

The first attempts at generating a transgenic strain of zebrafish involved the injection of plasmid DNA into fertilised eggs, which resulted in stable integration of this DNA into the genome (Stuart et al., 1988). However, the frequency of this occurrence was generally low due to the random nature by which the plasmid DNA integrated into the genome. If the DNA was integrated relatively early on, then a greater level of transgene expression would be observed as more cells would express it. If the integration occurred later, though, then fewer cells would express the transgene, resulting in a reduced level of expression (Grabher and Wittbrodt, 2008). Thus, this led to variegated expression, whereby not all cells that should express the transgene actually did (Stuart et al., 1990). Expression was also reduced over time and by adulthood there was sometimes very little expression. Additionally, there was a low frequency of germline transmission which occurred in a non-Mendelian manner (Stuart et al., 1988). Over the years, however, techniques that improved the rate of transgene integration into the genome were developed. One of these involved the use of I-SceI meganuclease, which cleaves specific recognition sites (Thermes et al., 2002). A plasmid vector containing the transgene flanked by these recognition sites would be injected along with mRNA encoding the meganuclease. The enzyme would cleave the vector at the
recognition sites and allow integration of the transgene into the genome. This approach gave a much higher rate of germline transmission than the previously used technique of injecting plasmid DNA alone into the fertilised eggs (Babaryka et al., 2009).

Another approach that relies on a similar principle to I-SceI meganuclease is that of transposon-based integration. Transposons are mobile genetic elements that can be transferred from one location to another through the activity of a transposase enzyme. The enzyme recognises and cleaves specific inverted repeat sequences and allows the integration of the excised DNA into the genome (Largaespada, 2003). One of the most commonly used transposon-based systems today is the Tol2 system which utilises Tol2 transposable elements identified in the medaka fish (*Oryzias latipes*) (Koga et al., 1996). The plasmid vector containing Tol2 elements and mRNA encoding the transposase are co-injected into fertilised eggs. The transposase then catalyses the excision of DNA flanked by the Tol2 sequences, allowing integration of the excised sequence into zebrafish germ cells (Kawakami et al., 1998; Kawakami and Shima, 1999; Kawakami et al., 2000). The Tol2 transposon system has a germline transmission rate of up to 50%, consistent with Mendelian inheritance and is, thus, currently the most reliable approach in terms of transmitting the transgene to progeny (Kawakami et al., 2004). It has been demonstrated that the minimal sizes of the Tol2 elements required for achieving this rate of germline transmission are 200bp (left element) and 150bp (right element), and that an insert of around 11kb can be effectively transmitted in this way (Urasaki et al., 2006).

A further advantage of the Tol2 system is that unlike other systems, the DNA integrated into the genome in this manner does not cause rearrangements of the surrounding DNA and thus no silencing effect is observed. Thus, subsequent generations continue to express the transgene at the same level as previous ones. Other approaches of transgenesis, however, can result in concatamers of plasmid DNA forming in the genome, which leads to gene silencing in future generations and thus expression being reduced or lost completely (Kawakami, 2005). In order to allow the integration of inserts greater than 11kb, the Tol2 system can be utilised in bacterial artificial chromosomes, which can carry inserts up to 350kb. However, the approach is exactly the same as for plasmid vectors, which has greatly enhanced the applicability of this technique (Suster et al., 2009). The principles of Tol2 transgenesis are summarised in Fig 5.1:
5. Attempting to Generate a Novel Transgenic Line

Figure 5.1: Generation of a Transgenic Line of Zebrafish Using the Tol2 Transposon System. Transposase mRNA is generated by *in vitro* transcription of cDNA and co-injected into fertilised eggs along with a Tol2 vector containing plasmid DNA encoding a transgene. The transposase catalyses the excision of the transgene flanked by the transposons and its integration into the genomic DNA. Injected eggs (founders) are then raised to adulthood and crossed to WT fish. The transgene is then transmitted in the germline to the progeny (F1) (Figure adapted from Kawakami, 2005).

An approach that has been used to enhance transgene expression is the GAL4/UAS system, originally employed in *Drosophila melanogaster* (Halpern *et al.*, 2008). It involves the use of two components: a regulatory protein GAL4 and an upstream activation sequence (UAS), each present in a separate construct. GAL4 is placed under the promoter of a gene of interest and an effector protein, usually a fluorescent reporter, is placed downstream of the UAS (Scheer and Campos-Ortega, 1999). Each of the two constructs is microinjected into separate groups of fertilised eggs and the resulting embryos are raised to adulthood. The adult lines, each expressing one of the two constructs, are then crossed to one another and the resulting progeny contain both constructs. Transgene expression occurs by GAL4 binding to UAS and driving expression of the effector gene (Halpern *et al.*, 2008). Thus, effector gene expression
can be regulated in a cell or tissue specific manner by GAL4 only being able to bind UAS when the promoter under which GAL4 is under the influence of, is activated. A further development in this approach is the use of a hybrid of GAL4 and VP16 of the herpes simplex virus, termed GAL4-VP16, which enhances gene expression more greatly than GAL4 alone (Sadowski et al, 1988). One limitation of the GAL4/UAS technique, however, is that transgene expression is not initially detected until the first generation of progeny (F1) of the injected founder fish (F0). This is because the constructs need to be injected into separate sets of eggs and raised to adulthood before crosses are carried out to obtain offspring expressing both constructs.

The ability to perform targeted ablation in a cell or tissue specific manner is a technique that has gained a lot of attention recently in zebrafish research. It allows the processes of development and regeneration to be studied, as well as behavioural interactions between cell populations in live fish. Several applications of this approach have been used in zebrafish in a variety of cell types. One method by which this technique is carried out involves the use of diphtheria toxin A (DTA), which encodes an ADP-ribosyl transferase that inhibits protein synthesis. DTA is fused to a cell or tissue-specific promoter of interest and causes ablation of the target cell population. However, undesired effects on other cells have been observed using this approach. Furthermore, in some cases, ablation did not occur in the target cells and the only effect observed on these cells was reduced cell growth (Kurita et al, 2003; Wan et al, 2006). Another strategy involves the use of a bacterial toxin, Kid, and an antidote against it, Cis. Kid is expressed under a cell or tissue-specific promoter and thus causes ablation of any cells or tissues expressing it. In contrast, Cis is expressed in the remainder of the organism, protecting it from the ablative effects of Kid (Slanchev et al, 2005). However, both the DTA and Kid/Cis approaches have only been successfully used in transient expression experiments.

