Understanding how neutrophils self-organise their migration to sites of inflammation in vivo

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text

It does not exceed the limit of 60,000 words prescribed by the Degree Committee for Biology.

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Summary

Neutrophils are major effectors of acute inflammation and microbial defence. Their infiltration and migration in injured tissue are critical for the inflammatory response. They are often observed navigating in a highly co-ordinated and directed manner leading to their aggregation at the target site of infection. This self-organised cell gathering is referred to as swarming. It is known that neutrophil swarming is driven by autocrine attractant production, notably of the lipid leukotriene B4 (LTB4). The decision to release attractants at the single-cell level is important and impacts the magnitude of the entire immune response. However, the precise mechanisms triggering this decision remain unclear.

In this study, I employed *in vivo* imaging of zebrafish larvae to reveal the molecular processes that trigger the release of LTB4 and initiation of swarms. I developed a 2-Photon laser wound assay to elicit and visualise neutrophil swarming. A major limitation in previous studies of neutrophil swarms was the lack of tools to understand cell signalling dynamics during the response. To overcome this, I generated a new biosensor to probe for calcium levels in neutrophils as it correlates with the production of chemoattractants. Using this new tool, I revealed that neutrophils clustering at the target inflammatory site are experiencing sustained high calcium elevation. Using a new probe to follow the production dynamics of LTB4, I demonstrated that the rise of intracellular calcium promotes the biosynthesis of this key attractant.

I further demonstrated that these calcium fluxes are triggered upon contact with necrotic tissue. This prompted me to interrogate the damage molecules driving these calcium signals in neutrophils. I discovered that the calcium fluxes were mediated by ATP that binds gated ion channels (P2X1) leading to rapid intracellular calcium uptake.

Surprisingly, I found that live neutrophils can also trigger this calcium flux in other neutrophils upon mutual contact. Using chemical and genetic inhibition, I found that connexin-43 (Cx43) hemichannels, through their ability to release ATP, enable amplification of the calcium signal leading to chemoattractant production and subsequent neutrophil recruitment. I concluded that activation of LTB4 synthesis is a group decision reached via Cx43-dependent communication in pioneer clustering neutrophils. As Cx43 inhibition significantly reduced neutrophil aggregation at the target site, I investigated if this inhibition could have consequences for wound defence. For this, I developed a wound colonisation assay with *Pseudomonas aeruginosa*. Using this approach, I showed that Cx43 was crucial for the protection of wounds from opportunistic bacteria.

Finally, I designed and generated an optogenetic tool to manipulate LTB4 biosynthesis dynamics *in vivo*. I demonstrated the effectiveness of this tool in immortalised cells and zebrafish larvae. The unique features of this tool make it very useful for a wide range of research applications on signalling dynamics.

In conclusion, I have shown that by reinforcing damage signalling, Cx43 channels coordinate attractant biosynthesis in pioneer neutrophils. This generates an effective chemoattractant gradient source and promotes targeted aggregation and defence. This study, therefore, reveals a new mechanistic principle of collective behaviour that could be exploited in future pathological research.

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GLOSSARY

5-Lipoxygenase
An enzyme that is important for the production of LTA4.

AA
Arachidonic acid is an omega-6 essential fatty acid.

BLT1
Member of the rhodopsin-like family of G-protein coupled receptors and is classically known as leukotriene B4 receptor.

Chemotaxis
The movement of a motile cell in the direction of an increasing gradient of an attracting molecule.

Connexin
Connexin Molecules can form hemichannel at the surface of cells to let small molecule under 1kDa to pass through the cytoplasm.

CRY2/CIB
CRY2 and CIB are two optogenetic domains that can dimerise upon blue light (488nm) exposure.

Cx43
Connexin 43 is a 43kDa connexin molecule that is expressed neutrophils.

CXCL-8
Also known as IL-8, is a chemokine that can attracts various immune cells including neutrophils.

DAMP
Damage Associated Molecular Pattern are host molecules associated with cell damage and that can activate immune cells.

Degranulation
The release of toxic granules by neutrophils.

fMLP
N-Formylmethionine-leucyl-phenylalanine is a potent chemotactic factor for neutrophils.

GCamP
Genetically encoded Calcium indicator

GPCR
G-protein coupled receptors.

H_2O_2
Hydrogen peroxide is a reactive oxygen species which is also a very potent neutrophil chemoattractant.

LAP2β
Lamina-Associated Polypeptide 2 is an integral membrane protein of the inner nuclear membrane.

LTA4
Leukotriene A 4 is a precursor of LTB4 and LXA4.

LTA4H
Leukotriene A 4 Hydrolase is the enzyme synthesising LTB4 from LTA4.

LTB4
Leukotriene B 4 is a very potent neutrophil chemoattractant.

Lyz
Lysozyme is an enzyme that can found abundantly in neutrophils and that has the capacity to break the cell wall of bacteria.

Macrophage
Innate immune cell well known for its phagocytic activity.

MO
Morpholino are nucleotide analogues that can bind RNA sequences and block gene expression.

Mpx
Myeloperoxydase is an enzyme that is found abundantly in neutrophils and that is important for the production of Reactive Oxygen Species.

NET
Neutrophil Extracellular Trap is a specific feature of neutrophils during which neutrophils trap bacteria in their DNA while releasing antimicrobial molecules.

Neutrophil
Innate immune cells involved in the inflammatory response to damage tissues and infections.

Optogenetic
A technique using light to control cellular processes.

P2X1
P2X1 is gated receptor of ATP with high calcium permeability.

PAMP
Pathogen Associated Molecular Pattern are molecules that can be found on pathogens and that can be recognised by immune cells.

Phagocytosis The capacity of a cell to engulf large particles such as a bacteria by using its plasma membrane.

PI
Propidium Iodide is a stain of late apoptotic early necrotic cells.

PLA2
Phospholipase A 2 is the enzyme releasing Arachidonic Acid from phospholipids.

PRR
Pathogen Recognition Receptor.

Pseudomonas aeruginosa

A gram-negative opportunistic bacterium that can infect humans, mice and zebrafish.

ROS

Reactive oxygen species.

TEM

TransEndothelial Migration is the process by which immune cells exit the bloodstream.

1. Introduction

1.1. The role of immune cells during inflammation

1.1.1. Introduction to inflammation

The word 'inflammation' comes from the Latin *inflammare* (to ignite), it was first described by Cornelius Celsus as "*Notae vero inflammationis sunt quattuor: rubor et tumour cum calore et dolore" (It is to note that* the signs of an inflammation are four: *redness and swelling with heat and pain)* (Aulus Cornelius Celsus, 47AD). Celsus regarded inflammation to be a beneficial response for the injury; based on this idea the bodily fluids were keeping the wound healthy. However, Virchow later described it as an inherently pathological response following his studies linking inflammation and cancer and the consideration or revelation of another sign of the inflammation: the loss of function of the inflamed tissue, *Functio laesa* (Virchow, Rudolf, 1863). Though the term 'Inflammation' originates from the associated clinical signs, today, we understand inflammation to be an immunological response to bodily injury. Previous signs of inflammation are now explained by increased blood flow (giving rise to redness and heat), immune cell migration and pro-inflammatory signalling (giving rise to swelling and pain) (Ferrero-Miliani et al., 2007).

At the forefront of the inflammation process, white blood cells composing the innate immune system are the first to respond to tissue injury and infection. Conversely to the adaptive immune system composed of B cells and T cells, innate immune cells do not develop and adapt targeted response against antigens they encounter and do not keep memory of the pathogen. Innate immune cells readily recognise pathogens in an innate manner, sensing conserved patterns of molecules at their surface. Neutrophils and macrophages are key effectors cells of the innate immunity; their infiltration into injured tissue is characteristic of acute inflammation.

Research on the inflammatory immune response gradually evolved since early studies in the late 19th century. An early description of innate immune cell movement appears in Elie Metchnikoff's book (Metchnikoff, 1901) in which he also famously described the process of macrophages eating bacteria: the phagocytosis. He notably wrote : "*Dans l'immunité naturelle, les phagocytes manifestent une chimiotaxie positive et cette forme de sensibilité est une condition indispensable pour que l'immunité existe et que les microbes disparaissent.* » ("In natural immunity, phagocytes exert a positive chemotaxis which is crucial for immunity and microbe clearance"). Highlighting the importance of chemical cues to guide immune cells to their target.

Chemotaxis is the movement of a cell or an organism in response to a chemical stimulant. The term was first coined by the German botanist Wilhelm Pfeffer and comes from chemo for chemical and the Greek taxis for "arrangement" (Pfeffer, 1881).

The chemical cues produced at inflammatory sites play a key role in recruiting immune cells to the damaged site. The first type of signals recognised by immune cells are PAMPs (Pathogen Associated Molecular Patterns) and DAMPs (Damage Associated Molecular Patterns). PAMPs are made up of molecules found in pathogens such as LPS (lipopolysaccharide) at the membrane of bacteria or β-Glucans found on the cell wall of bacteria. DAMPs are composed of host molecules such as fMLP or DNA and RNA from cells dying as a result of an injury. PAMPs and DAMPs are danger signals that can be directly recognized by receptors such as TLRs (Toll Like Receptors) and GPCRs (G protein coupled receptor) expressed at the surface of immune cells. Some of these GPCRs are receptors that can mediate chemotaxis (e.g. the fMLP receptor). On the other hand, TLRs do not regulate motility but rather regulate gene expression and the activation state of the cells. Upon sensing TLR ligands, resident cells undergo activation and start producing pro-inflammatory molecules. Those mediator molecules activate adhesive molecules on blood vessels, which promote neutrophil and macrophage arrest on endothelial cells and transmigration into the injured tissue through the process of 'extravasation'. Once in the target tissue, neutrophils and macrophages migrate to the site of injury following gradients of chemical cues (these are typically GPCR ligands).

1.1.2 The process of wound repair

As described above, inflammation is the body's attempt to preserve self-integrity, notably when a barrier is breached. The skin is a natural barrier against pathogens. When this barrier is broken as a result of an injury, opportunistic pathogens can invade the organism. Immune cells recruited to sites of injury are essential to protect the organism against external pathogens but also contribute to the wound healing process. Wound repair is a complex process resulting, in most cases, in scars, a fibrous tissue composed of collagen as the tissue it replaces. However, unlike the original tissue, the different alignment of collagen fibres in scars results in inferior functional quality than normal collagen (Xue and Jackson, 2015). This makes scars less flexible and weaker than the original tissue. This process happens in almost all tissues in response to destructive stimuli. Wound repair can be divided in 4 overlapping steps: haemostasis, inflammation, proliferation, and remodelling (Gurtner et al., 2008). During the **haemostasis** step, the bleeding is controlled by the aggregation of platelets and the deposition of coagulation factors leading to the formation of a blood clot (Furie and Furie,

2005). Attractants triggered at the wound site such as DAMPs and PAMPs are attracting white blood cells such as neutrophils which start migrating towards the wound. The accumulation of immune cells relaying pro-inflammatory signals is leading to the **inflammation**. The **proliferation** stage refers to the formation of new tissue consisting of blood vessels, immune cells and fibroblast allowing re-epithelialisation of the wound. Finally scar tissue formation happens during the **remodelling** step when new extracellular matrix consisting mainly of collagen is deposited by fibroblasts allowing the tissue to strengthen (Phillipson and Kubes, 2019). In some pathological cases, the proliferation and remodelling steps do not occur resulting in a non-healing wound. This can occur as a result of the colonisation of the wound by a pathogen such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* (Malone et al., 2017; Percival et al., 2012; Rahim et al., 2017). It can also be due to an altered immune system as it is the case in diabetic patients where immune function and blood circulation are altered (Moura Neto et al., 2013). Non-healing wounds are a serious burden and can, in some cases, result in amputation of the limb affected.

1.1.3 Immune cell migration

A critical step of the immune response to an injury is the migration of leukocytes towards the site of injury. This physiological process is crucial to defend the body against pathogens. Excessive or misdirected leukocyte migration can lead to pathologies such as Chron's disease (Thomas and Baumgart, 2012). After being activated by a diverse range of molecules, leukocytes can enter the affected tissue by crossing from the bloodstream through a process named TEM (TransEndothelial Migration), extravasation or *diapedesis* (Fig. 1.1). In the blood circulation, following the activation of leukocytes and endothelial cells by signalling molecules, endothelial cells start expressing adhesion molecules called selectin that will bind carbohydrates at the surface of leukocytes (Lorenzon et al., 1998). This initiates the rolling of leukocytes on the inner wall of the blood vessel composed of endothelial cells. During rolling, molecules released by both epithelial cells and leukocytes will switch surface integrins on leukocytes from low-affinity state to high-affinity state (Weber et al., 1996). At this point integrins will bind tightly their receptors on the surface of endothelial cells. This will strongly contribute to leukocyte adhesion and stopping on the epithelial cells. Next leukocytes spread over the endothelial cells by reorganising their cytoskeletons. The interaction of the leukocyte with the endothelial cells triggers the formation of gaps through which the leukocytes will squeeze to reach the next layer, the basement membrane, this is called the paracellular migration. Leukocyte use PECAM (Platelet Endothelial Cell Adhesion Molecule) proteins on their surface to interact and pull themselves through the epithelial cell wall (Vaporciyan et al.,

1993). Leukocyte can also get through the endothelial cell wall by directly crossing through an endothelial cell, this is referred to as transcellular migration and requires the redistribution of endothelial cytoplasmic organelles and the fusion of the apical and basal plasma membranes of the endothelial cells (Carman et al., 2007). At this point leukocytes still need to get through the basement membrane, a thin and fibrous extracellular matrix, to exit the circulation and reach the target tissue. The way leukocytes cross the basement membrane is still unclear, but they might digest the extracellular matrix with proteolytic enzymes or push through using sheer force or both (Rowe and Weiss, 2008). After crossing the basement membrane, leukocytes enter the interstitial tissue; a thick 3-dimensional fibrillar network of collagen fibres and densely packed cells. In those tissues, leukocytes follow gradients of chemical cues in order to navigate towards the site of inflammation.

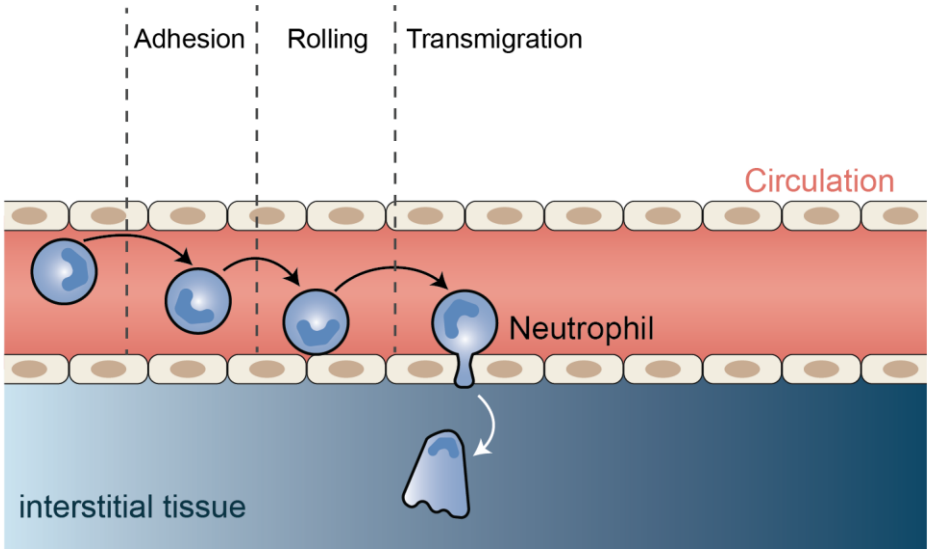


Figure 1.1. Leukocyte transendothelial migration.

Schematic of a leukocyte (in blue), adhering, rolling and transmigrating into the interstitial tissue (dark blue) through the circulation (red).

1.2. Immune cell motion in vivo

1.2.1. Molecular cascade

The first chemoattractants stemming from the sites of inflammation are DAMPs and PAMPs. Damage signals (DAMPs) are made up of a broad variety of molecules from cells in injured tissues. PAMPs are made up of molecules found in pathogens. fMLP (*N*-formylMethionyl-Leucyl-Phenylalanine), a formyl peptide released by mitochondria of dead cells or by bacteria is a well-studied DAMP/PAMP chemoattractant. It is among the first attractants to be detected by leukocytes after an injury (Wenceslau et al., 2013). The attractive potential of fMLP was first shown in leukocytes isolated from rabbit peritoneal exudates exposed to fMLP purified

from the bacteria *Escherichia coli in vitro* (Schiffmann et al., 1975). In this study, the authors used the boyden chamber assay, which involves loading cells in a compartment located above a chamber containing the chemoattractant of interest. Small pores (5µm wide) between the compartment containing the cells and the chemoattractant chamber allow the cells to migrate towards the chemoattractant chamber. The cell's chemotactic affinity with the attractant is assessed by calculating the number of cells that migrate to the chemoattractant chamber.

This assay was also used to demonstrate the chemotactic potential of proteins that are part of the complement system that can be activated as a result of an infection or an injury (Hartmann et al., 1997). The complement system is part of the immune system; its role is to enhance the anti-microbial activity of leukocytes and to promote inflammation. Proteins that constitute the complement system are produced by cells such as macrophages, neutrophils and epithelial cells. In this case the authors used HMC-1, an immortalised cell line of mast cells, which are leukocytes that are part of the innate immune system. Their data show that HMC-1 cells were attracted by the complement's molecules C5a and C3a.

Another early signal coming from the wound is H_2O_2. H_2O_2 is a reactive oxygen species generated by NADPH oxidase. It was shown in a model of zebrafish larva wounding at the tail that H_2O_2 was produced as a consequence of an injury and that neutrophils were attracted to it (Niethammer et al., 2009). To show this, the authors used transgenic animals expressing HyPer a genetically encoded probe for H_2O_2 and leukocytes expressing fluorescent proteins. Combined with confocal imaging, this allowed them to visualise the formation of a H_2O_2 gradient around the wound followed by leukocyte migration towards the site of injury. They were able to genetically inhibit this gradient and subsequent leukocyte migration by knocking down the NADPH oxidase responsible for H_2O_2 production.

Most DAMPs and PAMPs enhance the inflammation by stimulating the production of attractants by cells. For DAMPs, these include molecules such as DNA, RNA or ATP that are released from necrotic cells (Roh and Sohn, 2018). PAMPs include molecules such as LPS found on membranes of gram negative bacteria, nucleic acids from pathogens or peptidoglycan found on bacteria cell walls (Kumar et al., 2011). These molecules bind PRR (Pattern Recognition Receptor) receptors mainly expressed by immune cells. The discovery of this innate way to sense pathogens and damage gave rise to the Nobel Prize in Physiology or Medicine 2011. The idea of the innate immune sensing was first formulated by Janeway in 1989 at the Cold Spring Harbor symposium on quantitative biology during which he suggested "that there must be a system for the recognition of these inducers (bacterial structures such a lipopolysaccharide) of second signals, again based on pattern recognition rather than on the type of specificity we associate with the immune system" (Janeway, 1989). The first PRR were found by Bruno Le Maitre *et al* in 1996. For this the authors used a genetic approach to inhibit TLR (Toll Like Receptors) in drosophila and showed that the receptor was necessary to protect

flies against fungal contamination by inducing the activation cascade of NF-kB leading to the production of signalling molecules (Lemaitre et al., 1996). Nowadays, many other PRR has been found to be expressed at the surface of the cells and also in their cytoplasm.