A third technique involves using the bacterial enzyme nitroreductase (NTR), which has the ability to convert certain prodrugs into potent cytotoxic DNA cross-linking agents through a process of reduction. NTR can be fused to a cell or tissue-specific promoter and thus only NTR-expressing cells will be killed following administration of the prodrug (Curado et al, 2007). One prodrug that was initially used in vitro was CB1954, but this was found to be unsuitable as the converted product was cell permeable and thus able to enter neighbouring cells and kill them, resulting in a ‘bystander effect’ (Bridgewater et al, 1997). An alternative
prodrug that is commonly used today is metronidazole (Mtz). Unlike CB1954, the cytotoxic agent formed from Mtz by the action of NTR is not cell permeable and therefore is retained in the NTR-expressing cells. Thus, it is not able to enter neighbouring cells and exert a ‘bystander effect’. Additionally, upon removal from the prodrug, the effects of cell ablation are reversed, making this a very useful tool in regeneration studies (Curado et al, 2007). Stable transgenic lines expressing NTR constructs have also been generated, unlike the DTA and Kid/Cis techniques which are only used for transient studies (Gray et al, 2011). However, prodrug-mediated targeted cell ablation does have its limitations in that certain tissues might not be accessible to a prodrug and the prodrug itself may also be processed by the body. General limitations in targeted cell ablation are that a suitable promoter is required that is cell or tissue specific and some tissues might be resistant to ablation (Curado et al, 2008).

Mtz/NTR-mediated targeted cell ablation has been used to ablate cardiomyocytes, pancreatic beta (β) cells and hepatocytes in the context of tissue regeneration studies (Curado et al, 2007). It has also been used to study the effect of ablating one population of cells on the behaviour of another. This has been demonstrated by Gray et al, who use this approach to ablate macrophages and investigate the effect of this ablation on the response of neutrophils to acute injury (Gray et al, 2011). Given the apparent role of macrophages and neutrophils in the inflammation associated with feeding on a fat-enriched diet, it would be interesting to ablate one population of immune cells and study the effect this has on the behaviour of other immune cell populations. As there are multiple markers of immune cells in zebrafish, such as MPO, Pu.1, L-plastin and LyzC, the effect of ablation of any one of these cell populations on the behaviour of the remainder can be observed using techniques such as wholemount antibody staining. The current availability of transgenic lines for some of these immune cell markers means that there are therefore appropriate promoters that can be used to generate a stable transgenic line containing the ablation construct.
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5.2 Aim

The aim of this Chapter was to try to generate a stable transgenic line of zebrafish with an ablated population of immune cells. This was endeavoured through:

- Firstly, generating a Tol2 construct containing an ablation cassette of NTR fused to CFP located downstream of the LyzC promoter.

- Secondly, microinjecting this construct together with Tol2 transposase mRNA into WT larvae and attempting to establish a stable transgenic line.
5.3 Results

5.3.1 Generation of the pTol(2.4kb LyzC)CFP-NTR Construct

The pTol(2.4kb LyzC)CFP-NTR construct was generated from the pTol(2.4kb LyzC)eGFP and 14xUAS CFP-NTR plasmid vectors in three steps. The first step was restriction digestion of the pTol(2.4kb LyzC)eGFP vector to remove the GFP gene. The second step was PCR amplification of the CFP-NTR ablation cassette from the 14xUAS CFP-NTR vector. The final step was recombination of the digested vector with the PCR-amplified ablation cassette to generate the pTol(2.4kb LyzC)CFP-NTR construct. These steps are summarised in Fig 5.2:
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Figure 5.2: Schematic Representation of the Cloning Strategy Used for Generating the pTol(2.4kb LyzC)CFP-NTR Plasmid Vector. The pTol(2.4kb LyzC)eGFP plasmid vector (A) was digested with NcoI and XhoI (1) to remove the GFP gene (B) and result in a vector lacking GFP (C). The CFP-NTR ablation cassette (cyan) was amplified from the 14xUAS CFP-NTR plasmid vector (D) by PCR (2). This was achieved using primers that introduce sequences at the 5’ and 3’ ends of the resulting product (E) that are complementary to the NcoI/XhoI digested pTol(2.4kb LyzC)eGFP vector (C). The resulting PCR amplified product (E) and digested vector (C) were then recombined (3) to generate the pTol(2.4kb LyzC)CFP-NTR plasmid vector (F), which contains the CFP-NTR ablation cassette (cyan) immediately downstream of the 2.4kb LyzC promoter (red), in place of GFP.
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Restriction Digestion of the pTol(2.4kb LyzC)eGFP Plasmid Vector

In order to allow substitution of the CFP-NTR ablation cassette in place of GFP in the pTol(2.4kb LyzC)eGFP plasmid vector, the pTol(2.4kb LyzC)eGFP vector was digested with \textit{NcoI} and \textit{XhoI} to excise the GFP gene.

![Restriction Digestion of the pTol(2.4kb LyzC)eGFP Plasmid Vector.](image)

**Figure 5.3: Restriction Digestion of the pTol(2.4kb LyzC)eGFP Plasmid Vector.** The pTol(2.4kbLyzC)eGFP plasmid vector was digested with \textit{NcoI} and \textit{XhoI} in order to excise GFP from the vector. Approximately 100ng of the DNA from the digestion reaction was run on a 1% agarose gel along with 5\mu l of ladder (MW). Data shown for n = 1 experiments (number of times experiment was performed).

Restriction digestion of the pTol(2.4kb LyzC)eGFP plasmid vector with \textit{NcoI} and \textit{XhoI} resulted in the generation of two bands: one at approximately 700bp and another at approximately 6000 bp (**Fig 5.3**). The 700bp band corresponds to GFP and the 6000 bp band to that of the remaining vector. These results demonstrate that digestion of the pTol(2.4kb LyzC)eGFP with \textit{NcoI} and \textit{XhoI} vector results in excision of the GFP gene, as expected.
**PCR Amplification of the CFP-NTR Ablation Cassette**

After successfully excising GFP from the pTol(2.4kb LyzC)eGFP vector, the next step was to replace the excised GFP with the CFP-NTR ablation cassette. To achieve this, FWD and REV primers that introduced sequences complementary to the digested pTol(2.4kb LyzC)eGFP vector at the 5’ and 3’ ends of the generated product were used to amplify the CFP-NTR ablation cassette from the 14xUAS CFP-NTR vector.

![Image of gel with bands at 1400bp](image)

**Figure 5.4: PCR Amplification of the CFP-NTR Ablation Cassette.** The CFP-NTR ablation cassette was amplified by PCR using forward and reverse primers that introduce sequences to the 5’ and 3’ ends of the cassette that are complementary to the Ncol/Xhol digested pTol(2.4kbLyzC)eGFP plasmid vector. Three different primer concentrations were used (1µM, 0.5µM and 0.1µM) and 5µl of the PCR reaction was loaded onto a 1% agarose gel along with 5µl of ladder (MW). Data shown for n = 1 experiments (number of times experiment was performed).

Whilst a product at 1400bp, consistent with the expected size of the amplified sequence, was detected using all three concentrations of primers, a 1µM concentration produced primer-dimers observed at the bottom of the gel at around 100-200bp. However, 0.5µM and 0.1µM
did not produce primer-dimers. A concentration of 0.1µM primers appeared to give the clearest band at 1400bp, although the intensity of the band was lower than that of the product obtained using 0.5µM primers indicating a smaller amount of product (Fig 5.4). These results show that 0.1µM primer concentration gives the clearest quality band but results in a smaller amount of product than a concentration of 0.5µM.

Recombination of the Digested Vector and PCR-amplified Insert

Having obtained both the digested vector lacking GFP and the CFP-NTR ablation cassette containing complementary sequences at the 5’ and 3’ ends to that of the digested vector, recombination was required to anneal the digested vector and complementary insert. This was carried out using the InFusion kit and following this, a restriction digest was performed from minprepped DNA of positive colonies to confirm the presence of the CFP-NTR ablation cassette in the pTol(2.4kb LyzC)eGFP vector.
Competent DH5α cells were transformed with the Infusion reaction containing the linearised vector and insert. Positive colonies were then picked and grown overnight in LB media. The following day, DNA was miniprepped from the bacterial cultures and then digested with EcoRV. Additionally, the original pTol(2.4kbLyzC)eGFP plasmid vector was digested with EcoRV as a control (C). Approximately 100ng of DNA from the restriction digests were loaded onto a 1% agarose gel along with 5µl of ladder (MW). * denotes colonies that were positive for the presence of the recombined vector and insert. Data shown for n = 1 experiments (number of times experiment was performed).
Restriction digestion with EcoRV resulted in three bands for 12 out of 17 lanes (denoted by *) (Fig 5.5). This is consistent with the presence of the CFP-NTR ablation cassette, as there is a single EcoRV site approximately 750bp from the 5’ end of the cassette, which is observed as a band in these 12 lanes. The 2100bp band corresponds to two EcoRV sites contained within the 2.4kb LyzC promoter (one approximately 300bp from the 5’ end and another at the 3’ end) and the 4600bp band is the remaining vector. Thus, the total size of the product is approximately 7.4kb, which is consistent with the expected size (Fig 5.2F). Digestion of the original pTol(2.4kb LyzC)eGFP vector with EcoRV (denoted by C) produced two bands, as expected: one at 2100bp corresponding to the digestion at two EcoRV sites within the LyzC promoter and a band at 4600bp corresponding to the remaining vector (Fig 5.5). The absence of an additional band at 750bp is consistent with the lack of the CFP-NTR cassette in this vector. However, 4 lanes which were from positive colonies gave a digestion profile identical to that of the original vector (C) (Fig 5.5). An explanation for this is that the excised GFP may have recombined back into the digested vector during the InFusion reaction instead of the CFP-NTR cassette and thus regenerated the original pTol(2.4kb LyzC)eGFP vector. As the Ncol/Xhol digestion reaction was column purified, both the excised GFP and the remaining vector would have been present in the InFusion reaction. However, given that only 4 out of the 16 positive colonies showed this profile, the GFP may not have been present in the optimum amount required for annealing back into the vector. This accounts for why 12 out of the 16 positive colonies contain the CFP-NTR cassette and not GFP. These results indicate that 12 out of the 16 positive colonies contain the CFP-NTR ablation cassette and thus have been transformed with the pTol(2.4kb LyzC)CFP-NTR vector.

### 5.3.2 Attempting to Establish a Stable Transgenic Line of LyzC CFP-NTR Zebrafish

**Assessing the Presence of CFP Positive Cells in F0 Founder Larvae**

Following the successful generation of the pTol(2.4kb LyzC)CFP-NTR vector containing the CFP-NTR ablation cassette downstream of the LyzC promoter, which was confirmed by sequencing, the next step was to microinject this construct together with previously synthesised Tol2 mRNA into embryos at the single-cell stage. Larvae were microinjected and then at 3 dpf, the presence of CFP expressing cells was assessed.
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Figure 5.6: Assessing the Presence of CFP Positive Cells in F₀ Founder Larvae. Tra/Nac embryos were co-injected at the single cell stage with 25ng/µl of ablation construct and 25ng/µl Tol2 mRNA into the single cell. At 3 dpf, larvae were screened for the presence of LyzC positive cells in the posterior blood island. (A) 3dpf Tra/Nac larva co-injected with the ablation construct and Tol2 mRNA. (B) 3dpf non-injected Tra/Nac larva. The red rectangle outlines the area of interest and examples pigment cells are highlighted with a white arrow. Scale bar represents 0.4mm. Data shown for n =1 experiments (experiment performed 4 times).