PAMPs and DAMPs upon binding to PRR will activate a signalling cascade leading to the production of signalling molecules including attractants, that I will refer to as secondary attractants. These secondary attractants include small peptides called chemokines and eicosanoids lipid derived from arachidonic metabolism. All chemokines possess four conserved cysteines linked by disulfide bonds and are classified based on the spacing between those cysteines (Baggiolini et al., 1997). CXCL-8 or IL-8 (InterLeukin 8) was the first chemokine to be described. In 1987, researchers purified and identified IL-8 using HPLC (High-Performance Liquid Chromatography). Using the boyden chamber assay I previously described, they demonstrated the chemotactic potential of IL-8 for human leukocytes (Yoshimura et al., 1987). Subsequently, many other chemokines were discovered and it is now considered that they play a major role in leukocyte chemotaxis (Baggiolini et al., 1997).

These chemical cues contribute to the attraction and guidance of leukocytes towards the site of inflammation. For this, they bind GPCRs (G protein coupled receptors) expressed at the membrane of leukocytes initiating a cascade resulting in chemotaxis (Fig. 1.2). GPCRs are involved in many physiological processes and their discovery ultimately led to the Nobel Prize in chemistry in 2012. These receptors were found by attaching an iodine isotope to various hormones (Williams and Lefkowitz, 1976). Their importance for signal transduction was characterised later (Tuteja, 2009). Upon ligand binding, a conformational change occurs in the GPCR. Acting as a guanine exchange factor (GEF), the GPCR then activates its associate G protein by exchanging the GDP bound to the G protein for a GTP (Weis and Kobilka, 2018). The G protein can thereafter further interact with other proteins intracellularly. Signalling effectors such as Rho GTPases, PI3K (Phosphoinositide 3-kinase) are controlling cell movement triggering an intracellular signalling cascade leading to the reorganisation of the actomyosin cytoskeleton (Weis and Kobilka, 2018). The actomyosin cytoskeleton is important for adapting cell migration mode to the cellular environment and help leukocytes navigate in complicated tissues.

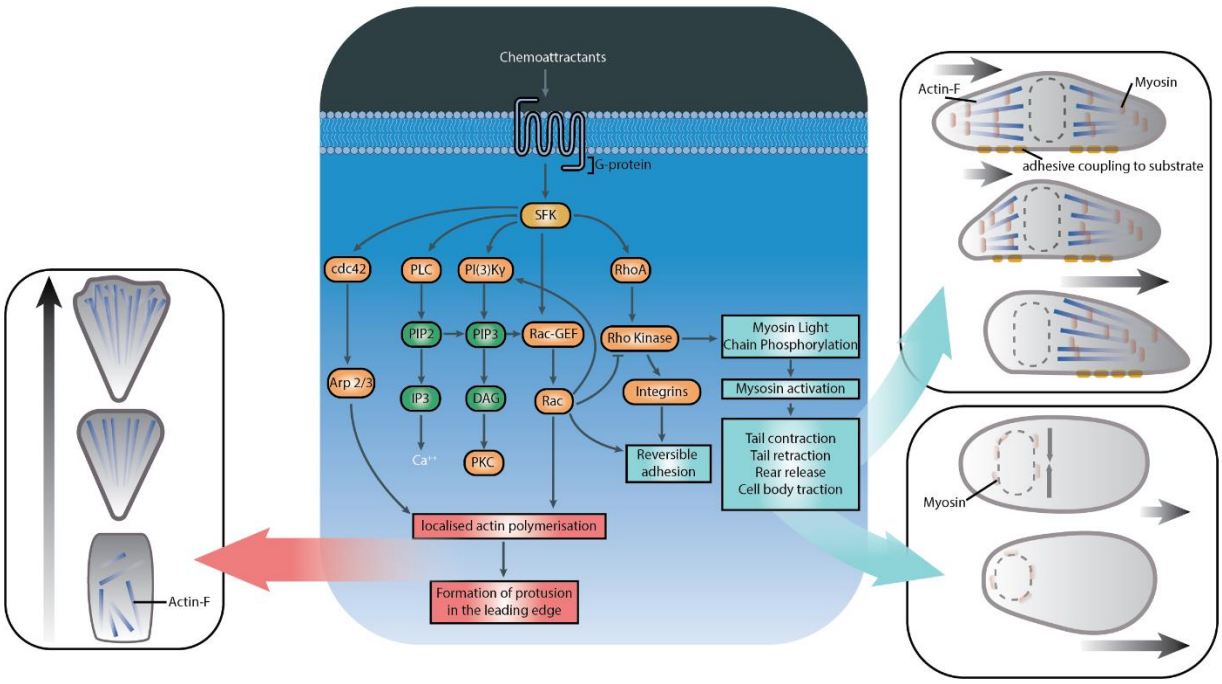


Figure 1.2. Molecular pathways associated with cell motility.

The molecular cascade leading to cell motility is triggered upon ligand binding a GPCR. Integrins, Rac and Rho kinase are involved reversible adhesion and cell contraction triggering cell movement (on the right). Arp2/3 and Rac lead to localise actin polymerisation and formation of protrusion at the leading-edge triggering cell movement (on the left).

1.2.2 Leukocyte motion in vivo

There are two main types of cell locomotion, the mesenchymal migration and the amoeboid migration. The mesenchymal locomotion relies on reorganisation of the actin distribution at the leading edge of the cell with formation of protrusions and adhesive interactions to the substrate, followed by retraction of the contractile cell rear to achieve cellular movement (Theriot and Mitchison, 1991). The amoeboid type of locomotion relies on the cell protruding and retracting extensions called pseudopods. The amoeboid locomotion is used by immune cells during the inflammation. There are also two distinct types of amoeboid locomotion, one relies on the contraction of the cell through blebbing and the other one relies on actin-polymerization-based gliding (Lämmermann and Sixt, 2009). In both cases, forces are driven by the actomyosin cytoskeleton. It was shown in tumour cells *in vitro* that the velocity of cells using the amoeboid type of locomotion is higher than the one using mesenchymal migration (Paňková et al., 2010). The ameboid type of migration is used by leukocytes to navigate in 3D

tissues and is therefore the type of locomotion used during inflammation. It has notably been shown in isolated human leukocytes that the mode of migration used in confined environments was non-adherent amoeboid locomotion (Malawista and de Boisfleury Chevance, 1997). In this study, cells were pressed between a slide and a coverslip to recreate a confined environment and erythrocytes were destroyed by a laser ruby microbeam to trigger leukocyte chemotaxis. Additionally, in another study, it was shown in interstitial tissue of mouse ears, *ex vivo*, imaged with a stereomicroscope that leukocytes were using an amoeboid type of migration (Lämmermann et al., 2008).

1.2.3 Chemoattractant gradients

The movement of the leukocytes towards signalling molecules is referred to as chemotaxis. Leukocytes can sense an increasing concentration of attractant referred to as attractant gradient. There are two theories regarding attractant gradient sensing by cells (Fig. 1.3) (Bourne and Weiner, 2002). The first postulates that chemoattractant concentration can be sensed at the leading edge of migrating cells, the protrusion that is exposed to the highest level of chemoattractant will be maintained and the migration will therefore be biased towards this gradient; this is referred to as **spatial sensing**. The second theory postulates that cell can sense gradient over time and is referred to as **temporal sensing**. This theory implies that cells are randomly moving but their speed increases upon sensing higher chemoattractant concentration biasing their migration along a gradient. Chemotaxis usually refers to cells migrating towards a gradient of soluble attractant in a fluid phase. Haptotaxis is the directional motility of cells along a gradient of substrate-bound chemoattractant or cellular adhesion site. The first study depicting gradient-guided interstitial leukocyte migration *in vivo* showed that leukocytes were following haptotactic gradients. More particularly, this was shown in a zebrafish larva model with transplanted cells expressing CXCL8 or a mutant of CXCL8 that cannot bind the substrate. In this study, zebrafish neutrophils expressing fluorescent proteins were observed accumulating around the transplanted cells producing CXCL8. However, a significant decrease in their accumulation was noted in conditions in which transplanted cells were expressing the mutant CXCL8, confirming the importance of haptotaxis for neutrophil migration *in vivo* (Sarris et al., 2012). A year later a study in mouse confirmed the importance of haptotactic gradient *in vivo*, this time for dentritic cell migrating along gradients of the chemokine CCL21 (Weber et al., 2013).

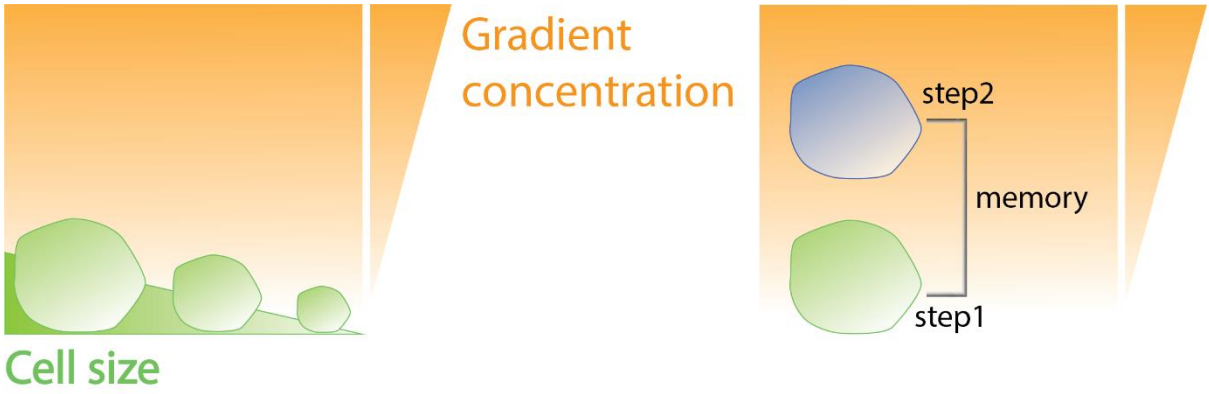


Figure 1.3. Gradient sensing.

Schematic describing the two ways cells can sense a gradient. Spatial gradient sensing is described on the left and temporal gradient sensing is described on the right.

Chemical cues can be secreted by cells, but cells can also leave “trails” enriched in chemoattractant behind them. A study showed that trails are pieces of cell membrane left by neutrophils adhering to a substrate during their migration. The trails were identified in a mouse model using a combination of confocal microscopy and electron microscopy. They were found guiding T cells to influenza infected trachea (Lim et al., 2015).

Another mode of migration observed *in vivo* is necrotaxis. This is a special type of chemotaxis in which cells are following a gradient generated as a result of apoptotic and or necrotic signals (Peters-Golden and Brock, 2003). In this case DAMPs such a fMLP are released from dead cells and form an attractant gradient leading leukocyte to the necrotic tissue. It was shown in a model of mouse injury *in vivo* that necrotaxis could be a mechanism that reinforce neutrophil recruitment at sites of injury (Lämmermann et al., 2013). It has been hypothesised that this mode of migration might be important for the recruitment of cells to wound focus (Lämmermann et al., 2013; Peters-Golden and Brock, 2003; Uderhardt et al., 2019).

With the development of new biophysical methods, a new type of cell attraction has been described: mechanotaxis (Li et al., 2002; Lo et al., 2000; Mak et al., 2016). Mechanotaxis refers to cellular migration triggered by physical forces applied to the cell such as fluidic shear stress, stiffness or confinement. Contrary to chemotaxis, this mechanism does require cue-dependent signalling. It was shown in a flow device, *in vitro*, that human lymphocytes adhering to blood vessels were steering themselves against the fluid flow. The direction of the flow appeared to be detected not by mechanosensors but by the cell protrusions (Valignat et al., 2014). It is however still largely unknown how mechanical cues play a role in leukocyte migration *in vivo*, but it is expected that upon injury or infection the mechanical properties of the tissues might be altered and might influence leukocyte migration to site of inflammation.

1.3. Neutrophils

1.3.1 The polymorphonuclear neutrophils

Neutrophils are crucial for the inflammatory process. The name 'neutrophil' originates from their staining characteristic with haematoxylin and eosin preparations; those cells being more easily stained by neutral dyes (neutro-phil = like neutral) (Schultze, 1865). They are also called polymorphonuclear neutrophils due to the varying shape of their nucleus. They are among the first immune cells to reach the site of injury. In human adults, neutrophils are formed from stem cells in the bone marrow and are the most abundant white blood cell type in the body representing 60 to 70% of the total leukocyte population. Neutrophils have a short lifespan of up to 12.5 hours for mouse cells, 5 days for resting neutrophils in zebrafish tissues (Dixon et al., 2012) and 5.4 days for human neutrophils (Pillay et al., 2010). Most of the neutrophils are found in the blood circulation and are triggering the early phase of the inflammatory response, particularly as a response to wounds, bacterial infection or cancer. After migrating through the blood by extravasation they chemotax towards target site of inflammation within the injured tissue. Formyl peptides such as fMLP are among the first chemoattractants of neutrophils that have been characterised. In 1982 it was shown *in vitro* with human isolated neutrophils that fMLP from mitochondria was a chemoattractant of neutrophils (Carp, 1982). It is now known that neutrophils follow cues of fMLP to migrate to site of injury (Zhang et al., 2010). LTB4 is another important and well documented cue guiding neutrophils to inflammation site. It has notably been shown that neutrophils follow LTB4 cues, *in vivo*, in a mouse model of inflammatory arthritis (Peters-Golden and Brock, 2003) and in a mouse model of injury (Lämmermann et al., 2013). Chemokines are also very important for LTB4 chemotaxis, notably CXCL8, formerly known as IL-8. It has first been described as a neutrophil chemotactic agent by Marco Bagglioni *et al*. For this the authors used the boyden chemotactic assay, to show that neutrophil were chemotaxing towards a source of IL-8 (Baggiolini et al., 1989). More recently, it was shown in a model of zebrafish larval wound that H_2O_2 was a potent chemoattractant of neutrophils, recruiting those cells to the site of injury (Niethammer et al., 2009). Neutrophils typically respond to an injury within minutes but are not always able to resolve the injury and as a result are subsequently helped by other leukocytes.

1.3.2 Neutrophil ontogeny

In adult human, neutrophils are produced in sinuses of the bone marrow. The process regulating stem cell maturation into neutrophil is referred to as granulopoiesis. Neutrophils and macrophages differentiate from a common progenitor. A pluripotent progenitor stem cell

differentiates into this granulocyte/macrophage progenitor. The principal molecule regulating granulopoiesis is G-CSF (granulocyte colony stimulating factor). G-CSF is essential for the development of neutrophil and its depletion results in neutropenia (Lieschke et al., 1994; Liu et al., 1996). GM-CSF, CXCL3, CXCL6 also stimulate granulopoiesis *in vivo* (Metcalf et al., 1986, 1987; Pojda and Tsuboi, 1990). After differentiation, a subset of differentiated neutrophils will form a reserve in the bone marrow while the others will be disseminated in the circulation and peripheric tissues.

1.3.3 Neutrophil role and response to pathogen

At the forefront of inflammation, neutrophils often encounter pathogens. They have three different methods to directly tackle pathogens: phagocytosis, degranulation and neutrophil extracellular trap. As a result, they express and secrete pro-inflammatory cytokines which in turn amplify the inflammation by recruiting other leukocytes.

A. Phagocytosis

Neutrophils can clear bacterial infections through phagocytosis. This process involves recognising and internalising pathogens coated with opsonins. These opsonin molecules are promoting phagocytosis by binding specific receptors on the surface of neutrophils. Such molecules include antibody and complement fragments (Owens and Peppas, 2006). Once internalised, pathogens are digested in lysosome/phagosomes containing hydrolytic enzymes and reactive oxygen species. Reactive oxygen species are generated during a so-called ‘respiratory burst’ involving the activation of the enzyme NADPH oxidase which produces superoxide, a reactive oxygen species. During this process, enzymes known as superoxide dismutases break down superoxide immediately into hydrogen peroxide which is further converted to hypochlorous acid (HClO) by the enzyme myeloperoxidase. HCl is mainly responsible for the anti-bacterial property of neutrophils. It has also been shown that neutrophils can phagocytose parasites such as *Plasmodium spp*, the pathogen responsible for malaria (Aitken et al., 2018).

B. Degranulation

Neutrophils can also release a range of molecules to fight pathogens through degranulation. Neutrophils contain four types of granules, secretory vesicles, azurophilic, specific or gelatinase granules. These granules are packed with antimicrobial molecules and can fuse

with phagocytic vesicles (Table 1). During degranulation, secretory vesicles have the highest propensity for extracellular release. This was shown after *in vitro* stimulation of neutrophils with fMLP (Sengeløv et al., 1993). In this case almost all secretory vesicles were discharged. With stronger stimulation using phorbol myristate acetate it was showed that the other types of granules were also released (Faurschou et al., 2002). The granules with the strongest anti-inflammatory content are the azurophilic granules and the specific granules. The content of these granules include molecules such as the small antimicrobial protein defensin (Barrera et al., 2012), the myeloperoxidase, an enzyme producing hypohalous acid that carry antimicrobial activity (Klebanoff, 2005), the lysozyme, an enzyme that can attack bacterial wall (Primo et al., 2018) and BPI (bactericidal/permeability-increasing protein), a protein with antibiotic properties (Elsbach, 1998). The release of the azurophilic and specific granules upon neutrophil stimulation contributes to the defence against pathogens. It was notably shown in vitro with rabbit neutrophils stimulated with *Salmonella typhimurium* that upon encountering this pathogen the specific and azurophilic granules were released (Joiner et al., 1989). The gelatinase granules contain enzymes that might play a role in the degradation of the basement membrane during neutrophil extravasation (Delclaux et al., 1996). Finally the role of secretory vesicles is not clear yet but it is hypothesised that those granules might contribute to neutrophil extravasation (Borregaard et al., 1994).

Granule	Molecules
Secretory	Leukolysin, Alkaline Phosphatase.
azurophilic	Myeloperoxidase, bactericidal/permeability-increasing protein (BPI), defensins, and the serine proteases neutrophil elastase and cathepsin G.
specific	Alkaline phosphatase, lysozyme, NADPH oxidase, collagenase, lactoferrin, histaminase and cathelicidin.
Gelatinase	Cathepsin, gelatinase and collagenase.

Table 1.1. List of neutrophil granules and their molecular content.

C. Neutrophil extracellular trap

NET (Neutrophil extracellular trap) is an antibacterial feature of neutrophil. Brinkmann and his colleagues discovered this phenomenon in 2004 and described it as: “[the] release [of] granule proteins and chromatin that together form extracellular fibres that bind Gram-positive and -negative bacteria” (Brinkmann et al., 2004). In this article, the authors analysed samples of Rabbit infected with *Shigella flexneri* and human appendicitis. They used a combination of immunofluorescence staining and electron microscopy to characterise the nature of NETs. They found that NET was composed of DNA, histones and antimicrobial azurophilic granules. The release of this content by neutrophil constitute an early event in cell death. They confirmed

the antimicrobial capacity of NET *in vitro* by observing human neutrophils forming NET to degrade *Salmonella typhimurium* and *Shigella flexneri.* NETs were also found to have antifungal activity against *Candida albicans*, *in vitro* and *in vivo* in a mouse model of fungal infection.

1.3.4 Neutrophil role and response to injury

With their wide range of microbicidal actions, neutrophils main role is to clear the wound from opportunistic pathogens. Their range of actions, however, is not limited to antimicrobial defence and neutrophils are known to have significant physiological relevance in the response to injury.