Within the PBI, there was autofluorescence in the pigment cells of both 3 dpf larvae injected with the construct and non-injected 3 dpf larvae (white arrows). However, there were no observable CFP-expressing cells in the PBI of larvae injected larvae compared to non-injected larvae indicating a lack of detectable expression of the injected construct in the F₀ founder larvae (Fig 5.6).

**Tail Transection of LyzC Transgenic Larvae to Assess the Expression of LyzC Positive Cells**

After observing a lack of detectable expression of CFP positive cells in the F₀ founder larvae, all injected larvae were raised to adulthood and then crossed to WT fish. 3 dpf offspring of such crosses were then tail transected together with 3dpf LyzC GFP and LyzC dsRed larvae to observe the presence of LyzC positive cells in the injured tails. This was to confirm whether the expression of the construct may have been very weak in the F₀ generation, but could increase in the F₁ generation through germline transmission.
Carrying out a tail transection on 3 dpf LyzC dsRed or LyzC GFP larvae resulted in the recruitment of fluorescent cells to the injury site 5h post-transection (Fig 5.7A and B). However, there was no observed recruitment of fluorescent cells to the wounded tails of F₁ LyzC CFP-NTR larvae (Fig 5.7D). Additionally, GFP expressing cells of a tail transected LyzC GFP larvae could be observed in the CFP channel, excluding the possibility of a high level of autofluorescence in this channel obscuring the visualisation of fluorescent cells (Fig
5.7C). Taken together, these results suggest a lack of detectable expression of CFP-expressing cells in the F₁ generation.

**Analysis of LyzC Expression in Transgenic Larvae by Flow Cytometry**

Unable to detect any CFP positive cells in either the F₀ founder larvae or the F₁ offspring, the expression of CFP in myeloid cells of adult potential transgenics was then analysed by flow cytometry.
Figure 5.8: Analysis of LyzC Expression in Transgenic Larvae by Flow Cytometry. Potential adult transgenic LyzC CFP-NTR fish, along with LyzC dsRed and Tra/Nac fish, were killed and had their kidneys removed. The kidneys were homogenised in PBS containing foetal calf serum to obtain a single-cell suspension and centrifuged. The resulting pellets were resuspended in PBS and then analysed by flow cytometry using both a 405/450 excitation/emission channel for analysis of Tra/Nac and potential LyzC CFP-NTR fish, and a 488/530 excitation/emission channel for analysis of LyzC dsRed fish. Profile for a Tra/Nac fish (A), a LyzC dsRed transgenic fish (B), and potential LyzC CFP-NTR transgenic fish (C) and (D). Data shown for n = 1 experiments (experiment was performed 3 times). Figure obtained courtesy of Fränze Progratzky.

Of the gated myeloid cell population, approximately 4% of cells in the Tra/Nac fish were CFP positive, indicating a low level of background fluorescence (Fig 5.8A) and
approximately 85% of cells were dsRed positive in the LyzC dsRed fish, as expected (Fig 5.8B). The majority of potential LyzC CFP-NTR transgenic fish gave a profile where a similar percentage of CFP positive cells were observed to that of the Tra/Nac control (Fig 5.8C), suggesting that these were not LyzC CFP-NTR transgenics. However, one of the potential LyzC CFP-NTR transgenic fish displayed a profile where 20% of gated myeloid cells were CFP positive, suggesting a potential transgenic (Fig 5.8D). These results suggest that the majority of the potential LyzC CFP-NTR fish are not transgenic.
5.4 Discussion

The results have demonstrated that whilst the pTol(2.4kb LyzC)CFP-NTR construct has been successfully generated, neither the injected F₀ founder larvae nor the F₁ larvae show any CFP positive cells consistent with LyzC expression. The majority of adult fish microinjected with the construct do not show any CFP positive cells within the myeloid population that is different to that of the Tra/Nac control fish when analysed by flow cytometry.

The approach used for attempting to generate the transgenic line involved application of the Tol2 system which results in a high level of stable integration of the transgene into the genome and germline transmission consistent with Mendelian inheritance, in that 50% of progeny from a positive founder crossed to a WT should be CFP positive (Kawakami et al, 2004). However, neither the founder larvae nor the F₁ offspring of the founder-WT cross displayed any CFP-positive cells. LyzC is a marker of myeloid cells and is expressed at 3dpf as shown by the recruitment of fluorescent cells to the site of injury in both LyzC GFP and LyzC dsRed larvae. However, no such recruitment was observed in the potential LyzC CFP-NTR transgenics, suggesting a lack of expression of the transgene in these larvae (Fig 5.7). The lack of observable expression was confirmed by the absence of CFP positive cells within the myeloid population in the majority of the potential adult LyzC CFP-NTR transgenics (Fig 5.8). The high level of stable integration of transgenes into zebrafish germ cells with the Tol2 approach is due to the co-injection of Tol2 mRNA along with the plasmid containing the transgene. The transposase encoded within the mRNA catalyses the excision of the transgene from the plasmid vector and its integration into the genome (Largaespada, 2003). Thus, the genomic integration of the transgene is an active process and not random, unlike approaches which involve the injection of plasmid DNA alone (Stuart et al, 1988; Stuart et al, 1990). Additionally, with the Tol2 system, there is no silencing effect observed with transgene expression over time due to the avoidance of rearrangements of surrounding genomic DNA. As a consequence of this, there is a high level of germline transmission to the offspring (Kawakami, 2005). Therefore, the results observed in using the Tol2 system in this case are not consistent with its reported success in the literature.
A possible explanation for the lack of observed expression of CFP in the F0 founder larvae may be that the transgene integrated relatively late into the genome following microinjection. Thus, only a small number of cells would express it, resulting in a very low, non-detectable level of expression. Alternatively, it is possible that the transgene may have failed to express following its genomic integration, resulting in no observable CFP-fluorescent cells or the integration into the genome may have failed completely. However, given the reported success of genome integration and germline transmission of transgenes using the Tol2 system, it is more likely that the lack of observable CFP fluorescence is due to a failure of expression rather than a failure of genome integration or genome integration occurring relatively late. Nevertheless, the latter two possibilities cannot be completely excluded.

The CFP-NTR ablation cassette has been previously used in combination with the Tol2 system by Curado et al to successfully generate stable transgenic lines of zebrafish expressing the cassette under the control of the cmlc2 promoter (cardiomyocytes) and the L-FABP promoter (hepatocytes) (Curado et al, 2007). A construct composed of NTR fused to mCherry under the influence of the insulin promoter has also been used to generate a transgenic line of zebrafish using the Tol2 system (Pisharath et al, 2007). Thus, the NTR ablation cassette has been used to successfully generate stable transgenic lines in the same way that it was used in this study. Furthermore, the 2.4kb LyzC promoter has been successfully used to drive GFP expression in a stable line of transgenic LyzC GFP zebrafish, which too was created using the Tol2 system (Kitaguchi et al, 2009). Therefore, the elements that were used to create the transgenic LyzC CFP-NTR line have been utilised in previous studies to generate stable transgenic lines, in that GFP expression has been driven by the 2.4kb LyzC promoter, and the cmlc2, L-FABP and insulin promoters have been used to drive expression of an NTR ablation cassette. However, this is the first time to our knowledge that the LyzC promoter has been used to drive expression of the CFP-NTR ablation cassette, which was ultimately unsuccessful.

5.4.1 Conclusion

Whilst the pTol(2.4kb LyzC)CFP-NTR construct was successfully created, it was not possible to generate a stable transgenic line of LyzC CFP-NTR zebrafish. This is most likely
due to a failure of expression of the transgene but also possibly due to a failure of the transgene to integrate into the genome or the integration occurring relatively late.
CHAPTER 6: FINAL DISCUSSION

The aims of this thesis were to establish zebrafish as a model for acute inflammation following feeding on a fat-enriched diet and to investigate the role of the NLRP3 inflammasome in this inflammation. The results have demonstrated that feeding larvae an enriched fat diet in the form of cream or in a more refined form as cholesterol leads to a recruitment of neutrophils and potentially macrophages to the intestine. Furthermore, in response to feeding on a cholesterol-enriched diet, the neutrophil accumulation appears to precede that of macrophages. Palmitic acid is capable of causing a localised recruitment of immune cells to the intestine whereas UFAs are unable to do so. Switching to a standard diet following feeding on a fat-enriched diet leads to an accelerated resolution of inflammation, possibly due to the anti-inflammatory effects of ω-3 FAs contained in the ZM. The recruitment of L-plastin positive cells to the intestine of cholesterol-fed larvae appears to depend upon the activities of cathepsin B, NADPH oxidase and caspase-1. Additionally, the expression of ASC in intestinal epithelial cells appears to drive the inflammatory infiltrate to the intestine, suggesting that the inflammation associated with feeding on a cholesterol-enriched diet is at least partly dependent upon signalling through the NLRP3 inflammasome.

Very little is known about the acute inflammation associated with diseases such as obesity, type II diabetes and atherosclerosis, and the findings of this thesis potentially shed some light on this. It is known that neutrophils transiently infiltrate AT of mice after 3 days of feeding on a high fat diet and then disappear after 14 days, following which macrophages appear (Elgazar-Carmon et al., 2008). These findings indicate a role of neutrophils and potentially macrophages following a 6h feeding period on a fat-enriched diet, which suggests that even after a period of a few hours, localised inflammation in the intestine has already begun. The peak accumulation of neutrophils appears to precede that of macrophages, consistent with an initial infiltration of neutrophils followed by macrophages in AT following high fat feeding and in other known cases of inflammation (Elgazar-Carmon et al., 2008; Soehnlein and Lindbom, 2010).

To further investigate the role of macrophages recruited to the intestine 18h following removal from the cholesterol-enriched diet, the phenotype of these immune cells could be
studied by examining the expression of markers associated with M1 and M2 macrophages. This would provide information as to whether these macrophages are responding to the cholesterol or to apoptotic neutrophils as part of the resolution phase. A marker of M1 macrophages is TNF-α and arginase is an M2 macrophage marker. Wholemount triple antibody staining can be carried out using antibodies against L-plastin, TNF-α and arginase, which are each stained with secondary antibodies conjugated to different, non-overlapping fluorophores. L-plastin positive cells positive for TNF-α are M1, those that are positive for arginase are M2 and any L-plastin positive cells that are negative for both TNF-α and arginase are neutrophils. This approach has been used by Feng et al in 7dpf larvae but in place of an arginase antibody, a fluorescent in situ probe was used (Feng et al, 2010). The inability to detect certain cytokines at all in the zebrafish larval intestine through qRT-PCR greatly hinders the understanding of the inflammation associated with feeding on a fat-enriched diet. To circumvent this, WISH could be used to detect changes in expression of cytokines in the intestine of larvae fed a fat-enriched diet compared to control larvae. This could be carried out at various time points both during the feeding period itself and following removal from the diet to investigate the inflammatory and resolution phases of the response to feeding on a fat-enriched diet.