A. Detrimental role of the neutrophil in the response to injury

It is still largely debated whether neutrophils have a physiologically beneficial or detrimental role during wound repair. Neutrophils play an important role during inflammation by releasing pro-inflammatory molecules leading to the activation and recruitment of leukocytes but they might also delay wound healing and further damage the wounded site. A study in diabetic mice notably reported that neutrophil depletion accelerated sterile wound healing (Dovi et al., 2003). In this study, the authors used rabbit anti-neutrophil serum to deplete the neutrophils in mice. The authors suggest that proteases released by neutrophils could delay wound healing. Such proteases including elastase and proteinase 3 that can cleave a variety of ECM protein and subsequently affect function such as migration and proliferation. Supporting this hypothesis for a detrimental role of neutrophils in wound repair, another study using human skin and human neutrophils, *in vitro*, showed that neutrophil proteases and notably the elastase could degrade the epidermal-dermal junction and therefore inhibit the re-epithelialisation of the tissue (Briggaman et al., 1984). In another study*, in vitro*, TNFα-pretreated neutrophils caused the detachment of cultured keratinocytes (cells that constitute 90% of the epidermis) from the substratum composed of collagen (Katayama et al., 1994). Another protease that is released by neutrophils, MMP-9 (Matrix MetalloProteinase-9) can alter the ECM. Interestingly, this protease has been shown to alters the structure of collagen basement membrane and delay wound re-epithelialisation in a murine wound model injected with MMP-9 (Reiss et al., 2010). Neutrophils could therefore play a detrimental role in wound healing by inhibiting the process of re-epithelialisation of the injured tissue.

B. Beneficial role of the neutrophil in the response to injury

It is to be noted that although in some conditions neutrophils might have a detrimental role in wound healing, they are still essential to protect the organism against external pathogen. Notably, neutropenic patients have a higher risk of mortality from infection (Newburger and Dale, 2013).

In recent years there has been an increase in studies that point towards a positive role for neutrophils in wound healing. A study in mice notably reported that CXCR2 (CXCL8 receptor which is essential for the recruitment of neutrophil to wounds) deficiency led to delayed re-epithelialisation (Devalaraja et al., 2000). In this study CXCR2 knock out led to defective neutrophil recruitment to the site of injury. The authors noted: "the remarkable lack of neutrophil recruitment in CXCR2 knockout mice remains the hallmark of this initial wound healing" and suggested that the defective neutrophil recruitment was most likely responsible for the delay in wound healing.

An important process in wound healing is the re-vascularisation of the wounded tissue. Neutrophils are known to secrete VEGF (Vascular Endothelial Growth Factor) and to play a role in angiogenesis (Gargett et al., 2001; Heryanto et al., 2004), which would be beneficial for tissue healing.

Neutrophils and macrophages can also secrete anti-inflammatory and pro-resolution molecules to help the return to homeostasis that has been perturbed by the injury (Lämmermann et al., 2013; Tauber, 2003). A recent study described the first neutrophils reaching the wound as **N1** (pro-inflammatory) **neutrophils** expressing IL-1 and TNFα (Tumor Necrosis Factor alpha) and also described a different type of neutrophil subsequently arising, the **N2** (anti-inflammatory) **neutrophils** secreting IL-10 (Ma et al., 2016) like anti-inflammatory macrophages. This was shown using flow cytometry analysis of neutrophils isolated from the left ventricle of a mouse following myocardial infarction. This anti-inflammatory type of neutrophil is accelerating the resolution of the inflammatory response by releasing anti-inflammatory molecules that block pathways such as NF-κB signalling that is involve in neutrophil activation and production of cytokines. Another study by Wang *et al* also described the restoring role of neutrophils using a mouse model of sterile injury. In this case, using intravital microscopy, the authors observed neutrophils dismantling injured vessels and creating channels for new vascular regrowth (Wang et al., 2017a).

As shown in different studies of neutrophils *in vitro* or in models of sterile injury in diabetic mice *in vivo*, neutrophils might have an aggravating role by delaying wound healing (Dovi et al., 2003). However, it has been shown in another study that the presence of neutrophils might be important for wound healing (Devalaraja et al., 2000). Both cases are different as, in the first

study the authors performed a 2cm incisional wound on diabetic mice depleted for neutrophils whereas in the second study, the authors performed a 3mm excisional punch on mice knocked out for CXCL2, which is important for the recruitment of neutrophils to the site of injury but which might also play a role for keratinocytes (which are important for wound repair). The role of neutrophil in wound repair might therefore be detrimental or beneficial depending on the type of wound or the specific context of those injuries.

1.3.5 Neutrophil disorders

As neutrophils are an important arm of the inflammatory response, neutrophil disorders are a concern, especially in children in which they are a notable cause of mortality (Lakshman and Finn, 2001). Such disorders result in a reduced (neutropenia) or increased (neutrophilia) number of neutrophils in the blood or in defective neutrophil function. People affected by neutropenia are more likely to contract bacterial infections which can lead to death in the case of neutropenic sepsis (Hughes et al., 2002) confirming the crucial role of neutrophils to fight bacterial infections. Neutropenia is either congenital or acquired. Congenital neutropenia is mainly due to mutations in the gene *ela2* which is encoding for elastase (Horwitz et al., 2013). Mutations result in the misfolding of elastase and lead to endoplasmic reticulum stress and neutrophil apoptosis (Köllner et al., 2006). Acquired neutropenia is due to antibodies that target neutrophil's antigens (Schwartzberg, 2006).

Neutrophilia is often observed in response to an infection or chronic inflammation (Stockley et al., 2013). In this case, neutrophilia is caused by the overproduction of inflammatory signals that trigger the proliferation of hematopoietic stem and multipotential progenitor. Another cause of neutrophilia is LAD (Leukocyte Adhesion Deficiency). The hallmark of LAD-induced neutrophilia is mutations on sLe^{X}, a gene coding for the selectin ligand (important for extravasation) on leukocytes (Phillips et al., 1995). As a result of this mutation neutrophils cannot exit the blood circulation and accumulate in the organism.

Other disorders linked to neutrophil function are the cause of severe chronic infections confirming the important physiological role of neutrophils (Table 2). Such disorders include MPO (myeloperoxidase) deficiency, which results in major systemic infections in patients due to neutrophils impaired killing properties (Parry et al., 1981). MPO is an enzyme that produces antibacterial products such as hypochlorous acid during the respiratory burst (the release of antimicrobial reactive oxygen species by neutrophils) (Hampton et al., 1998). Other defect such as chronic granulomatous disease, Severe (glucose-6-phosphate dehydrogenase) deficiency and Glutathione synthetase deficiency also affect the respiratory burst and

therefore, the capacity of neutrophils to kill pathogens (Al-Jishi et al., 1999; Cappellini and Fiorelli, 2008; The International Chronic Granulomatous Disease Cooperative Study Group, 1991). A further understanding of neutrophil biology could reveal important insights to fight those diseases.

Neutrophil disorder	Symptoms	Citation
SCN (Severe Congenital Neutropenia)	Neutropenia, G-CSF mutations	(Kostman, 1975)
Reticular dysgenesis	Neutropenia, defect in the development of haematopoietic stem cells	(Alonso et al., 1972)
Cyclical neutropenia	Neutropenia	(Chusid et al., 1986)
Antibody mediated neutropenia	Neutropenia	(Jonsson and Buchanan, 1991)
LAD	Neutrophilia, non-functioning neutrophils	(Marlin et al., 1986)
MPO (Myeloperoxidase) deficiency	Defective MPO	(Parry et al., 1981)
chronic granulomatous disease	Defective reactive oxygen species	(The International Chronic Granulomatous Disease Cooperative Study Group, 1991)
Severe (glucose-6-phosphate dehydrogenase) deficiency	Reduced or absent respiratory burst	(Cappellini and Fiorelli, 2008)
Glutathione synthetase deficiency	Reduced or absent respiratory burst	(Al-Jishi et al., 1999)
Chediak Higasi syndrome	Degranulation disorder	(Barrat et al., 1996)

Table 1.2. Neutrophil disorders.

1.4. Neutrophil migration

1.4.1 Introduction

A very important part of the inflammation response is the infiltration of cells and their migration to target sites of injury. Neutrophils are mostly found in the circulation and must go through TEM (TransEndothelial Migration) to reach the newly injured tissue. TEM is crucial for neutrophil's action; people affected by LAD due to a mutation on the gene coding for selectin ligand have impaired neutrophil infiltration and are therefore more susceptible to infection (Phillips et al., 1995). Neutrophil recruitment from the blood circulation into the inflamed tissue relies on a multistep mechanism. As for other leukocytes, the first step of extravasation requires an initial selectin-mediated rolling on the epithelial cells composing the cell wall of the

blood vessel. This Is followed by neutrophils' activation by chemokines such as CXCL8, and other chemoattractants including complement C5a, leukotriene LTB4, platelet activating factor and bacteria-derived formylated peptides (Choi et al., 2009). The chemokine activation of neutrophils induces integrin dependent firm adhesion which leads to subsequent transendothelial migration (Rot and von Andrian, 2004). After crossing the epithelial cells composing the blood vessel wall, neutrophils cross the basement membrane through a mechanism that is unclear but that might involve the release of gelatinase granules and secretory vesicle to ease neutrophil's crossing by remodelling the ECM of the basement membrane (Reiss et al., 2010).

After that, neutrophils reach the interstitial tissue. Once in the complex 3D environment of the interstitial tissue, neutrophils follow cues of various chemoattractants towards sites of inflammation and get activated by PAMPs and DAMPs. Upon activation, neutrophils produce chemoattractants including chemokines such as CXCL8 and pro-inflammatory lipid such as LTB4 (Silva, 2010). This leads to the recruitment of further leukocytes to the inflammation. In order to move in the intricate interstitial tissues more efficiently, neutrophils are able to squeeze their nucleus and propel themselves in the tissue, using an amoeboid mode of migration and reaching a speed of up to 20µm/s (Lämmermann et al., 2008; Salvermoser et al., 2018).

At the end of inflammation, neutrophils play a role in the resolution of the migration by secreting pro-resolution anti-inflammatory molecules including lipoxins (Serhan et al., 2014) and either reverse migrate to the circulation (Nourshargh et al., 2016) or die from apoptosis. In the latter case, they end up being cleared from the inflammation by efferocytosis performed by macrophages (Greenlee-Wacker, 2016).

1.4.2 Neutrophil Swarming

A. Swarming definition

In interstitial tissues, neutrophils can exert a highly coordinated behaviour during their migration to a target site in response to an injury or an infection; migrating in waves or continuously migrating. Once at the target site of injury, neutrophils stop and aggregate forming tight cell clusters at a very precise focus site (Fig. 1.4). This specific type of behaviour is referred to as neutrophil swarming, a term coined by Chtanova and colleagues working in Ellen A Robey's lab in 2008 upon observation of the neutrophil behaviour in response to Toxoplasma *gondii* in mouse lymph node using two-photon laser imaging *in vivo* (Chtanova et al., 2008). Swarming is a collective behaviour that is exhibited by various entities such as bacteria, plant, fish and birds (Bouffanais, 2015). The swarming mode of migration carries

many benefits, such as better aerodynamic for fish and birds' migration, protection from predators and better resource foraging.

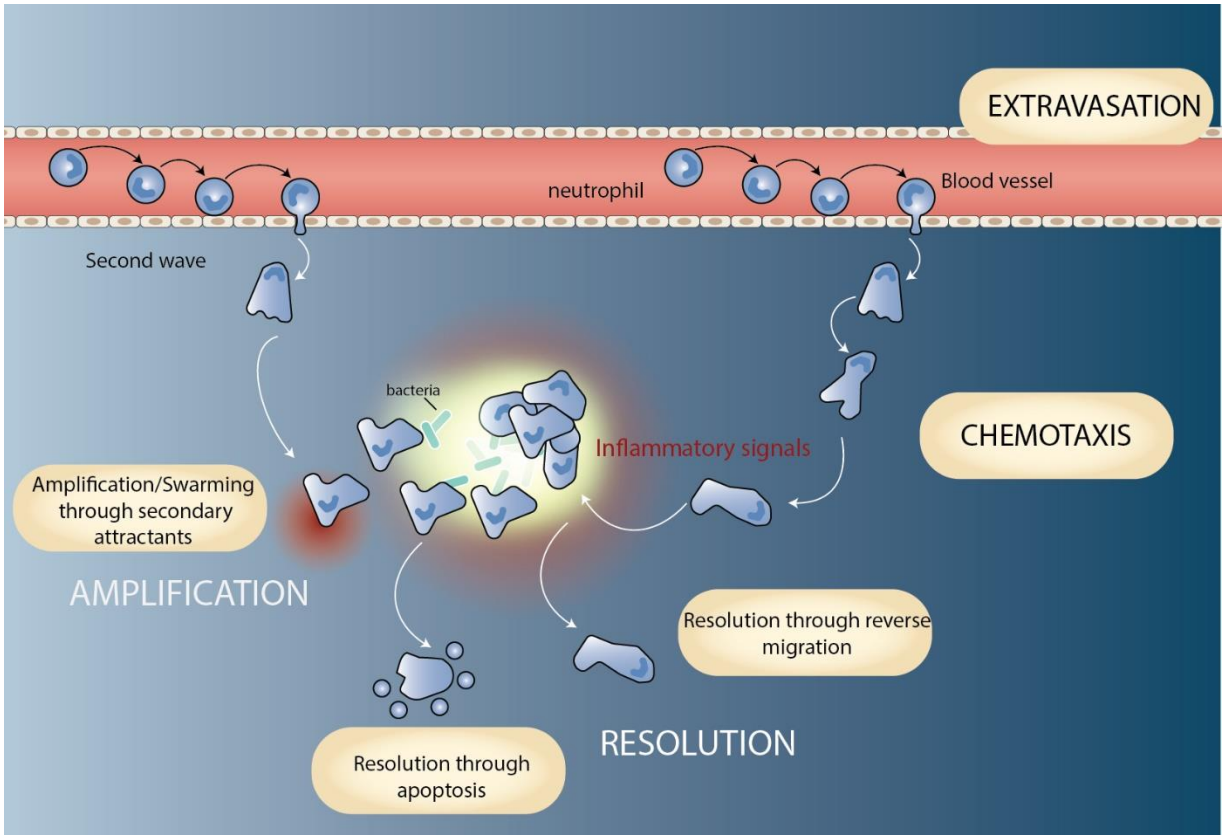


Figure 1.4. Mechanism of neutrophil swarming *in vivo*.

Schematic describing the mechanism of neutrophil swarming *in vivo*. Neutrophils reside in the blood and are reaching the tissue through extravasation. In the interstitial tissue, neutrophil follow cues of inflammatory signals (chemotaxis) and start aggregating to the site of inflammation (red gradient). Neutrophils also amplify the signal by producing LTB4. This leads to the recruitment of further neutrophils. The inflammatory response is resolved through neutrophil apoptosis or reverse migration.

B. Swarming in neutrophils

Other the past 10 years, researchers have been actively investigating the cellular and molecular mechanisms leading to neutrophil swarming.

Based on a study on human neutrophils *in vitro* it is believed that fMLP might be one of the primary attractants leading to neutrophil migration and activation triggering the subsequent release of attractants from neutrophils leading to an amplification of pro-inflammatory signalling (Dahinden et al., 1988). More specifically, it was later shown in isolated human and mouse neutrophils *in vitro*, that neutrophils stimulated with fMLP are in turn producing LTB4 (Afonso et al., 2012). In this study, mouse neutrophils knocked out for fMLP receptors FPR1 were still observed to migrate directionally towards the source of fMLP by following the cues

of LTB4 produced by WT neutrophils. This led to the conclusion that during their migration towards sites of inflammation, neutrophils can self-amplify their migration via LTB4.

In 2013 Lämmermann *et al* developed a laser induced wound assay in mouse, targeting tissue in the ear to specifically trigger swarms. This is the first major study investigating the mechanisms underlying neutrophil swarming *in vivo* (Lämmermann et al., 2013). In order to have a swarm of broad amplitude with many neutrophils recruited at the site of inflammation, the authors applied mechanical pressure on the mouse ears prior to the laser wound injury to stimulate neutrophil infiltration in this tissue. Interestingly, it was later confirmed in another study using a similar model that a threshold number of 10 neutrophils was required to reach the site of injury in order to trigger neutrophil swarms (Park et al., 2018). LTB4 is a very potent attractant of neutrophils and it was not only shown to trigger neutrophil's attraction *in vitro* but it was also shown to trigger neutrophil accumulation and aggregation at sites of inflammation *in vivo*. It was notably shown that knock out of LTB4 receptor BLT1 in a mouse model of inflammatory arthritis was reducing the overall number of neutrophils accumulating at sites of inflammation (Peters-Golden and Brock, 2003). Based on this knowledge, Lämmermann *et al* studied the impact of LTB4 in swarms also using mice knocked out for BLT1 and demonstrated a major role for LTB4 in swarms. They notably analysed the behaviour of WT and BLT1 KO neutrophils co-injected in a mouse ear. For this, they analysed the directionality of neutrophils and their speed towards the site of injury. BLT1 KO neutrophils had a very low chemotactic index (the measure of neutrophil directionality towards the wound) and a low velocity and were not observed aggregating like WT neutrophils. During swarms, cells accumulate and stop at the cluster. To determine whether integrins were involved in neutrophil swarming, the authors used mice knocked out for Talin, a molecule crucial for integrins activation (Calderwood and Ginsberg, 2003). Using their laser wound assay, they observed that Talin KO neutrophils could not stop and cluster at the site of inflammation and concluded that integrins were required for neutrophils to access the wound they generated. The requirement of integrins for reaching the injury might be because collagen fibres are destroyed by the laser wound (as they observed with second harmonic imaging).

C. Neutrophil swarming current hypothetical model

Although it is now known that LTB4 is a major determinant of neutrophil swarming, the LTB4 signalling dynamics leading to the formation of neutrophil swarms *in vivo* are still unknown. The current hypothesis postulates that a signal relay of LTB4 is taking place while neutrophils migrate towards the site of inflammation (Fig. 1.5). This is based on the observation of isolated human and mouse neutrophils migrating towards a gradient source of fMLP *in vitro* (Afonso et

al., 2012). In this study, the authors demonstrated that LTB4 produced by neutrophils enhanced their migration towards the source of fMLP by chemically and genetically inhibiting LTB4 receptor, fMLP receptor and an enzyme important for LTB4 production (5-LO; 5-lipoxygenase). They notably established with an under-agarose migration assay that neutrophil migration was enhanced by LTB4 and that LTB4 produced by WT neutrophils could rescue the migration of cells knocked out for fMLP receptor. Conversely when cells knocked out for fMLP receptors were mixed with cells knocked out for 5-LO, their migration was not rescued. Based on these data the authors hypothesised that LTB4 was being relayed from neutrophil to neutrophil while they were migrating along the fMLP gradient source, effectively forming a signal relay of LTB4. According to this hypothesis, individual neutrophils would need to secrete LTB4 in a polarised fashion towards the rear of the cell in order to reach out to neutrophils located further away. Interestingly, using human neutrophils, *in vitro*, Majumdar *et al* observed with a combination of fluorescence microscopy and electron microscopy that, enzymes involved in the production of LTB4 were located towards the rear of neutrophils migrating towards a source fMLP (Majumdar et al., 2016). Additionally, the authors observed the secretion of exosomes packed with LTB4 producing enzymes and LTB4 itself at the trailing edge of the neutrophils. Based on these evidences it has been suggested that the production of LTB4 by neutrophils is polarised.