The results suggest that both cholesterol uptake into intestinal epithelial cells and the expression of ASC in these cells may be required for localised inflammation in the intestine to occur. Thus, it is possible that cholesterol uptake into epithelial cells may activate the NLRP3 inflammasome leading to IL-1β release. The receptor through which IL-1β exerts its pro-inflammatory effects, IL-1RI, is expressed on epithelial cells in the intestine and potentially SMCs due to IL-1β inducing IL-6 release in these cells (Khan et al, 1995; McGee et al, 1996). Therefore, it is possible that the released IL-1β may engage its receptor on these cells resulting in the production of chemokines such as IL-8 and MCP-1, leading to the recruitment of further immune cells (Stienstra et al, 2012). Given the dependence of immune cell recruitment upon cathepsin B activity, it appears that cholesterol activates the inflammasome in a lysosomal-dependent manner, consistent with its mechanism of uptake into intestinal epithelial cells (Betters and Yu, 2010).

To gain further insight into the expression of ASC in the intestine, WISH could be used with an RNA probe against zebrafish ASC. This can be combined with transverse sectioning to examine the localisation of ASC expression in the cells of the intestine at the histological
level. In order to investigate ASC expression in intestinal macrophages and neutrophils, WISH can be carried out with L-plastin antibody staining. The expression of other components of the NLRP3 inflammasome can also be examined in this way. The possibility of ASC knockdown occurring in the intestinal epithelial cells assumes that the FITC fluorescence is a measure of the localisation of the MO and thus the FITC and MO are still conjugated following feeding. To see if this is the case, the effect of feeding the ASC-specific splice-blocking MO on expression of ASC studied with WISH can be compared to that of untreated larvae and provide further information as to where the MO is localising when administered in this way. If the MO is localising in the region where the FITC fluorescence is observed, then this region should display a reduced level of ASC expression. Thus, information that could not be gained from the qRT-PCR experiments studying ASC expression in the intestine following MO feeding could potentially be obtained in this manner. Additionally, the effects of MO feeding on ASC expression in the intestine can be compared to that of MO microinjection in terms of the cell types that are affected by both methods of delivery. This may help to resolve the different outcomes observed in terms of recruitment of L-plastin positive cells and detection of expression by qRT-PCR when delivering the MO by these two methods.

Despite the proposed ability of cholesterol to activate the NLRP3 inflammasome, an unanswered question is the mechanism of transduction of the priming signal required to generate the pro-form of IL-1β following feeding on a cholesterol-enriched diet. It is known that oxLDL is capable of providing this priming signal by engaging the TLR4/6 heterodimer in a CD36-dependent and CD14 independent manner (Stewart et al., 2010). However, the possibility that activation of the NLRP3 inflammasome is occurring in the intestinal epithelial cells means it is unlikely dietary cholesterol will have formed oxLDL at this stage as it has not yet been processed by the organism. Thus, cholesterol itself may provide this priming signal possibly through an unknown mechanism. Oxysterols have been shown to induce secretion of IL-1β from human macrophages, suggesting that possible oxidation of the dietary cholesterol following ingestion by the larvae may result in generation of pro-IL-1β (Palozza et al., 2011). Treatment with ezetimibe has been demonstrated to decrease IL-1β expression in rat livers, suggesting that cholesterol is capable of inducing IL-1β expression in hepatocytes (Nomura et al., 2009).
The inflammation associated with feeding on a palmitic acid-enriched diet also appears to be dependent upon caspase-1 activity, suggesting a potential dependence upon the NLRP3 inflammasome. Furthermore, palmitic acid is capable of activating TLR4 and thus could potentially provide the priming signal required to generate pro-IL-1β and the second signal through activation of the NLRP3 inflammasome (Kim et al., 2007; Davis et al., 2008; Maloney et al., 2009). Interestingly, studies by Wen et al. demonstrated that palmitic acid was incapable of inducing mature IL-1β release from macrophages in the absence of LPS, indicating that LPS was required to provide the priming signal and palmitic acid activates the inflammasome providing the second signal (Wen et al., 2011). However, these results suggest that palmitic acid alone is capable of providing both signals as the inflammation associated with feeding on a palmitic acid-enriched diet appears to be dependent upon caspase-1 activity without any further inflammatory stimuli required. An explanation for the requirement of LPS to provide the priming signal is that palmitic acid used in studies by Wen et al. was conjugated to BSA to enhance its solubility. This may have affected its ability to activate TLR4 signalling and thus provide the priming signal (Wen et al., 2011). However, because activation of TLR4 by palmitic acid is likely to be dependent upon CD14 and MD-2, the priming signal is unlikely to occur in this manner in zebrafish and another pathway may be involved. It is known that the LPS response in zebrafish occurs independently of TLR4 signalling and MyD88, yet the expression of genes induced in response to LPS treatment are MyD88-dependent such as IL-1β, IL-12 and TNF-α (Sepulcre et al., 2009). This suggests the involvement of another pathway that results in expression of the same genes. A similar scenario may be the case for the inflammation associated with feeding on diets enriched in cholesterol or palmitic acid.

An alternative source of the priming signal may be the microbiota of the intestine. It is known that there is a low level of inflammation in the intestine during its colonisation by the microbiota, which results in a recruitment of neutrophils. LPS produced by the microbiota is responsible for this and the infiltration occurs in a MyD88 and TNF-α dependent manner. This is required to establish a homeostatic population of these immune cells in the intestine (Bates et al., 2007). The NF-κB pathway has also been shown to be activated following colonisation of the zebrafish gut with microbiota (Kanther et al., 2011). It is possible that the microbiota may lead to induction of expression of the pro-form of IL-1β potentially in a MyD88-dependent manner through TLR signalling. Given that the expression of other pro-inflammatory cytokines such as TNF-α and IL-6, and chemokines such as IL-8 and MCP-1
are also induced in this manner, the level of expression of these genes needs to be relatively low to prevent an overactive immune response in the resting state. There are regulatory mechanisms in place to prevent an excessive inflammatory response to the microbiota. One example of this is intestinal alkaline phosphatase, expressed in intestinal epithelial cells, dephosphorylating LPS and thereby reducing its immunogenic properties (Bates et al, 2007). TLR2 and TLR4 are also expressed at relatively low levels in intestinal epithelial cells to minimise recognition of commensal bacteria (Cario and Podolsky, 2000). Thus, if the microbiota alone were to provide the priming signal, it would need to involve a predominant expression of pro-IL-1β compared to other cytokines and chemokines. This is because it would be of no benefit to the host to have an inflammatory response against the resident intestinal flora. It is also possible that the induction of pro-IL-1β expression by the microbiota may occur in a MyD88-independent manner as was observed by Sepulcre et al following LPS stimulation (Sepulcre et al, 2009).