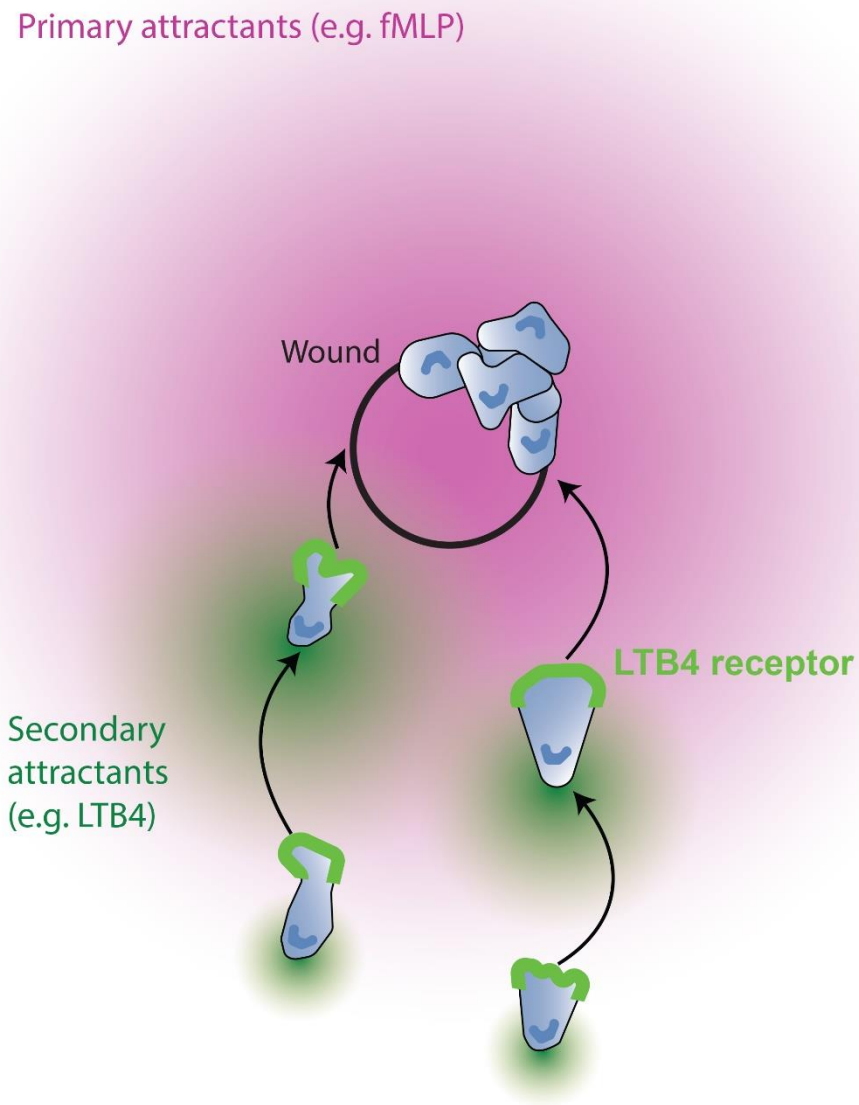


Figure 1.5. Current model for neutrophil swarming dynamic.

Schematic representation of the current model for neutrophil swarming. Neutrophils are migrating towards the site of injury by chemotaxing along a primary attractant gradient (in pink) stemming from the wound. LTB4 (in green) production and sensing is polarised, respectively towards the leading edge and the back of the neutrophils migrating towards the site of injury.

It has also been suggested that necrotaxis (the migration of neutrophils towards molecules released by dead tissue) could play a role in neutrophil swarming. It has been observed in mouse after a laser wound injury that pioneer neutrophils reaching the wound were dying from apoptosis and that this was associated with further neutrophil recruitment (Lämmermann et al., 2013; Uderhardt et al., 2019).

Neutrophil swarming appears to be evolutionarily conserved and has been observed in zebrafish larvae at infection loci (e.g. *Pseudomonas aeruginosa* (Deng et al., 2013) and *Streptococcus iniae* (Harvie et al., 2013)). Although neutrophil swarming has never been observed in humans, human neutrophils transplanted into a mouse ear has been observed to be swarming alongside mouse neutrophils upon laser injury (Lämmermann et al., 2013).
It remains unclear how LTB4 controls neutrophil swarming, but the dynamics of its production are likely to be a major determinant. As explained above, *in vitro* work with human neutrophils suggests that neutrophils produce LTB4 as they migrate towards fMLP, amplifying the range of recruitment ('signal relay' model) (Afonso et al., 2012). Such autocrine/paracrine control of population behaviour has been observed in other organisms. During *Dictyostelium discoideum* development, periodic production of cAMP triggers chemotactic aggregation of individual amoebae (Das et al., 2011; Goldbeter, 2006). Rather than forming stable, spatial gradients (Sarris et al., 2012; Weber et al., 2013), the attractant is produced in a coordinated wave-like fashion. Dictyostelium can interpret such signalling waves through 'temporal sensing' (Skoge et al., 2014). Another related phenomenon, 'quorum sensing', is observed in bacteria which sense changes in population density by the accumulation of an autocrine signal and respond to concentration thresholds by altering gene expression (Waters and Bassler, 2005). One of the possible quorum sensing phenotypes is bacteria swarming. Swarming motility in bacteria often requires the production from bacteria of their own biosurfactants (compounds lowering surface tension) such a glycolipid and or lipopeptide. Biosurfactants gradients are formed by leading swarming bacteria and act as wetting agent by reducing tensions leading the migrating pack of bacteria in the same direction.

1.4.3 LTB4 production and pathway

A. Biosynthesis of LTB4

LTB4 (Leukotriene B4) is a small lipid that act as a proinflammatory signal for neutrophil. LTB4 is required for neutrophil swarming (Lämmermann et al., 2013). LTB4 is the product of AA (Arachidonic Acid) metabolism which takes place at the nuclear membrane (Fig. 1.6). PLA2 (Phospholipase A2) metabolise AA after calcium-dependent translocation from the nuclear lumen to the nuclear membrane. This was shown by using membrane vesicle made from immortalised COS-1 and CHO cell lines transfected with a recombinant PLA2 protein and by submitting the vesicles to an increasing calcium concentration (Clark et al., 1991). The following step in the synthesis of LTB4 also occurs at the nuclear envelope membrane (Luo et al., 2003). This was demonstrated in NIH 3T3 cells using a fluorescent reporter and mutant

for 5-LO (5-lipoxygenase), an enzyme known to be important for the production of LTB4 (Funk, 2001). The authors notably showed that following a rise of intracellular calcium concentration triggered by a calcium ionophore 5-LO translocate from the nuclear lumen to the nuclear membrane. The authors also tracked AA by fluorescently labelling it and showed that it strongly colocalised with the activated 5-LO. 5-LO converts AA into LTA4 and might require the help of FLAP (5-LO activating protein), an integral nuclear membrane protein (Peters-Golden and Brock, 2003). It is assumed that LTA4 diffuse from the nucleus to the cytoplasm. LTA4 is then further metabolised into LTB4 by LTA4 hydrolase (Brock et al., 2001; Iversen et al., 1994). It was shown in human neutrophils by performing immunostaining and analysing the cellular content after fractionation that LTA4 hydrolyse is located in the cytoplasm (Brock et al., 2001).

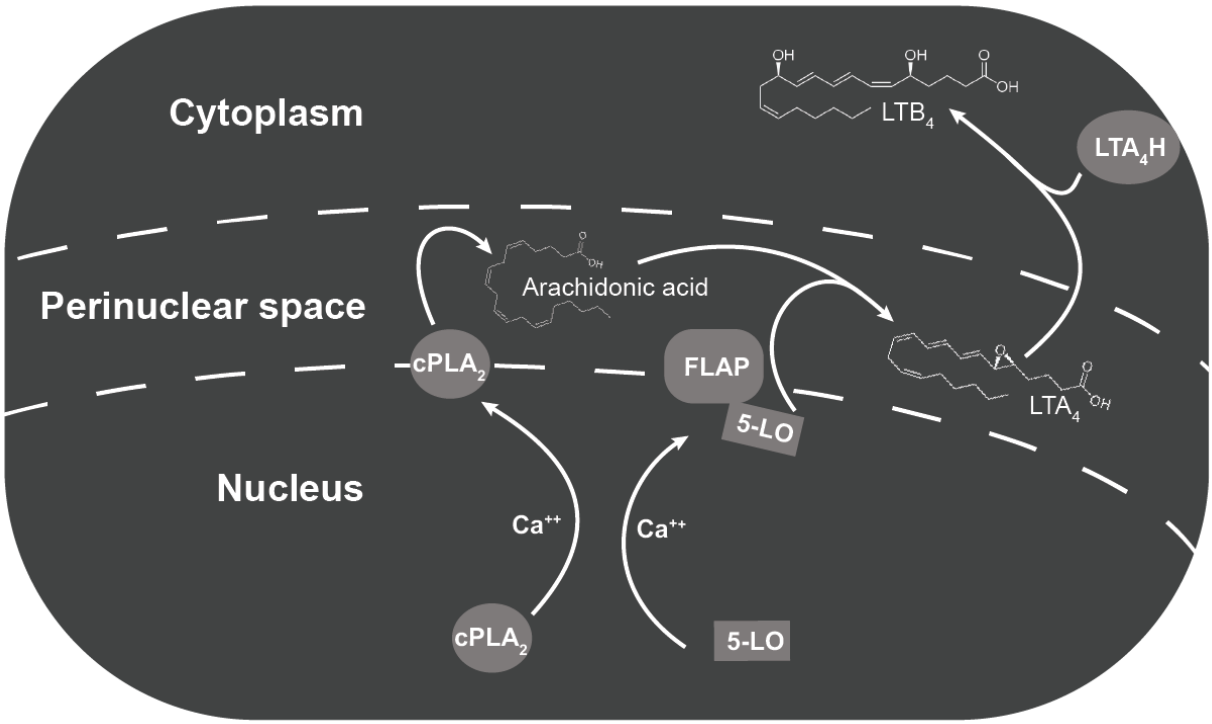


Figure 1.6. LTB4 biosynthesis.

Schematic representation of LTB4 biosynthesis. 5-LO (5-Lypoxygenase) and cPLA2 (Phospholipase A 2) are translocated from the nucleus lumen to the inner membrane of the nucleus following an intracellular calcium elevation. At the nuclear membrane, phospholipase A 2 generates arachidonic acid which is turned into LTA4 by 5-LO which is stabilised by FLAP (Five-Lypoxygenase Associated Protein). LTB4 is produced from LTA4 by LTA4H in the cytoplasm.

Other lipid molecules can be produced as a result of the processing of AA by 5-LO, notably lipoxin A4 and lipoxin B4. Lipoxin A4 can directly be synthesised from LTA4 by either 12- or 15-lipoxygenase as it has been shown in isolated trout macrophages (Pettitt et al., 1991). Those lipoxins are specialised resolving molecules that have anti-inflammatory properties

leading to the resolution of the inflammation (Qu et al., 2015). Aside from neutrophils, LTB4 can also attract eosinophils, differentiated T cells, and some subsets of macrophages and dendritic cells (Peters-Golden and Brock, 2003).

B. LTB4 receptors

LTB4 signals through two GPCR receptors in humans, BLT1 (high affinity) and BLT2 (low affinity) (Peters-Golden and Brock, 2003). These receptors were first identified by Yokomizo *et al* in 1997 in HL-60 cells, a human neutrophilic leukaemia cell line in which LTB4 binding activity is increased during differentiation by retinoic acid. The authors isolated two cDNA that had homology for chemokines receptors. They demonstrated that they were indeed LTB4 receptors by expressing them in CHO cells lacking endogenous for expression for LTB4 receptor. After stimulation with LTB4, the authors observed an increase in intracellular calcium, D-myo-inositol-1,4,5-triphosphate accumulation, and inhibition of adenylyl cyclase, characteristic of LTB4 downstream signalling in these CHO cells expressing exogenous LBT4 receptors (Yokomizo et al., 1997). BLT1 is expressed in leukocytes and BLT2 is expressed in a variety of cell types including epithelial cells. Multiple *in vivo* studies in mice model demonstrate a major role for BLT1 in inflammatory diseases. BLT1 knocked out mice were notably used to show the major role of this receptor for neutrophil accumulation during acute ear inflammation (Haribabu et al., 2000) or its requirement in the auto-immune condition of allergen-induced airway hyperresponsiveness (Terawaki et al., 2005) or its role for recruiting neutrophils in the auto-immune disease arthritis (Peters-Golden and Brock, 2003).

Chemoattractants		Receptors		Citation
Human	**Zebrafish**	**Human**	**Zebrafish**	
Chemokines				
CXCL1	CXCL1	CXCR2	CXCR2	(Powell et al., 2017)
CXCL2	CXCL2	CXCR2	CXCR2	(Powell et al., 2017)
CXCL8	CXCL8a/CXCL8b	CXCR1/CXCR2	CXCR1/CXCR2	(Oehlers et al., 2010)
CCL7	CCL38.1	n/a	n/a	(David et al., 2002)
CCL9	CXCL11.1	CXCR3	CXCR3.2	(Torraca et al., 2015)
CXCL12	CXCL12a/CXCL12b	CXCR4	CXCR4b	(David et al., 2002)
n/a	CXCL18b	n/a	CXCR2	(Torraca et al., 2017)
Peptides/Cytokines				
C5a	C5a	C5aR	C5aR1	(Natarajan Niranjana et al., 2018)
C3a	C3a	C3aR	C3aR1	(Carmona-Fontaine et al., 2011)
Formylated peptides (e.g. fMLP)	Formylated peptides	FPR1	FPR1	(Yang et al., 2012)
Eicosanoids				
LTB4	LTB4	BLT1	BLT1a/Blt1b	(Okuno et al., 2015)
Other				
H_2O_2	H_2O_2	Lyn	Lyn	(Yoo et al., 2011)

Table 1.3. List of functional and verified chemoattractants and receptors in zebrafish neutrophils and their human ortholog

1.5. The zebrafish model

1.5.1 The zebrafish model in development and function of the immune system.

A. Introduction

To study neutrophil swarming, I used the zebrafish (*Danio rerio*) larva model. Zebrafish are teleost, freshwater fish native of south Asia. The use of zebrafish in science was pioneered by Charles W. Creaser at Wayne State University who suggested that this fish would be suitable to study embryology (Creaser, 1934). Forty years later, George Streisinger successfully cloned homozygous zebrafish (Streisinger et al., 1981), pioneering the use of this vertebrate animal model in developmental biology. Zebrafish started being widely used because the quick embryonic developmental period and the transparency of the embryos. Indeed, when kept in PTU (1-phenyl-2-thiourea), zebrafish larvae do not develop pigments and remain transparent allowing non-invasive imaging to be performed. In 2001, the Sanger Institute started the sequencing project of the genome of the Tübingen strain of zebrafish subsequently revealing that “71.4% of human genes have at least one zebrafish orthologue”(Howe et al., 2013). The

larvae I used were between 3- and 4-days post fertilisation. The first leukocytes present in zebrafish are macrophages from the so-called “primitive wave” lineage arising 24 hours post fertilisation from the yolk sac (Lichanska and Hume, 2000). Those cells then quickly spread throughout the mesenchyme of the embryo (Yang et al., 2012). Neutrophils arise after 32hours and are abundant in the caudal hematopoietic tissue (CHT) which is, at 48hpf, the site of haematopoiesis. A wide array of tools has been developed to study immunity, infection and inflammation in this model.

B. Zebrafish neutrophils

Zebrafish neutrophils share morphological, biochemical and functional features with mammalian neutrophils. The larval zebrafish neutrophils have first been documented using electron microscopy (Lieschke et al., 2001). In this study, the authors observed that larval zebrafish possess a polymorphic nucleus, granules and myeloid peroxidase (mpx) like their human counterpart. Zebrafish neutrophils also have similar antimicrobial properties than their human counterpart. They were notably observed phagocytosing *Escherichia coli* bacteria, using live imaging microscopy (Colucci-Guyon et al., 2011). It was shown in whole kidney from adult zebrafish that upon calcium ionophore stimulation, NETs and granules are released from zebrafish neutrophils (Palić et al., 2007). More recently, it was also shown that NETs were released in larval neutrophils reaching a fin injury (Isles et al., 2019). Zebrafish neutrophils are usually tracked using either Sudan black staining that stains their azurophilic granules (Peters-Golden and Brock, 2003) or by expressing fluorescent reporter probes under the control of lysozyme C (Peters-Golden and Brock, 2003) or myeloperoxidase (Elks et al., 2011), two neutrophil specific reporters. Similar to human neutrophils, zebrafish neutrophils chemotax along CXCL8 gradients *in vivo*, as observed with live imaging of neutrophils accumulating around transplanted cells expressing CXCL8 in 3dpf larvae (Sarris et al., 2012). Zebrafish neutrophils also recognize other classical cues such as LTB4. It was notably shown that LTB4 induces neutrophil motility in zebrafish larva incubated in the bath of LTB4 (Deng et al., 2013). It has been found that zebrafish genome codes for the highest number of chemokines found in all vertebrate species so far with 100 chemokines being present in zebrafish (Nomiyama et al., 2008). Zebrafish neutrophils recognise chemoattractants that are similar to those detected by their human counterpart (Table 3). Studying neutrophils in larval zebrafish allows insight into innate immune cell functions independently of the adaptive immune system which develop later (Davis et al., 2002).

C. Zebrafish neutrophil ontogeny

In zebrafish embryos, the first leukocytes arising from the yolk sac are the primitive myeloid cells. These morphologically homogeneous hematopoietic progenitors of the primitive macrophages give rise to macrophages and neutrophils in similar numbers (Yang et al., 2012). With the advent of blood circulation at 26hpf, some of the primitive myeloid cells from the yolk sac are taken away by the blood flow (Yang et al., 2012). Using a cell tracer, it was shown that many of these cells stop in the caudal vein and the surrounding mesenchyme (Murayama et al., 2006). These cells then differentiate in macrophages in the tissue they colonise. Others start differentiating into neutrophils by 32 to 35hpf; this is one of the two origins of the larval neutrophils (Peters-Golden and Brock, 2003). From 33hpf onward, definitive hematopoietic stem cells born in the trunk seed the CHT in which they expand and differentiate. This leads to a steady larval granulopoiesis until at least 9dpf; this is the second origin of the larval neutrophils (Murayama et al., 2006). After 48hours post fertilization, neutrophils are the major leukocyte type found in the larva. At this stage they are mostly found in the trunk and in the tail (Willett et al., 1999). From 48 to 72hpf the population of neutrophils in the zebrafish larva is doubling. As for mammals, G-CSF is essential for zebrafish granulopoiesis throughout the life of the zebrafish, its depletion resulting in zebrafish neutropenia (Basheer et al., 2019).

1.5.2 Pathogen and wound studies using zebrafish larvae

Originally used as a model organism for developmental biology, the zebrafish larva model has subsequently been used to study haematopoiesis, inflammation (Renshaw et al., 2006) and various host-pathogen interaction. Stephen Renshaw *et al.* were the first to establish an inflammatory wound model in zebrafish performing caudal fin tail transection on 3-5 dpf larvae and observed neutrophil expressing GFP migrating towards the inflicted wound (Renshaw et al., 2006). Anna Huttenlocher's team also demonstrated the same year that neutrophil expressing GFP in 2dpf zebrafish larvae were chemotaxing to ventral fin wound (Yang et al., 2012). Using this model, Anna Huttenlocher's team was the first to show neutrophil reverse migration from the injury in 3dpf zebrafish larvae using transgenic zebrafish expressing the photoconvertible dye dendra2 in neutrophils (Peters-Golden and Brock, 2003). Key insights in early and late wound signalling were found using the zebrafish larva wound model, such as the role of H_2O_2 (Niethammer et al., 2009; Yoo et al., 2011) or the role of ROS (Reactive Oxygen Species) and vimentin (a cytoskeletal component) in orchestrating the formation of collagen during the scaring process (LeBert et al.). The number of articles published per year and accessible on PubMed with the words: "zebrafish+wound" rose from 5 or less per year in

2000 and before to consistently more than 50 per year after 2010 showing an increase of the use of this model organism to study wounds (Fig. 1.7, A).