A priming signal may potentially be generated by the microbiota in combination with feeding diets enriched in either cholesterol or palmitic acid, reminiscent of what is observed in inflammatory bowel disease (IBD) whereby an inflammatory response is also mounted against the microbiota (Wlodarska and Finlay, 2010). In a chemically-induced colitis model in zebrafish, it has been demonstrated that the inflammation observed with TNBS treatment is dependent upon the microbiota. It is also proposed that TNBS may disrupt function of epithelial cells affecting their contact with commensal bacteria (Oehlers et al, 2011). In intestinal epithelial cells taken from human patients suffering from IBD, there is an increased expression of TLR4 (Cario and Podolsky, 2000). It is possible that cholesterol and palmitic acid may disrupt the regulatory mechanisms in place to suppress the inflammatory response against the microbiota, which leads to a state of intolerance of the gut flora and a hyperinflammatory response against it. This may be due to interfering with intestinal alkaline phosphatase activity or expression, or potentially upregulating expression of TLRs. Thus, this would lead to a priming signal within the intestinal epithelial cells as a result of altered recognition of commensal PAMPs by the epithelial cells. IBD is also characterised by an alteration in the composition of the gut flora which leads to an immune response against it, but in the short timescale of acute feeding, it is unlikely that such a scenario is occurring (Peterson et al, 2008). Therefore, it is possible that the microbiota may be responsible for generation of the priming signal as a result of feeding on a diet enriched in either cholesterol or palmitic acid given the known dependence of gastrointestinal inflammation associated
with IBD on the resident intestinal flora. It is also possible that cholesterol and palmitic acid may generate the priming signal without any involvement of the microbiota, or the microbiota alone may provide the signal. However, the data in this thesis does not indicate that any one of these three possibilities is more likely than the others.

Studies investigating the role of microbiota in inflammation often use germ-free animals, which lack resident bacteria in the gastrointestinal tract, or treatment with antibiotics to deplete the microbiota. It is not however applicable to use these approaches in the context of an acute fat feeding model to investigate the role of the microbiota in generating the priming signal. The microbiota plays an important role in regulating the proliferation of intestinal epithelial cells, which precedes their differentiation and is required for development of the larval intestine (Crosnier et al., 2005). ASC is also expressed in mature, highly differentiated intestinal epithelial cells near the lumen and not immature, undifferentiated cells (Masumoto et al., 2001; Yokoyama et al., 2003). Furthermore, full development of the larval intestine occurs by 5 dpf, which coincides with the onset of feeding (Ng et al., 2005). Thus, depleting the microbiota may interfere with the development of the intestinal epithelia which could affect ASC expression and also the ability of intestinal epithelial cells to take up cholesterol or palmitic acid. If an effect is observed following acute fat feeding as a result of depleting the microbiota, it may be due to absence of the microbiota-dependent priming signal or possibly due to a reduction or absence of ASC expression, or a reduced ability for epithelial cells to take up dietary fat. Thus, there could be a number of effects of depleting the microbiota in addition to potentially abrogating the priming signal, which may also be responsible.

However, given the possible dependence on TLRs in providing the priming signal, an approach to investigate this could involve MO knockdown of zebrafish TLRs. As both NF-κB and MAPK signalling occur downstream of IL-1β signalling as well as through TLR signalling, it would not be possible to distinguish whether NF-κB and MAPK signalling are required for the priming signal as well as the downstream signalling through IL-1β, or just the downstream IL-1β signalling, if these two signalling pathways were targeted. Thus, the targeting of the TLRs ensures that only a pathway that occurs potentially in parallel to NLRP3 signalling, and not downstream of it as well, is affected. This could potentially provide information about whether the priming signal is occurring in a TLR-dependent manner by observing the effect of gene knockdown on immune cell recruitment to the
6. Discussion

Furthermore, the effect of MO knockdown of TLRs on IL-1β expression in the intestine can be examined using WISH.

Whilst feeding on a palmitic acid-enriched diet results in recruitment of immune cells to the intestine, feeding on diets enriched in UFAs such as linoleic acid and oleic acid do not, suggesting that these FAs are non-inflammatory. This is consistent with the inability of oleic acid to impair insulin signalling or induce TNF-α expression or secretion, and the failure of linoleic acid to induce MCP-1 and IL-6 expression compared to palmitic acid (Chavez and Summers, 2003; Bradley et al, 2008; Wang et al, 2009). Oleic acid and linoleic acid are also capable of antagonising the inflammatory effects of palmitic acid, suggesting an anti-inflammatory effect of these UFAs (Weigert et al, 2004; Kennedy et al, 2009). This is further supported by the observation of switching to a standard diet enriched in ω-3 FAs following feeding on a fat-enriched diet results in an accelerated resolution of inflammation. ω-3 FAs are converted to protectins and resolvins which are involved in the resolution of inflammation through blocking neutrophil transmigration and promoting the phagocytosis of neutrophils by macrophages (Schwab et al, 2007; Serhan et al, 2008; Soehnlein and Lindbom, 2010). Thus, supplying such FAs in the diet immediately following feeding on a fat-enriched diet means that the processes involved in resolving inflammation occur more quickly. Therefore, FAs vary in their ability to induce acute inflammation in the intestine and this may have consequences on chronic inflammation associated with metabolic diseases. For instance, obese patients have high levels of SFAs such as palmitic acid which can predispose to type II diabetes through interference with insulin signalling (Reynoso et al, 2003; Yerlikaya et al, 2011).