Increasing interest in zebrafish infection model also arose during the 21st century consistently reaching more than 100 publications per year containing the key words “zebrafish+infection” after 2013 (Fig. 1.7, B). Zebrafish and mammals can be infected with similar pathogens with bacteria such as *Listeria monocytogenes* (Menudier et al., 1996)*, S. pyogenes* (Neely et al., 2002)*, Staphylococcus aureus* (Prajsnar et al., 2008) *or Pseudomonas aeruginosa* (Torraca and Mostowy, 2018). The most notable example of the use of the zebrafish model to study bacterial infection being *Mycobacterium marinum* in zebrafish which is closely related to M. tuberculosis infection in human. The outcome of this bacterial infection in both zebrafish larvae and humans is similar in many aspects, notably the formation of granulomas (Davis et al., 2002). The infection of zebrafish larvae can be instigated by adding bacteria in the medium (Valenzuela et al., 2018); by micro-injections (Benard et al., 2012); or by microgavage (Cocchiaro and Rawls, 2013). Zebrafish have also been widely used to study some of the most prominent human fungal infection such as *Candida albicans*, *Aspergillus fumigatus* or *Cryptococcus gattii* (Rosowski et al., 2018). More recently zebrafish larvae have been used to study human viral infection such as HSV (Herpes Simplex Virus) (Antoine et al., 2014) and even parasitic infection caused by trypanosoma (Akle et al., 2017).

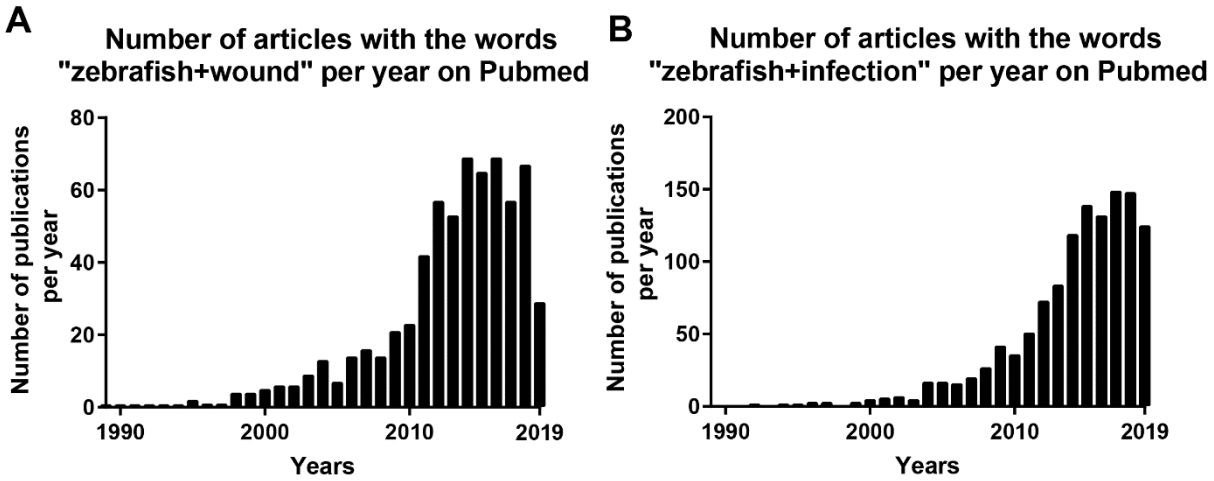


Figure 1.7. Increasing number of scientific articles on zebrafish infection and injury.

A. Number of scientific articles, per year, with the words “zebrafish + wound”. B. Number of scientific articles per year, with the words “zebrafish + infection”.

1.5.3 Imaging zebrafish larvae

A. Transparent embryos and larvae

Zebrafish embryos start developing pigments around 24 hours post fertilisation, however, several techniques have been used to keep the embryos and larvae completely transparent by inhibiting pigment formation. Zebrafish eggs can be incubated with 1-phenyl 2-thiourea (*PTU*), a tyrosinase inhibitor (tyrosinase catalyse the reaction of melanine) commonly used to inhibit melanogenesis (Millott and Lynn, 1966). Additionally, genetically modified lines have been generated in order to obtain transparent fish such as *casper* and more recently *crystal* (Antinucci and Hindges, 2016). There are three kinds of pigmented cell types in zebrafish (or chromophores): iridophores, melanophores and xantophores. The *crystal* mutant is a transgenic line carrying multiple knock out genes involved in the formation of melanophores and iridophores.

B. Microscopy imaging

Many techniques were developed over the last decades using the zebrafish larva model to study immunity. Notably transgenic lines expressing protein under the control of leukocyte specific promoters, wounds and infection assays and imaging techniques. Imaging cell motion using differential interference contrast (DIC) microscopy with polarised light to increase the contrast started in the 1980s (Allen et al., 1981). Video-enhanced differential interference contrast (VE-DIC) use in zebrafish was pioneered by the Herbomel's lab in the 1990s (Yang et al., 2012). The development of zebrafish in vivo imaging led to the identification of macrophages and neutrophil populations, their migration and the tissue they reside in. Using this type of microscopy, the leukocytes were differentiated based on their morphology. The zebrafish is a model that is genetically tractable. This is a crucial element for *in vivo* imaging that is facilitated by the use of fluorescent tags expressed under the control of neutrophil specific promoters lyz (lyzozome C) and mpx (myeloperoxidase). Time-lapse images generated using laser microscopy on confocal microscope or more recently two photon microscopy and light sheet imaging, give detailed videos of leukocytes migration. With software such as *Imaris* and *Fiji*, cells can also be automatically tracked, their fluorescent intensity, speed, directionality and other mathematical measurements and data can be extracted for further analysis.

C. Confocal laser scanning microscope

Marvin Minsky created confocal microscopy in 1957 (Minsky, 1988). Confocal microscopy uses point illumination to excite the sample and a pinhole in the same optical configuration plane, hence the name confocal (having a common focus). Standard laser confocal microscopes use mirrors controlling the laser in X and Y direction for scanning the sample. When the sample is fluorescent, part of the light will pass back in the objective lens, travelling through the same path as the laser and towards the detector. The high resolution three-dimensional images obtained with confocal microscopy led to an improvement of our understanding of the cellular structure (Wallace et al., 2001). Indeed, with confocal microscope, 3D images such as the reconstruction of the cytoskeletal dynamics were obtained by using genetically modified cells expressing fluorescent tagged protein (Gerisch and Müller-Taubenberger, 2003). This imaging technique has been used in zebrafish but is now used mostly on fixed samples (Walters et al., 2009) or low time resolution videos. Other types of microscopy are more suited for the time resolution needed for imaging leukocyte dynamics (see below).

D. Spinning-disk microscopy

Improvements have been made on the confocal microscope in order to acquire both high resolution images and a fast time scales to capture fast events such as cell motion or calcium dynamics. In the spinning disc a series of pinholes on a disk that is spinning are used to scan multiple areas in parallel hence reducing the acquisition time and decreasing the excitation energy needed to illuminate the sample resulting in less phototoxicity and photobleaching. This type of microscopy allows for acquisition of rapid actin dynamics and has been extensively used to study Dictyostelium cytoskeleton dynamics, cell motion and chemotaxis. (Affolter and Weijer, 2005; Diez et al., 2005; Weijer, 2004). This microscopy technique was also used to image neutrophils migrating along CXCL8 gradients in live zebrafish larva (Sarris et al., 2012).

E. 2-Photon laser scanning microscopy

2PM (2-Photon microscopy) is a type of fluorescent microscopy that was created in Watt W. Webb lab in Cornell in 1990 (Denk et al., 1990). It uses a longer wavelength (near infrared) to excite the fluorophores. Using such wavelengths is limiting the diffraction of light in the tissue

resulting in less photobleaching than in classical confocal microscopy. This multi-photon technology can be used to generate localised cellular disruption. It is possible to photodynamically destroy biological structure with 2PM laser. Single cell ablation or larger injury have been generated in the past to answer various biological questions, including questions related to neutrophil swarming (Lämmermann et al., 2013; Muto and Kawakami, 2018). The multiphoton absorption also allows deeper penetration of the light in the tissue imaging. 1.6 mm penetration depth was reported by 2PM, in the cortex of a mouse brain (Kobat et al., 2011). The ability to image in deeper tissues has been used to study cells in their "natural environment" *in vivo* instead of glass dishes (Helmchen and Denk, 2002, 2005; Stephens and Allan, 2003). 2PM is therefore extensively used to study the migration of cells in organisms. In zebrafish 2PM was used to image neuronal populations (Renninger and Orger, 2013).

1.5.4 Optogenetics

A. Introduction

Optogenetics is a combination of optics and genetics to control molecules with light. This technique is used to observe and perturb the spatiotemporal dynamics of signals in living cells and organisms (Tischer and Weiner, 2014). It relies on proteins which change conformation upon exposure to light. Three of these types of proteins are based in plants (cryptochromes and phytochromes from *Arabidopsis thaliana* (Liu et al., 2008; Peters-Golden and Brock, 2003) and light-oxygen-voltage (LOV) domains from *Adiantium capillus-veneris* (Christie et al., 1999)*,* one is based on the fluorescent protein Dronpa from *coral Pectiniidae* (Ando et al., 2004). There are various strategies to control molecular processes by illuminating the cells. Two protein domains can bind to each other upon light exposure, or a single protein can change conformation. Optogenetic tools were first used to study neuroscience to control the opening and closing of ion gated channels in neurons with light (Zemelman et al., 2002). Optogenetic tools have subsequently been used for various application such as modulating gene expression (Wang et al., 2012), protein clustering, sequestration based inhibition (Bugaj et al., 2013) of proteins or controlling the release of chemokine (Sarris et al., 2016).

B. Optogenetics in zebrafish

The transparency and the ease to genetically edit the zebrafish larva makes this organism a good model for the usage of light-based protein tools. The easy optical access allows the use of optogenetic tools in those intact developing larvae. This allows optogenetic studies in embryonic and larval zebrafish to be carried out in a high-throughput fashion. Ehud Isacoff

and his team pioneered the use of optogenetic tools in zebrafish by generating larvae expressing the engineered light-gated ionotropic glutamate receptor (LiGluR) in sensory neurons to control the neuronal activity (Szobota et al., 2007). Soon after neuroscientists started developing genetically modified zebrafish expressing a variety of optogenetic tools such as the light-activated cation channel, Channelrhodopsin-2 (ChR2) (Douglass et al., 2008) or halorhodopsin (NpHR), a light-driven microbial chloride pump (Arrenberg et al., 2009). With this study they demonstrated a powerful tool to manipulate biochemical pathways with light. Subsequently development biologists started using optogenetic tools in zebrafish larvae as well; cryptochrome 2 was transiently expressed in zebrafish larva to control transcription through the manipulation of the bHlH transcription factor (Liu et al., 2012). Light Oxygen Voltage protein was used to study the β-catenin pathway by photoactivating it (Bugaj et al., 2013). In our department Clare Buckley used the phytochrome B system to manipulate apical polarity in zebrafish embryo (Yang et al., 2012).

Optogenetic tools have also been developed to study immunology in zebrafish. Anna Huttenlocher's team generated stable transgenic line expressing the previously engineered PA-RAC (photoactivable Rac) protein containing a photoreactive LOV (light oxygen voltage) domain (Yoo et al., 2010). This construct expressed under the control of a neutrophil promoter was used to study neutrophil motion in zebrafish larvae. Our lab previously generated an optogenetic tool using an engineered version of the UVR8 system allowing the release of chemokine IL8 upon UV light exposure. This tool was tested *in vivo* in zebrafish by transplanting HEK293T cells expressing the optogenetic construct (Sarris et al., 2016).

Optogenetic tools and approaches have been widely used and demonstrated using the zebrafish model, yielding important new knowledge on neutrophil circuits, developmental pathways and cell migration.

1.6. This study

The overarching aim of my PhD project was to understand the signalling dynamics leading to neutrophil swarming in injured tissue *in vivo*. I have used zebrafish larvae as a model system to answer the following questions:

1. What are the cellular and molecular events leading to neutrophil swarming *in vivo*? What is triggering LTB4 production?

2. How does LTB4 autocrine/paracrine signalling affect polarity and directional migration? Is the production of LTB4 polarised or uniform? Is the current neutrophil swarming model valid?

3. Crucially, what are the physiological or pathological consequences of swarming? Can we develop tools to trigger LTB4 production in individual neutrophils *in situ* and would this be sufficient to generate swarms?

2. Materials and Method

Breeding and injection of one-cell stage eggs were performed in the fish facility in the Physiology Building of the PDN (Physiology, Development and Neuroscience) Department in downing site. The rest of the experiments were done in the Anatomy Building of the PDN Department in Downing site.

2.1 Cloning

2.1.1 Molecular cloning

Constructs were cloned in either PCS2+, pcDNA or Tol2 vectors. For constructs insertions into the plasmids, 2µg DNA was digested with restriction enzymes (New England Biolabs) at 37°C overnight in a digesting solution (18µL dH2O, 2µL cut smart buffer (New England Biolabs)).
After digestion, fragments were ligated with the Rapid DNA ligation kit (Roche) as described in the manufacturer's instruction. E. coli TOP SHOT chemically competent cells were then transformed by heat shock with the ligated product (30min on ice followed by 30sec at 42 °C). Cells were then spread on agar culture plate containing ampicillin. After that, they were grown in 100mL of LB medium containing 100 µg/mL of ampicillin, 25µg/mL Chloramphenicol or 50µg/mL Kanamycin and under agitation at 220rpm, 37°C in a shaker overnight. Midi preps were then performed on the resulting cultures with the QIA filter plasmid midi prep kit (Qiagen) as described in the manufacturer's instruction. DNA concentration was assessed with the NanoDrop1000UV (thermo scientific).

2.1.2 PCR

For PCR, we used the KOD hot start kit (Takeda) according to the manufacturer's protocol along with the DNA engine Tetrad 2© thermocycler.

2.1.3 Electrophoresis

DNA ran on 1 to 2% agarose gel red in TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA) depending on their size at 110V for 25min.

2.2 RNA

RNAs were prepared with linearised (digested with Not1) DNA plasmid containing a SP6 promoter. The mMessage mMachine SP6 transcription kit (Invitrogen) was then used to transcript our plasmid DNA in mRNA based on manufacturer's protocol. Poly Adenylation of the produced mRNA was performed using a Poly (A) tailing kit (Invitrogen). RNAs were then purified with the RNA purification kit (Invitrogen).

2.3 Tissue Culture

2.3.1 Cell lines

MycoAlert mycoplasma detection kit (Lonza) was used on all cell lines according to manufacturer's protocol to assure that cell lines were mycoplasma free. Human embryonic kidney (HEK)-293T cells and NIH 3T3 cells were kept at 37°C with 5% CO2 in DMEM (Life Technologies) containing 5%Hepes, 10%FBS and 100µg/mL of penicillin/streptomycin antibiotics.

2.3.2 Transfections

The day before transfection, cells were seeded in imaging plates. Cells were transfected with 1µg/mL of plasmid for single plasmid transfection and 0.5µg/mL of plasmid for double transfection using lipofectamine 2000 (Life Technologies) as described in the manufacturer's protocol. Cells were then incubated overnight in opti-MEM (Life Technologies) then washed with dPBS (Life Technologies) and incubated in DMEM without red phenol +10%FBS for imaging. Transient transfectants were evaluated microscopically with an Olympus (MVX10) fluorescent microscope, live, 16h after transfection.

2.4 Zebrafish work

2.4.1 Zebrafish

Zebrafish were maintained in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act 1986. Adult zebrafish were maintained under project licence 70/8255, which was reviewed by the University Biomedical Services Committee. Animals were maintained according to ARRIVE guidelines. Zebrafish were bred and maintained under standard conditions at 28.5 ±0.5°C on a 14h light: 10h dark cycle. Embryos were collected from natural spawnings at 4-5 hours post-fertilization (hpf) and thereafter kept in a temperature-controlled incubator at 28 °C. Embryos were grown at 28°C in E3 medium, bleached as described in the Zebrafish Book (Westerfield M) and then kept in E3 medium supplemented with 0.3 µg/ml of methylene blue and 0.003% 1-phenyl-2-thiourea (Sigma-Aldrich) to prevent melanin synthesis. For live-imaging of neutrophils expressing fluorescent receptors, methylene blue was omitted from E3 medium to minimize tissue autofluorescence. All embryos were used between 2.5-3.5 dpf thus before the onset of independent feeding. For live imaging or fixation, larvae were anaesthetised in E3 containing 0.04% MS-222 (Sigma). Where indicated, larvae were treated with 50µM Calcium ionophore A23187 (Sigma), 10µM NF279 (BIO-TECHNE LTD), 50 µM Carbenoxolone (Sigma) in E3, propidium iodide 50µg/mL (Sigma).

2.4.2 Generating transgenic zebrafish line

All DNA expression vectors for transgenesis use a backbone vector with a Lysozyme C promoter (*lyz*) for neutrophil-specific expression and minimal Tol2 elements for efficient integration and a SV40 polyadenylation sequence (Kwan et al., 2007). Sequences clone in this backbone vector are represented in table 1.

Name	Sequences	Origin
GCamp6F	(Chen et al., 2013b)	From Dr. Nachiket Kashikar
***lta4h*-EGFP**	ENSDART00000028171.7	Genewiz
***blt1b*-YFP**	ENSDARG00000032631.10	Genewiz
tRFP-*5lo*	ENSDART00000079884.6	cDNA library
***cx43*_T2a-mCherry**	ENSDARG00000041799	Genewiz
***cx43*dn_T2a_mCherry**	(Oyamada et al., 2002)	Genewiz
Cry2phr-mCherry5-LO-codon-opt	(Taslimi et al., 2014)	Genewiz/Chandra Tucker

Cry2WT-mCherry-5-LO	(Taslimi et al., 2014)	From Chandra Tucker
Cry2phr-mCherry-CPLA2	(Taslimi et al., 2014)	From Chandra Tucker
CIBN-YFP-LAP2β	(Taslimi et al., 2014) /ENSRNOT00000012715.4	Genewiz/Chandra Tucker

Table 2.1. Constructs cloned in Tol2 vector.

For transgenesis, 0,5 nL of solution containing 25 ng/µL DNA plasmid and 35 ng/µL were injected into the cytoplasm of one-cell stage embryos. Transposase mRNA was synthesized from pCS2-TP (citation Tol2kit) by *in vitro* transcription (SP6 message machine, Ambion). Injected embryos were stored at 28°C until 5dpf and thereafter were raised in the fish nursery according to standard rearing protocols. At 3 months old, F0 fish were outcrossed to a wildtype (TL) line to screen for germline transgenesis. The subsequent outcross of the F0 was then grown to give rise to F1 fish. F1 fish were outcross with homozygous Tg(*lyz*::dsred2) in order to select for the best expression pattern in neutrophils among F1 fish. Selected F1 fish were then outcross to WT (TL) fish to give rise to the F2 generation. Adult fish were used for up to 2 years (Fig. 2.1).

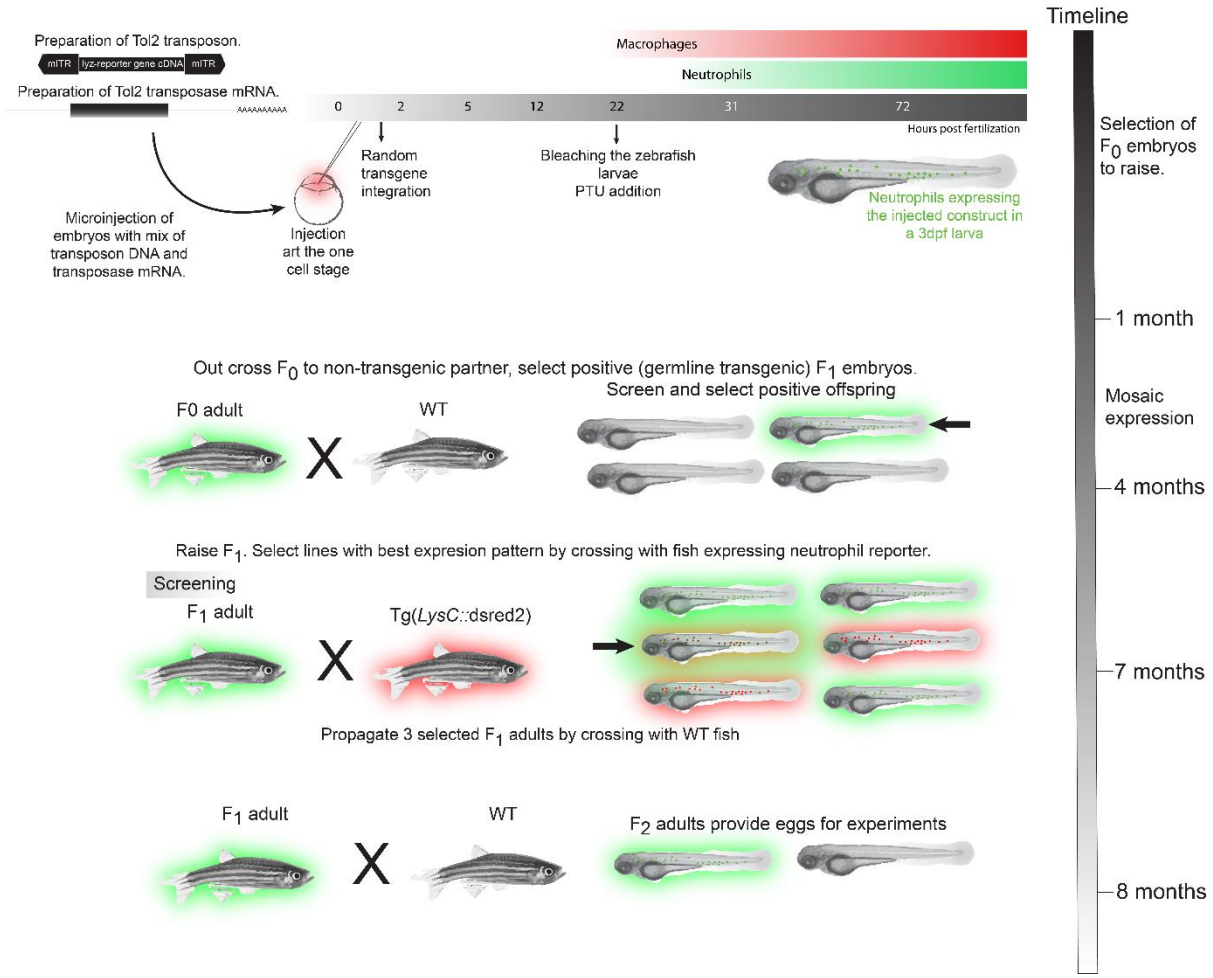


Figure 2.1 Generation of stable transgenic zebrafish.

Schematic describing the transgenesis and subsequent generation of transgenic fish to generate a stable transgenic zebrafish line.

2.4.3. Whole-Mount Immunohistochemistry

GFP^{+} neutrophils and tRFP-5-LO were detected using chicken anti-GFP (abcam) at 1:500 and rabbit anti-tRFP (Evrogen) at 1:500. Secondary antibodies used were anti-chicken-Alexa488 (Invitrogen) and anti-rabbit-Cy3 (Jackson) at 1:500. 3dpf larvae were fixed overnight in 4% formaldehyde (ThermoFisher) at 4°C, washed twice in PBST (PBS, 0.1% Tween-20) and permeabilized overnight in methanol 100% at -20°C. Larvae were progressively rehydrated by decreasing methanol concentration, heated 15min at 70°C and fixed in ice cold acetone during 20 min at -20°C. After blocking in 10% sheep serum (Sigma-Aldrich), proteins were probed with primary antibody and revealed using secondary antibody. Nuclei were stained with DAPI at 0.5µg/mL (Sigma Aldrich). Subsequently larvae were embedded in 80% glycerol and mounted for confocal observation (Olympus Fluoview FV1000).

2.5 ELISA

HEK293T cells transfected with optogenetic constructs were exposed to 470nm blue LED light (LIU470A, THORLABS) and incubated at 37°C for 15min, in DMEM supplemented with 10µM arachidonic acid (Sigma) and 50µM of calcium ionophore A23187 (Sigma) depending on the condition. Supernatant from those cells ($10.^6$ cells/mL) were harvested and used to dose LTB4. Leukotriene B_4 ELISA kit (Cayman Chemical) was used according to the manufacturer's protocol to determine LTB4 concentration.

2.6 Western blot

For western blotting, 10 larvae (3dpf) of each genotype were collected. Larvae were then lysed in 100µL of OCG buffer (0.3M NaCl, 2.5µM EDTA pH8, 0.9M Tris HCl pH7.5, protease inhibitors, phosphatase inhibitor) with 1mm glass beads (BioSpec) for 3x20sec of sonication. Qubit protein assay kit (Invitrogen) was used to obtain protein concentration. Proteins (25µg) were resolved on a Bolt 10% Bis-Tris Plus Gel (Invitrogen), blotted onto nitrocellulose membrane using iBlot2 transfer stacks (Life Technologies). Proteins were probed with rabbit anti-Cx43 (1:2000) (Sigma-Aldrich) and rabbit anti-ß-Tubulin antibodies (1:2000) (abcam) after saturation in PBST (PBS, 0.1% Tween-20) containing 5% of milk. Proteins were then revealed using an enhanced chemiluminescence detection system (Pierce ECL Plus Western Blotting Substrate, Invitrogen) with goat anti-rabbit HRP antibody (1:2000) (abcam).

2.7 Wounding zebrafish larvae

Many neutrophils can be observed in the CHT (Caudal Haematopoietic Tissue) on the ventral side of the larvae. The CHT is the equivalent of the human foetal liver (the first site of definitive hematopoiesis in mammals) and contains most of the neutrophils present in the larvae allowing us to image many neutrophils at once. Laser wounding on or near the CHT or cutting the ventral fin near the CHT with a sterile scalpel (Swann-Morton) generally triggers neutrophil clustering towards the site of injury. Neutrophils are also often observed migrating in interstitial tissues towards a wounded tail after tail transection performed with a sterile scalpel, but this method rarely generate neutrophil clustering.

2.8 Imaging

2.8.1 Mounting

Larvae were mounted onto a glass-bottom plate in 1% low melting agarose (Invitrogen) or a custom-built coverslip chamber (for when using an upright scope). Agarose-embedded embryos were covered with 2ml E3 medium (supplemented with tricaine) (Fig. 2.2).

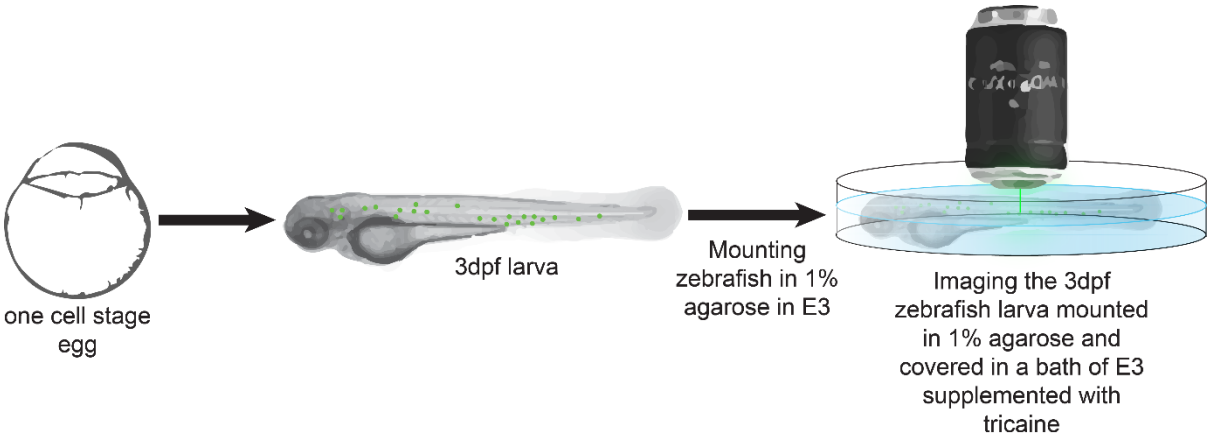


Figure 2.2 Zebrafish larva imaging.

Schematic describing the mounting and subsequent imaging of a zebrafish larva.

2.8.2 Bacteria injection

When indicated, 3dpf larvae were injected with Crimson or CFP fluorescent *Escherichia coli* in the interstitial tissue between the otic vesicle and the eye. This is a strategic area to visualise local neutrophil recruitment and migration towards site of infection (Sarris et al., 2012) because neutrophils are constitutively present and motile in this area making it convenient as a control for random movement in the absence of stimulation.

2.8.3 Laser wound

Laser wounding was performed on a two-photon scanning microscope (LaVision Biotec TriM Scope II). A tunable ultrafast laser (Insight DeepSee, SpectraPhysics) was tuned to 930 nm and the laser power adjusted to approximately 500mW. A square region of interest (ROI) of ~40µm in width was defined in one focal plane followed by single laser scan across the ROI at a pixel spacing of 240nm and dwell time of 13 µs. Confocal stacks were acquired

immediately after, using a 25x/1.05 NA water-dipping lens. GFP was imaged with 930nm and DsRed was imaged with a 1040nm line. For imaging 5-LO translocation, the resolution of imaging with the two-photon microscope was limiting. Larvae were thus transferred (within 10-20 min) for imaging onto an upright Nikon E1000 microscope coupled to a Yokogawa CSU10 spinning disc confocal scanner unit with a 40x/0.80W water objective (Nikon). In some cases, propidium iodide (50 µg/ml) was added to the medium 30 min prior to imaging. PI penetration was observed only with superficial laser wound.

2.8.4 Fin wound

Larvae were wounded on the ventral fin near the CHT with a sterile scalpel in a petri dish containing E3+tricaine. After wounding larvae were immediately mounted as previously described. They were then imaged either on i) an inverted PerkinElmer UltraVIEW ERS, Olympus IX81 spinning disk confocal microscope with a 30x/1.05 NA silicon (Olympus) or 40x/1.25 NA silicon objective (Olympus) and 488nm for GFP excitation and 561 for tagRFP or mCherry or ii) on an upright Nikon E1000 microscope coupled to a Yokogawa CSU10 spinning disc confocal scanner unit with a 20x/0.75 NA air objective (Nikon) or 10x/0.5 NA air objective (Nikon) and illuminated using a Spectral Applied Research LMM5 laser module (491 nm for GFP excitation; 561 nm for Ruby or TagRFP or mCherry). Confocal stacks using a 2 µm z spacing were acquired every 20-40 sec.

2.8.5 Optogenetic

All samples were kept in the dark and manipulated under a yellow lamp (Pro-Lite). Larvae were mounted as described above, immortalized were seeded on imaging plates as described before. Time-lapse fluorescence images were acquired with a laser-scanning confocal microscope (Olympus Fluoview FV1000) or a confocal spinning disk (Nikkon Eclipse E1000). Photoactivation experiments were performed using the 488nm laser from the confocal microscope, activating a region of interest for 1second at 25 µW or when indicated using a blue LED light (472nm) (THORLABS) at 4mW at a distance of 10 cm away from the sample.

2.9 Morpholino

Morpholinos were ordered on geneTOOLS LTD, their names, sequences, types and origins are indicated in table2. MO Cx43.4 was designed on geneTOOLS. All morpholinos were injected in one cell stage eggs in a morpholino injection solution (120mM KCl, 20mM HEPES, 0.1% phenol red). For Cx43 knock down we used MO Cx43.3 (Bhadra and Iovine, 2015) (main isoform of Cx43) and MOCx43.4 (another isoform of Cx43 in zebrafish); 1nL of 0.2mM of each morpholino was injected (0.4mM total). As injection control we used 0.4mM of Negative Vivo-Morpholino control oligo. For immune cell depletion, 1nL of a solution containing 0.5mM of each (1mM total) gcsfr (Feng et al., 2012) and PU.1 (Rhodes et al., 2005) morpholinos were co-injected. For LTA4H knockdown I injected 3 nL of 0.5mM MO LTA4H (Vincent et al., 2017).

Name	Sequence	Type	Origin
MO Cx43.3	5'GTTCTAGCTGGAAAGAAG TAAAGAG3'	Translation	GeneTools (Bhadra and Iovine, 2015)
MO Cx43.4	5'ACTTCTCCATCTCCGTTAT ATTTTG3'	Splice	GeneTools
MO Ctr	5'CCTCTTACCTCAGTTACAA TTTATA3'	Control	GeneTools
MO gcsfr	5'GAAGCACAAGCGAGACGG ATGCCAT3'	Splice	From Jean-Pierre Levraud (Feng et al., 2012)
MO PU1	5′-GATATACTGATACTCCATTG GTGGT3′	Translation	From Jean-Pierre Levraud (Rhodes et al., 2005)
MO LTA4H	5'CAGTCTGATCAAGAGAAAGA CTCGA3'	Splice	GeneTools (Vincent et al., 2017)

Table 2.2. List of morpholinos injected.

2.10 Wound colonisation assay

3dpf larvae were anaesthetized with 0.04% MS-222 (Sigma) and wounded in the ventral fin or tail fin using a sterile surgical scalpel blade (Swann-Morton, 23). Larvae were subsequently incubated 2 hours at 33°C in Ringer (145 mM NaCl, 2 mM KCl, 1.5 mM K_2HPO_4, 1 mM $MgSO_4$, 10 mM HEPES, 2 mM $CaCl_2$ and 10 mM glucose, pH7.2) medium with 1.10^7/mL *P. aeruginosa* PAO1 strain (provided by Dr. Martin Welch). Following incubation, larvae were washed 5 times

in PBS and separated in individual wells containing 100µL of PBS on a 96 well plate, to avoid transmission of infection across larvae. Survival and bacterial burden were monitored at 2, 6 and 18 hours post wounding. For determining the bacterial burden, larvae were homogenised with a pestle gun (Anachem LTD) in 100µL PBS in a 4.5mL Eppendorf tube. Serial dilutions of these homogenates were then plated on P. aeruginosa isolation agar (Scientific laboratory supplies LTD) supplemented with cetrimide (200 mg/L) and nalidixic acid (15 mg/L) (CN supplement; Scientific laboratory supplies LTD) and incubated 24h at 37°C. Colonies were counted to determine the bacterial burden characterised by the number of colony forming unit contained in one fish.

2.11 Tail fin wounds and Sudan Black staining

The tail fin was amputated up to the level of the notochord and larvae were fixed 3 hours later in 1ml of 4% ethanol-free formaldehyde (Polysciences, Warrington, PA) in phosphate-buffered saline (PBS; Sigma-Aldrich) overnight at 4ºC with agitation. Fixed larvae were rinsed in PBT (PBS with 0.1% Tween-20; Sigma-Aldrich) twice for 5 minutes and incubated in 1ml Sudan Black (Sigma-Aldrich) for 15 minutes. Following staining, larvae were washed in 70% ethanol for several hours and passaged into 30% ethanol overnight at 4ºC with agitation. Larvae were washed in PBT for ten minutes, passaged into increasing concentrations of glycerol and stored in 80% glycerol at 4ºC. Larvae were imaged on an optical microscope Stemi 2000-CS (ZEISS) mounted with axiocam ERcs 5s (Zeiss).

2.12 RT-PCR of Cx43 genes in neutrophils cDNA

RNA extraction: Larvae were dried and snap freeze in liquid nitrogen. 10 larvae were used, and RNA was extracted with RNAeasy minikit (QIAgen) according to manufacturer's instruction. RNA was then reverse transcribed to DNA with SuperScript™ III Reverse Transcriptase (Invitrogen) according to manufacturer's instruction. PCR were performed on the cDNA with the KOD hot start (Novagen, TOYOBO) kit according to manufacturer's instruction.

Primers used for probing for detection of splicing in the case of LTA4H Cx43.3 and Cx43.4 detection are indicated on table 3.

Primer name	Sequences
Cx43.3 Forward	GCTCTCCA CTCTTTACTTCTTTCCAG
Cx43.3 Reverse	GTATTGCACTTGAAAGCTGACTGC
Cx43.4 Forward	GAGTCGTCATCGCGAGACATTGA
Cx43.4 Reverse	GTCTATGAGTCTCAATCAAGCATGGATCC
LTA4H Forward	TCTGAGAAGGAATATGTGGATGAA
LTA4H Reverse	CAGCAAGAGATCTGTCTCCA

Table 2.3. List of primers used for RT PCR

2.13 Image Analysis

2.13.1 Detection and scoring of 5-LO translocation in zebrafish neutrophils

We trialled automated and manual detection of 5-LO translocation events described in Enyedi *et al*. (Enyedi et al., 2016) . Automated detection was inaccurate for these cells due to the irregular shape of the nucleus and their movement. We thus used visual inspection of the time-lapse videos on ImageJ (National Institutes of Health, Bethesda, MD) and representative sample videos were confirmed by two viewers. Only unambiguous translocation events were scored.

2.13.2 Analysis of 5-LO translocation in relation to distance or GCamp6F intensity

Frames in which 5-LO translocation events were detected were duplicated and thereafter analysed with MATLAB 2018b (The MathWorks, Inc., Natick, MA) in an automated fashion. Individual cells were segmented using marker-based watershed segmentation and intensity thresholding. Mean fluorescence intensities of the GCamp6F signal in segmented neutrophils were subsequently computed. For each neutrophil, the fluorescence intensity was normalised to the most fluorescent cell in the corresponding frame to allow pooling of value across embryos with different imaging settings. The wound centre was manually inputted and the distance of individual neutrophil centroids from the wound centre was automatically computed.

2.13.3 Analysis of GCamp6F in neutrophil cell-cell contacts

Contacts between bright and dim GCamp6F+ neutrophils were counted and categorised according to whether a sharp increase of fluorescence was observed in the dim cell upon contact or not. These events were quantified using visual inspection of the time-lapse videos on ImageJ. Only unambiguous events were scored.

2.13.4 Extraction of cell trajectories

Analysis of neutrophil trajectories was performed in Imaris v8.2 (Bitplane AG, Zürich, Switzerland) on 2D maximum intensity projections of the 4D time-lapse videos. Unless otherwise indicated, analysed trajectories were extracted from the area covered by the fin and part of CHT that there was neutrophil immobilisation. A track duration threshold of 3 time-frames was defined to exclude short-lived tracks. Manual track corrections were also applied where needed. Instantaneous (x,y,t) coordinates over time were exported into Microsoft Excel 2016 spreadsheets files (Microsoft Corporation, Redmond, WA). For speed analyses embryos results are pooled from transgenic Tg(*mpx*:GFP) or Tg(*lyz*:GCamp6F) distributed across the different conditions (no treatment, CBX and morpholino-treated).