The chronic inflammation associated with diseases of the metabolic syndrome is well established and interconnected in that inflammation resulting from obesity can pre-dispose to type II diabetes and atherosclerosis. These disorders are increasingly prevalent today with a lifestyle heavily influenced by an unhealthy diet and a lack of exercise. The inflammation is systemic and affects multiple sites in the body, such as AT, muscle, liver and the vasculature. The results of this thesis demonstrate an acute inflammation localised to the gastrointestinal tract after feeding on a fat-enriched diet for a period of a few hours. Thus, it appears that this highly localised inflammation precedes the systemic chronic inflammation associated with the metabolic syndrome. As very little is known about the acute inflammation associated with feeding on a fat-enriched diet, the findings of this thesis may open up avenues for
therapeutic potential. The results suggest that the NLRP3 inflammasome is at least partly responsible for the inflammation observed in feeding on a cholesterol-enriched diet through the use of pharmacological inhibitors. As the targets of inhibition are conserved in mammalian systems, possible drugs could be designed against the human orthologues of these. The inflammatory effect resulting from feeding on a fat-enriched diet is resolved more quickly upon switching to a standard diet which also may have therapeutic potential in preventing the chronic inflammation associated with obesity. Western diets typically have a high content of SFAs and are often lacking in UFAs. Thus, switching to healthier diets especially high in ω-3 FAs may reduce the incidence of obesity and the metabolic complications arising from it. Given the similarity in both the development and function of the innate immune system in zebrafish together with the manner in which dietary fat is taken up and processed in zebrafish compared to mammalian systems, the findings of this thesis may well be relevant to human disease. Complications arising from type II diabetes and atherosclerosis claim many lives each year and being able to target possible early causes of the inflammation associated with these diseases at the molecular level could be of great benefit to global health.
Figure 7.1: Attempting to Detect H$_2$O$_2$ in the Intestines of Cholesterol-Fed Larvae. 6 dpf WT larvae were treated with either 50µM fluorogenic H$_2$O$_2$ probe or DMSO for 1h. They were then fed either a standard diet (ZM), or a cholesterol-enriched diet or were unfed for 10 mins. Following this, larvae were anaesthetised and imaged under an epifluorescent microscope for fluorescence in the intestine. (A) Cholesterol-fed larva pre-treated with DMSO. (B) Unfed, (C) ZM-fed and (D) cholesterol-fed larvae pre-treated with fluorogenic H$_2$O$_2$ probe. Larvae are oriented from anterior (left) to posterior (right). Scale bar represents 0.4mm. Data shown for n = 1 experiments (number of times experiment was performed).
Figure 7.2: Attempting to Detect Caspase-1 Activity in the Intestines of 7dpf Larvae. (A) 6 dpf WT larvae were treated with either 10µM, 40µM or 100µM fluorogenic caspase-1 substrate (Z-YVAD-AFC) for 1h. They were then anaesthetised and imaged under a wide-field microscope for fluorescence in the intestine. (i) Untreated, (ii) DMSO-treated and (iii) 10 µM Z-YVAD-AFC-treated larvae. (B) In a separate experiment, 6 dpf WT larvae were fed for 6h on either a standard diet (ZM) or a cholesterol-enriched diet. At the end of this feeding period, larvae were anaesthetised and then treated for 30 mins in 1 x FLICA, followed by a 30 min wash in PBS. Larvae were then imaged for fluorescence in the intestine. (i) Untreated larva. (ii) and (iii) ZM-fed larva treated with FLICA. (iv) and (v) cholesterol-fed larva treated with FLICA. Larvae are oriented sagitally from anterior (left) to posterior (right). Scale bar represents 0.4mm. Data shown for n = 1 experiments (number of times experiment was performed).
Figure 7.3: Lack of a Correlation Between Fluorescence Intensity of FLICA and Recruitment of L-Plastin Positive Cells to the Intestine. 6 dpf WT larvae were fed for 6h on either a standard diet (ZM) or a cholesterol-enriched diet. 3h after removal from the diet, larvae were anaesthetised and then treated for 30 mins in 1 x FLICA, followed by a 30 min wash in PBS. One group of cholesterol-fed larvae were not treated with FLICA (Untreated). Larvae were then imaged for fluorescence in the intestine and scored on the brightness of this fluorescence. The larvae were fixed and stained, and numbers of L-plastin positive cells in the intestine were then counted in each of the groups. Comparison of the numbers of cells in individual larvae (A) and mean numbers of cells in larvae (B) fed either ZM or a cholesterol-enriched diet and scored on the brightness of the intestine. Data shown for n = 1 experiments (number of times experiment was performed).
Figure 7.4: Quantifying the Fluorescence Intensity of FLICA Following Treatment with Inhibitors of the NLRP3 Inflammasome. 6 dpf WT larvae were pre-treated with either DMSO, 100µM caspase-1 and 5 inhibitor (N-Acetyl WEHD-al), 100µM cathepsin B inhibitor (Ca-074-Me) or were untreated for 30 mins. Following the pre-treatment, larvae were either fed for 6h on a standard diet (ZM) or a cholesterol-enriched diet in the presence of the inhibitors. At the end of this feeding period, larvae were removed from the diet and 3h following removal were anaesthetised and then treated for 30 mins in 1 x FLICA, followed by a 30 min wash in PBS. Larvae were then imaged for fluorescence in the intestine which was quantified. Comparison of the fluorescence intensity in individual larvae (A) and mean fluorescence intensity in larvae (B) fed either ZM or a cholesterol-enriched diet under the different pre-treatment conditions. Comparison of the fluorescence intensity in individual larvae (C) and mean fluorescence intensity in larvae (D) fed either ZM or a cholesterol-enriched diet and untreated or pre-treated with DMSO. Statistical significance was determined using a Kruskall-Wallis test followed by a Dunn’s post test. *** denotes p < 0.001, and * p < 0.05. Error bars represent 95% confidence intervals. Data shown for n = 1 experiments (number of times experiment was performed).
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