2.13.5 Extraction of cell surface data

Analysis of neutrophil size and calcium signal was performed in Imaris. Neutrophils were segmented as surfaces and manual surface splitting or merging was applied where needed. Instantaneous neutrophil size and calcium signal intensity were exported into Microsoft Excel 2016 spreadsheets files.

2.13.6 Definition of (mechanical or laser) wound

The perimeter of the wound (either mechanical or laser wound) was manually defined as a set of points in MATLAB, using a time-projection of the 2D maximum intensity projection.

2.13.7 Quantification of GCamp6F levels

Calcium signal values were extracted from Imaris and imported into MATLAB for plotting. Calcium values for individual segmented neutrophils were normalised to the mean calcium value of the neutrophils in the whole area outside the wound, prior to wounding. For fin wound videos, the calcium values for individual neutrophils were normalised to the mean calcium value of the neutrophils in the whole area outside the wound, at the first timepoint of imaging. For the laser wound experiments, the first 3-9 frames post-wounding were excluded to eliminate distortion of the data by the tissue-scale calcium wave.

2.13.8 Quantification of GCamp6F levels with neutrophil size

Neutrophil size and calcium signal values were computed in MATLAB and plotted against each other. A threshold of 100 pixels on the size of detected objects was applied to eliminate false detections.

2.13.9 Calculation of neutrophil radial speed

Radial speed was calculated in MATLAB using the following equation (Lämmermann et al., 2013):

$$u_r = u \times cos\theta$$

where u is the instantaneous speed of neutrophil between two successive positions and θ is the angle between the vector of the movement and the vector that connects the position with the wound. The angle θ was calculated using the vector between the neutrophil position (centroid) and its nearest point to the wound. When the cosine of θ has value +1, the neutrophil migrates directly towards the wound while when it has value -1, the neutrophil migrates directly away from the wound. To uncover trends in directionality of motion in embryos with different speed levels, we used a normalisation. Instantaneous speed values for individual neutrophils were divided by the mean instantaneous speed value of the corresponding embryo. Normalised radial speeds were computed with the equation:

$$ur_{norm} = u_{norm} \times cos\theta$$

Normalised radial speed values were binned every 7.5 minutes. For the laser wound experiments, the first 3-9 frames post-wounding were excluded for consistency with the calcium signal calculation.

2.13.10 Quantification of neutrophil GCamp6F levels and speed upon contact with necrotic cells

Individual neutrophils were visually inspected to determine the time-point that they touched the PI-stained necrotic cells. This time-point was considered as the time-point 0. The neutrophils were tracked for 180 seconds before and after this time-point. Individual neutrophil calcium values were normalised with the calcium value of the first time-point of the track.

2.14 Statistics

All error bars indicate S.E.M. All p values were calculated with two-tailed statistical tests and 95% confidence intervals. t-test (pairwise comparisons) and one-way ANOVA (multiple group comparisons) were performed after distribution was tested for normality otherwise non-parametric tests were performed (Mann-Whitney for two-way comparisons and Kruskal-Wallis with Dunn's post-test for multiple comparisons). Statistical tests were performed in Prism8 (GraphPad Software Inc., La Jolla, CA). The statistical test and the n number are indicated in the figure legends. The error bars show standard error of the mean across individual embryos (reflecting embryo variation). Live imaging experiments were acquired in minimum three independent experiments. In figure panels, * corresponds to $p<0.03$, ** to $p<0.002$ and *** to $p<0.0002$.

3. Dynamics of Neutrophil swarming at wound sites.

3.1 Introduction

Guided leukocyte migration is a key feature of the inflammatory response. It is required for fighting infection and cancer and plays a major role in auto-immunity and wound repair. Neutrophils are major effectors of acute inflammation and microbial defence (Kolaczkowska and Kubes, 2013). To reach sites of inflammation, neutrophils cross vascular endothelium through a well-studied cascade of events (Nauseef and Borregaard, 2014). Less is known about how neutrophils navigate within target tissues. Neutrophil interstitial migration appears to be guided by various chemoattractants, such as chemokines and other small molecules such as fMLP (*N*-formylMethionyl-Leucyl-Phenylalanine) and LTB4 (Leukotriene B4), all of which are sensed through GPCRs (G-protein-Coupled Receptors) (Kolaczkowska and Kubes, 2013). Upon infection or wounding, neutrophils often exhibit highly co-ordinated and directed migration with adhesion dependent aggregation at the target site of infection or tissue damage (Chtanova et al., 2008; Deng et al., 2013; Lämmermann, 2015; Lämmermann et al., 2013; Peters et al., 2008). This phenomenon is referred to as neutrophil swarming. It has been shown that LTB4 and integrins were crucial for neutrophil swarming in mice (Lämmermann et al., 2013). In this study, the authors triggered neutrophil swarms by performing laser injury and used mice knocked out for LTB4 receptor BLT1 and Talin (a protein linking integrins to the cytoskeleton) to demonstrate the importance of LTB4 and integrins.

Neutrophil swarms were first observed *in vivo* in mice infected with the parasite *Toxoplasma gondii* (Chtanova et al., 2008) or the protozoan *Leishmania major* (Peters et al., 2008). In both cases the authors described the highly coordinated migration pattern of those cells, with neutrophils migrating very directionally, in streams, towards the site of infection. In zebrafish larvae, neutrophil swarms have been observed upon localised bacterial infection with *Pseudomonas aeruginosa* (Deng et al., 2013) and *Streptococcus iniae* (Harvie et al., 2013). Most of the data we possess on neutrophil swarming come from experiments on mouse models. Neutrophil swarming has never been directly observed in human. However, human neutrophils were reported to swarm, *in vitro*, in a similar fashion to mouse neutrophils. Indeed, a study using time-lapse video bright-field microscopy showed neutrophil accumulation following the destruction of erythrocytes with a ruby laser microbeam (Malawista et al., 2008).

Highly directed chemotaxis and accumulation of neutrophils at the site of erythrocyte death was described in this article. In another study, human neutrophil swarms were observed in mouse, after human neutrophils transplantation in the mouse earflap followed by a laser injury (Lämmermann et al., 2013). In another study, using non-invasive reflectance confocal microscopy on psoriasis lesion of patients, Wolberink *et al* were able to identify neutrophil accumulation in psoriasis lesion of human patients *in vivo*. this accumulation could be the result of neutrophil swarming (Wolberink et al., 2014). Based on these evidences, neutrophil swarming appears to be evolutionarily conserved across species from human to zebrafish and is observed both in response to a pathogenic infection and tissue injury.

Our lab is using three days post fertilisation zebrafish larvae as a model to study neutrophil migration. At this stage, this organism is transparent allowing high-resolution, sub-cellular dynamics to be followed (Deng and Huttenlocher, 2012; Minina et al., 2007). Moreover, imaging is entirely non-invasive in contrast to mouse intravital imaging, which often requires surgical exposure of tissues (which in turn affects neutrophil migration). Finally, zebrafish neutrophils are easy to genetically manipulate and arise quickly during development (at 2 days post fertilisation) in contrast to mammalian neutrophils.

Swarming behaviours have been previously observed in 2-4dpf zebrafish larvae after a sterile injury of the caudal fin performed with a scalpel (Niethammer et al., 2009). However, this assay does not consistently trigger neutrophil swarms and when it does, it triggers swarms of low amplitude with few neutrophils being recruited. Therefore, I decided to develop a sterile injury assay that could consistently trigger neutrophil swarms of large amplitude.

Along with developing a new assay, I decided to create new probes reporting for molecular events happening in neutrophils during swarms. Neutrophil intracellular calcium dynamics are important molecular events happening during neutrophil migration. In zebrafish, calcium dynamics have been observed in epithelial cells during injury (Enyedi et al., 2013) and in neutrophils migrating in a solitary manner (Beerman et al., 2015), but not in swarming neutrophils. Calcium is the earliest signal in the wound inflammatory response (Razzell et al., 2013). Many signalling mechanisms in immune cells rely on calcium as a secondary messenger (Dixit and Simon, 2012). Production of pro-inflammatory molecules, notably LTB4, is associated with intracellular calcium elevation (Dixit and Simon, 2012; Luo et al., 2003). Resting leukocytes maintain a relatively low calcium concentration, however, during inflammation, elevated intracellular calcium fluctuations can be observed in immune cells as a result of antigen recognition or detection of pro-inflammatory molecules, (Vig and Kinet, 2009). Visualising calcium patterns in neutrophils during wound inflammatory response is therefore extremely informative and can be used as an indicator of immune cell activation.

Molecular dynamics of the cytoskeleton are also crucial for neutrophil migration. While migrating within interstitial tissue, immune cells mostly use non-adherent migration

(Lämmermann and Germain, 2014; Weber et al., 2013). This type of migration relies on myosin and actin, which generates forward propulsion forces by friction with the environment. Actin dynamics during tissue injury is particularly interesting as supracellular actin ring are formed by epithelial cells surrounding the injury. A study in drosophila using laser ablation to generate wounds showed that these actin ring were important for wound repair (Abreu-Blanco et al., 2011). In their study, the authors used embryos expressing sGMCA (spaghetti squash–driven, GFP, Moesin α-helical–Coiled, Actin binding site) a fluorescent actin reporter to analyse actin rings. They used *sgh* mutants characterised by lower level of myosin than WT, in those mutants the formation of actin ring was incomplete, and the wound stayed open. Visualising actin dynamics in migrating, aggregating and stopping neutrophils at the site of injury could tell us more about the role neutrophils' cytoskeleton plays for cell migration, wound closure and wound repair during swarms.

In this section, I describe a novel methodology to visualise and analyse neutrophil swarming in zebrafish larvae *in vivo* using time lapse imaging of neutrophils expressing fluorescent probes. I use this technique to dissect the dynamics of neutrophil behaviour at the wound. Using reporters for calcium dynamics, actin dynamics and cell death I interrogate the basic molecular mechanisms associated with neutrophil swarming.

3.2 Dynamics of neutrophil behaviour at the wound

To visualise neutrophils, I used 3dpf larvae from a stable transgenic zebrafish line expressing GFP under the control of the myeloperoxidase promoter which induces gene expression in neutrophil only Tg(*mpx*::GFP) (Fig. 3.1, A). To trigger neutrophil swarms, I developed a two-photon laser wound assay. I performed wounds of various sizes up to 150x150µm and down to 20x20µm targeting different tissues in the zebrafish looking to elicit the most consistent neutrophil swarming behaviour. Swarms are more likely to occur under high neutrophil density (Lämmermann et al., 2013; Park et al., 2018; Reátegui et al., 2017), and it is in the caudal hematopoietic tissue (CHT), a tissue rich in neutrophils, that I visualised the most consistent neutrophil swarming behaviour after 45x45µm laser wound injury. Within minutes, neutrophils begun migrating to the wound in a highly directional and coordinated manner, culminating in clusters at the wound core by 0.5 hours post wounding (Fig. 3.1, B). This type of wound shows laser-induced autofluorescence as it can be seen on figure 3.1 B at time 0 during wounding. This was a useful indicator of the location of the injured tissue and of the efficiency of the laser injury.

Alternatively, I triggered neutrophil swarms by performing mechanical wounding with a sterile scalpel on the ventral fin of 3dpf embryos near the CHT (Fig. 3.1, C). Mechanical wounds to

recruit neutrophils are usually performed at the tip of the tail of the zebrafish embryo. Tail wounds are a good way to observe neutrophils migrating over a long distance but are rarely triggering neutrophil swarms (Henry et al., 2013). Here, by performing a wound near the CHT I was able to obtain more consistent generation of neutrophil swarms. Using this type of tissue injury, I was able to use other microscopes than the 2-Photon microscope to visualise neutrophil swarming, such as the fast imaging, high resolution, spinning disc confocal microscope.

In the case of mechanical wounding, the neutrophils recruitment was delayed in comparison to laser wounding. The first neutrophils were recruited between 30 and 45min post wounding instead of 10min for the laser wound. With mechanical wounds, in some cases, and as it is shown in this example, I could observe two waves of recruitment, the first one at around 60 minutes post wounding and second at around 90 post wounding (Fig. 3.1, C). When performing laser wound, however, only one continuous wave of neutrophils migrating to the site of injury was observed.

To quantify the dynamics of neutrophil migrating towards the site of injury, we devised an analysis approach based on the previous study of neutrophil swarming (Lämmermann et al., 2013). For this, we analysed the radial speed of neutrophils with respect to the centre of the wound. It is the rate of change of the distance between neutrophils and the wound. It describes the speed with which neutrophils move away from or approach the centre of the wound. In our case, we pooled data obtained from different embryos with various neutrophil speed levels to uncover the trend in the directionality of neutrophils. We normalised those data by dividing instantaneous speed values of individual neutrophils by the mean instantaneous speed value of neutrophils in the corresponding embryo.

Quantification shows that, in the case of the laser wound assay, during the first half hour post wounding, neutrophil migration is highly directional, with a high normalised radial speed. Normalised radial speed quickly decreases after 30min which corresponds to neutrophil clustering and a diminished recruitment of neutrophils to the wound (Fig. 3.1, D).

To conclude, I developed two wound assays in 3dpf zebrafish larvae that consistently result in the generation of a neutrophil swarm at the site of injury. Laser wound presented more advantages over mechanical fin wound triggering swarms more quickly and eliciting a markedly clearer neutrophil migration wave following the injury. I therefore decided to proceed with laser wound injury experiments to elicit neutrophil swarms.

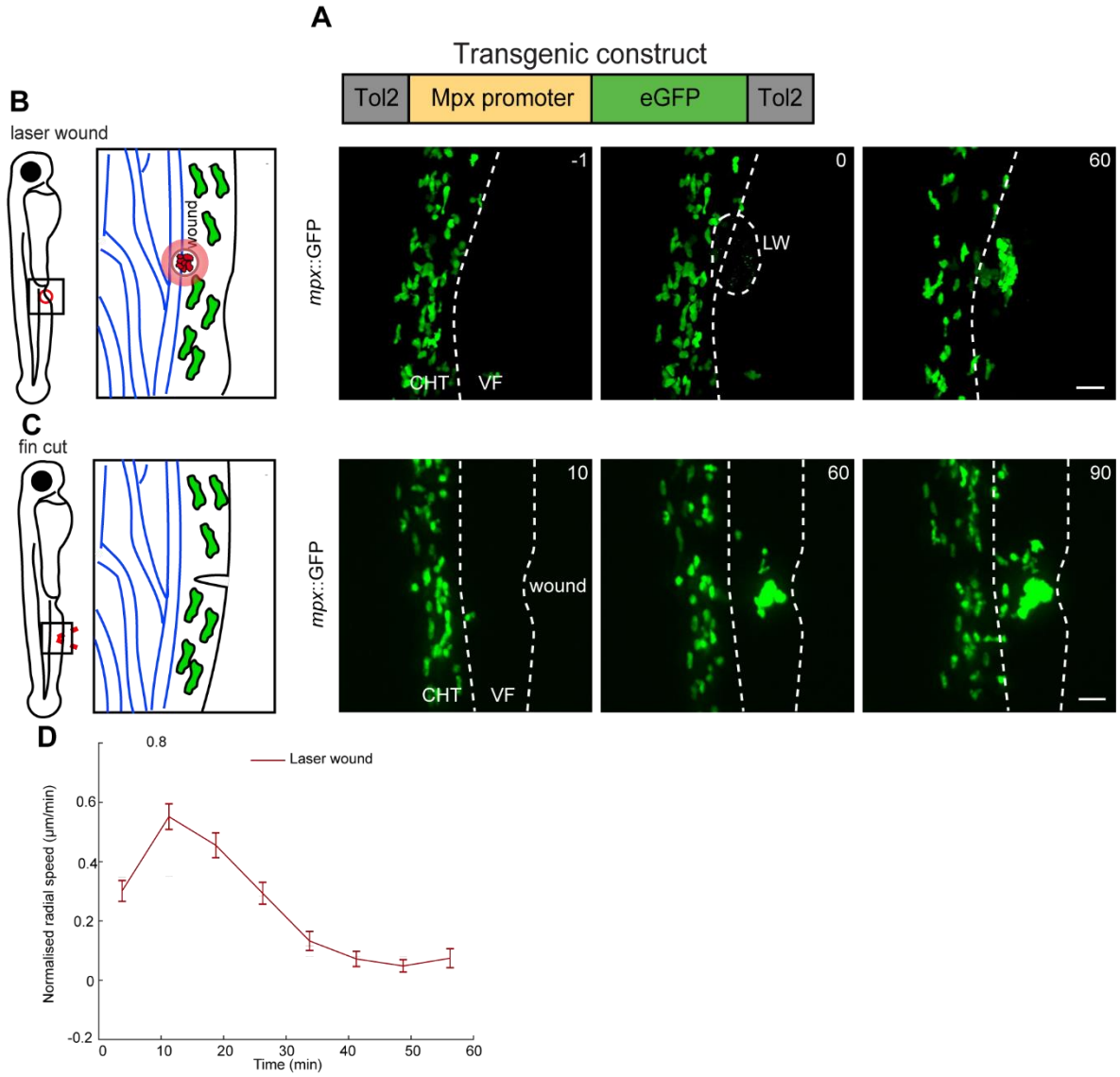


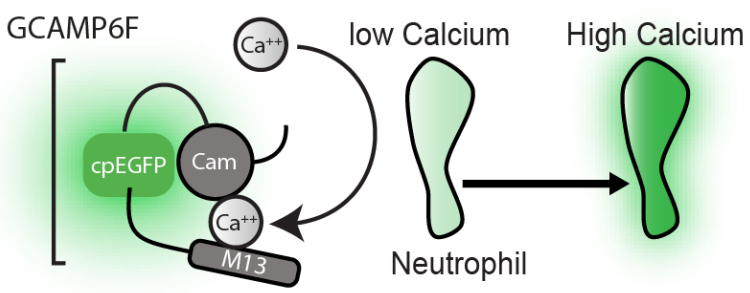
Figure 3.1. Neutrophil swarming towards sites of sterile injury

A The eGFP construct is expressed under the control of the myeloperoxidase promoter (mpx). B Schematic of a 3dpf zebrafish larva showing the area imaged and location of two-photon laser wound damage. Images show neutrophils (expressing eGFP in green) within the caudal hematopoietic tissue (CHT) migrating towards the laser wound damage (LW), induced at the border between the Ventral Fin (VF) and the CHT. Dotted line outlines wound. Scale bar, 25µm. C Schematic of a 3dpf zebrafish larva showing the area imaged and location of the mechanical sterile injury. As described above, images show neutrophils within the caudal hematopoietic tissue (CHT) migrating towards the mechanical wound (wound), performed on the Ventral Fin (VF) near the CHT. Scale bar, 25µm. D Quantification of normalised radial speed of neutrophils migrating towards a laser wound overtime. n=12 larvae. E Same quantification as in D for neutrophils migrating towards a mechanical wound. n=8 larvae. Quantification was performed by Antonios Georgantzoglou.

3.3 Calcium dynamics of neutrophils migrating to the wound

Being able to generate neutrophil swarms in 3dpf zebrafish larvae, I moved on to further characterise this neutrophil response to sterile injury. For this, I decided to study neutrophil's calcium dynamics. In order to follow calcium fluctuation in neutrophils, I generated a zebrafish transgenic line expressing GCamp6F, a GECI (Genetically Encoded Calcium Indicator), under the control of the lysozyme C promoter (Box. 3.1 and Fig. 3.2, A) (Akerboom et al., 2009; Wang et al., 2008). When I chose GCamp6F, it was the latest generation of GECI, with a high cellular fluorescence change and spiking kinetic of 50-75ms per spike of fluorescence (Chen et al., 2013b). A neutrophil expressing GCamp6F will be dim when its intracellular calcium concentration is low and bright upon increase of intracellular calcium concentration.

Box 3.1. GCamp6F, a Genetically Encoded Calcium Indicator



GCamp6F is composed of a circularly permutated GFP (N- and C-termini are fused, creating a new terminus in the middle of the protein fused with calmodulin and M13. Upon binding of Calcium ions, the calmodulin undergoes a conformational change and binds M13 a peptide sequence from myosin light chain kinase. In the absence of Calcium, the circularly permutated GFP exists in a poorly fluorescent state due to a water pathway that enables protonation of the chromophore and poor absorbance at the excitation wavelengths. Calcium binding to the calmodulin moiety results in a structural shift that eliminates this solvent pathway, rapid de-protonation of the chromophore, and bright fluorescence (Akerboom et al., 2009; Wang et al., 2008).

Genetic sensors have yet to surpass the sensitivity of synthetic probes such as fluo-4AM. This synthetic probe has a spiking kinetic of 4ms and allows a better detection of calcium intracellular calcium kinetic in organelles (Hagen et al., 2012). However, chemical probes have to be delivered via invasive physical or chemical methods and while fluo-4 can diffuse into the embryo and label cells (Enyedi et al., 2016) and it would not be possible to observe fluo-4 in in a specific tissue unlike genetic probes that can be expressed under the control of a tissue specific promoter.

To visualise calcium dynamics in swarming neutrophils I used the laser wound assay because it allowed me to monitor the entirety of the neutrophil response and the calcium dynamics before, after and during the wounding process. After I performed a laser wound on a live 3dpf zebrafish larva (Fig 3.2, B), I observed a calcium wave (CW) propagating in neutrophils in less than 30 seconds (Fig 3.2, C). This is a well characterised phenomenon that might contribute to the early stage of cell migration during the course of the immune response and which is likely to be due to cell to cell transfer of ATP (Sieger et al., 2012). Neutrophils reached the site of injury within the first 10min post laser wounding. Upon reaching the wound, neutrophils cluster and exhibit high GFP chromophore brightness associated with strong intracellular calcium elevation that is on average sustained across the cluster but fluctuates within individual cells (Fig 3.2, C). This indicates that neutrophil arrest is associated with a sharp rise of intracellular calcium concentration.

Quantification showed that mean calcium intensity in clustering cells was sustained at high levels through the first hour post wound in comparison to migrating cells (Fig. 3.2, D). By plotting the distribution of cluster size and calcium intensity we found a positive association between the two parameters (Fig. 3.2, E). I concluded that in neutrophils at the cluster the calcium concentration is significantly higher than for the average neutrophil in the whole area. All neutrophils stopping at the cluster showed a similar calcium pattern and the overall GCamp6F fluorescence was maintain among cells in the cluster and gradually increased overtime, which is consistent with our observation that newly recruited neutrophils display a high intracellular calcium concentration.

All in all, our data highlighted an unusual calcium pattern in neutrophils clustering at the wound, characterised by a sharp intracellular and overall sustained calcium elevation. This calcium increase was associated with neutrophil stopping.

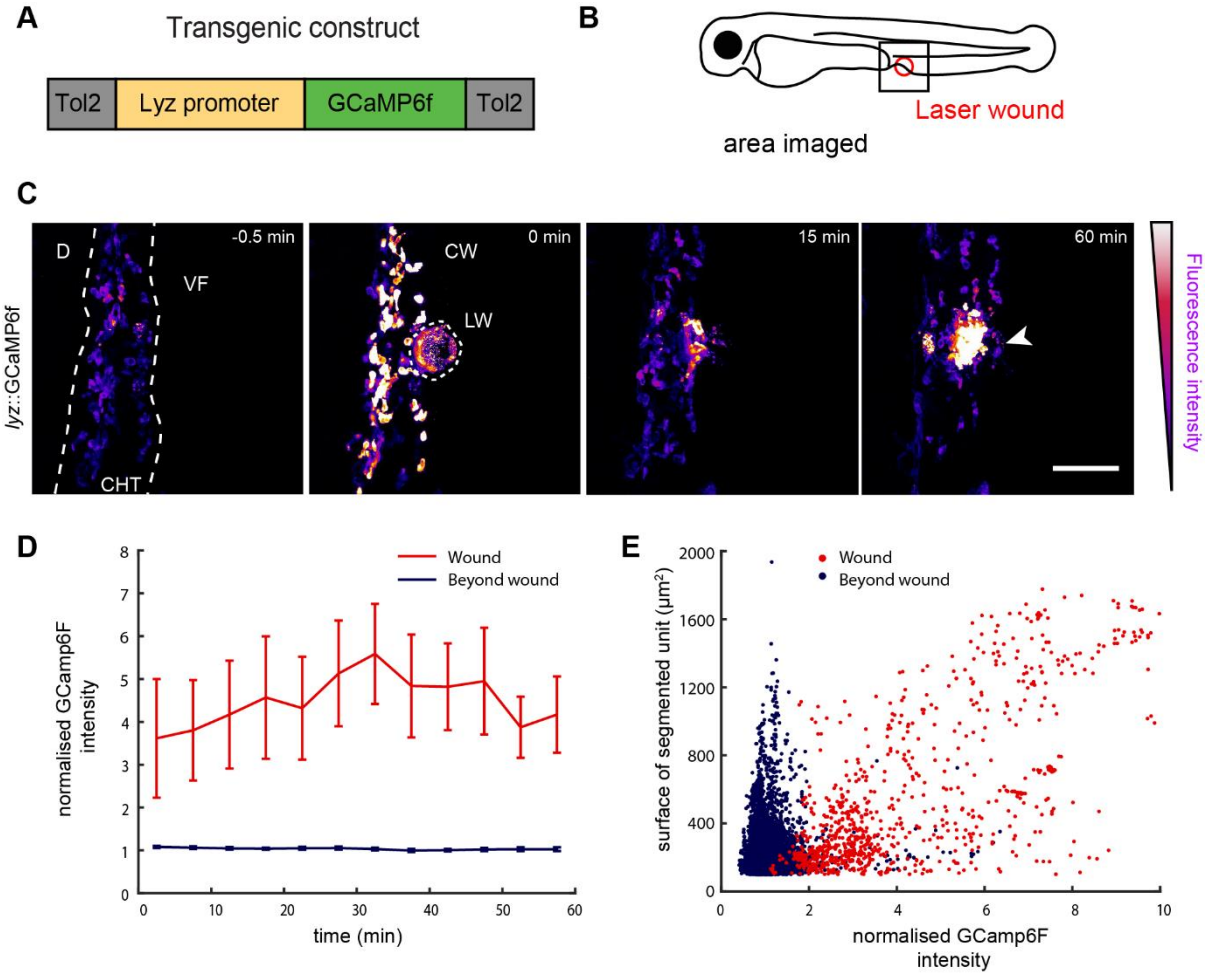


Figure 3.2. Calcium dynamics in neutrophils during swarming

A Schematic of a 3dpf zebrafish larva showing the area imaged and location of two-photon laser wound damage. B The GCamp6F construct is expressed under the control of the lysozyme C promoter (Lyz). C Maximum intensity z projection time-lapse sequence of neutrophils in a Tg(*lyz*::GCamp6F) larva. Images show neutrophils (colour-coded for GCamp6F intensity) within the caudal hematopoietic tissue (CHT) migrating towards the laser wound damage (LW), induced at the border between the Ventral Fin (VF) and the CHT. The calcium wave (CW) is indicated in the second panel and the neutrophil cluster is indicated with a white arrow. Dotted line outlines wound. Scale bar, 50µm. D Quantification of GCamp6F intensity over time in neutrophils at the wound versus neutrophils beyond the wound. n=8 larvae in 8 imaging sessions. E Quantification of GCamp6F intensity in relation to the surface area of segmented neutrophils. Raw intensity values were normalised to the mean intensity of segmented neutrophils prior to wound, to account for variation in imaging settings. Individual dots represent single cells or clustered cells at the wound (red) or beyond the wound (blue). As an indication, the surface area for single neutrophils is up to 400 µm^2. Values scale further with clustering. n=8 larvae in 8 imaging sessions. Error bars indicate standard error of the mean. Quantification was performed by Antonios Georgantzoglou.

3.4 Cells stop after contact with necrotic tissue

Sustain elevation of intracellular calcium concentration is generally associated with apoptosis and autophagy within a variety of cell types (Giorgi et al., 2018). This led me to think that cell death could occur in clustering neutrophils at the damage site. To understand the interactions of neutrophils with the necrotic tissue and to determine if neutrophils within the cluster were alive despite maintaining a very high intracellular calcium concentration, cell death in response to a laser wound injury was assessed.

I monitored cell death using PI (Propidium Iodide), a fluorescent intercalant DNA agent that is not permeant to live cells (Johnson Simon et al., 2013). Propidium iodide stains nucleic acids in early necrotic cells which membranes are readily compromised and late apoptotic cells. While PI cannot penetrate the skin of the zebrafish larva, wounding allowed transient interstitial access to the dye and staining of the local necrotic cells.

Tg(*lyz*::GCamp6F) larvae were embedded in 1% agarose in the continuous presence of PI in the bath and were subsequently subjected to laser wound injury in the area of the CHT (Fig. 3.3, A). In less than 30sec after performing the wound on the larva, necrotic cells in the laser wound area were stained with propidium iodide (Fig. 3.3, B). I found that neutrophils underwent a calcium flux upon direct contact with necrotic tissue concomitant with cessation of migration (Fig. 3.4, A, B). We monitored the speed and fluorescent intensity of GCamp6F expressing neutrophils 2 minutes before and 2 minutes after entering in contact with the necrotic tissue (Fig. 3.4, C). While neutrophils' speed decreased upon contact with dead cells, neutrophil's intracellular calcium concentration increased. This indicated that neutrophils were stopping upon contact with necrotic tissue. As I previously described, stopping was associated with a rise of intracellular calcium concentration within the neutrophils.

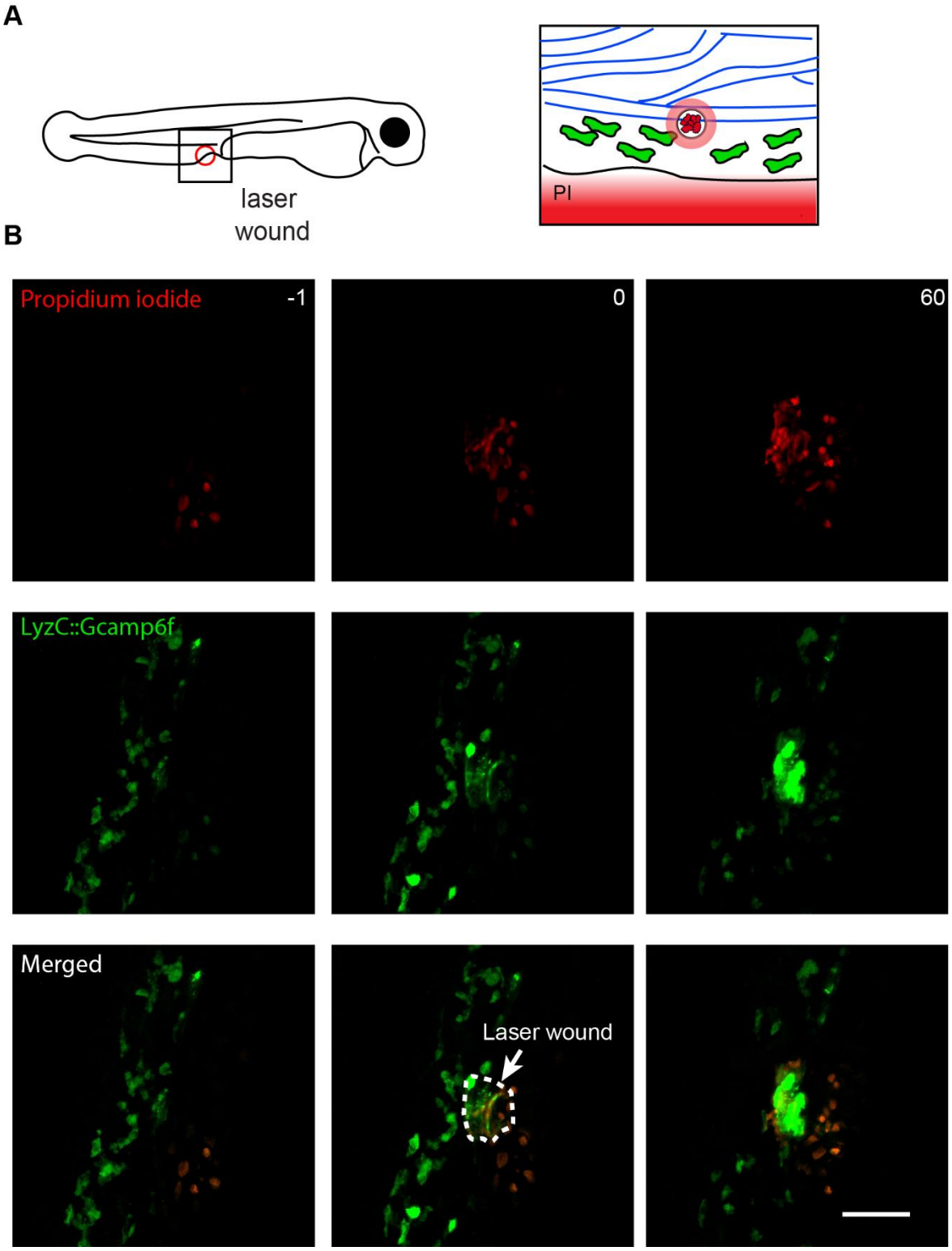


Figure 3.3. cell death during neutrophil swarming

A Two-photon laser wounding was performed on 3dpf larvae in the continuous presence of propidium iodide (PI) in the embryo medium. B Confocal time-lapse projections of neutrophils expressing GCamp6F (green) in 3dpf larvae incubated in PI (red). The time is indicated in minutes and is relative to the laser wound injury. Dotted line indicates laser wound. Scale bar = 50µm.

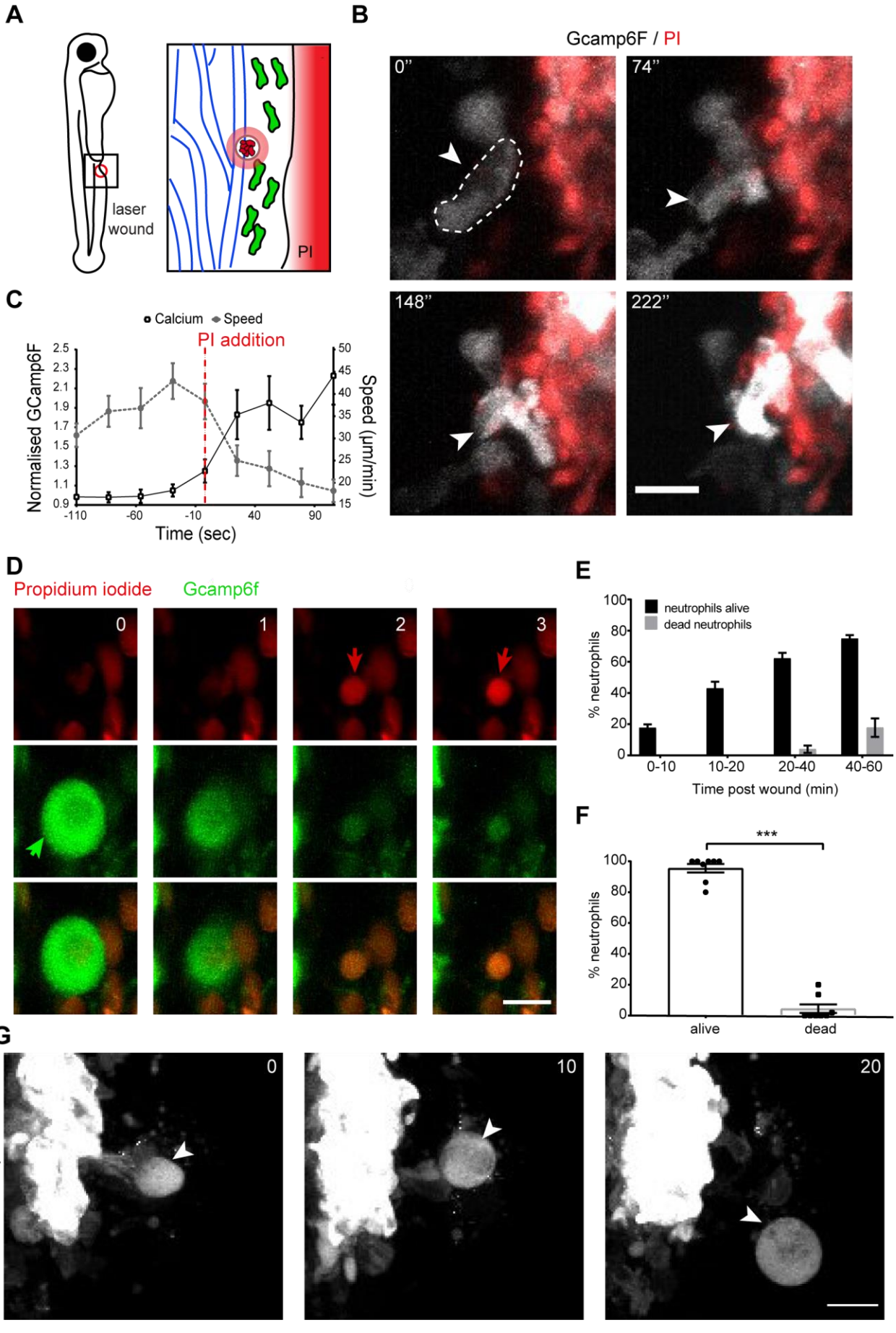

A
laser
wound
PI
B
Gcamp6F / PI
0''
74''
148''
222''
C
Calcium
Speed
PI addition
Normalised GCamp6F
Speed (µm/min)
Time (sec)
D
Propidium iodide
Gcamp6f
0
1
2
3
E
neutrophils alive
dead neutrophils
% neutrophils
0-10
10-20
20-40
40-60
Time post wound (min)
F
***
% neutrophils
alive
dead
G
Gcamp6f
0
10
20

Figure 3.4. Kinetics of neutrophil death in the first hour post wound

A Two-photon laser wounding was performed on 3dpf larvae in the continuous presence of propidium iodide (PI) in the embryo medium. B Sequence of maximum intensity projection images of a GCamp6F-expressing (white) neutrophil (indicated with an arrow) entering a contact with PI^+ tissue cells (red); Time in relation to the first frame is indicated in seconds. Scale bar, 10µm. **C** Quantification of speed and calcium ratio in neutrophils before and after their contact with propidium iodide, n= 3 experiments (4 larvae). D Zoomed in example of an apoptotic neutrophil. The green arrow indicates a neutrophil with an apoptotic shape in the GCamp6F channel. The red arrows indicate the nucleus of this neutrophil subsequently up taking PI. Scale bar = 10µm. E, F Percentage of dead or alive neutrophils over time E or averaged through the first hour post-wound F. Data pooled from 8 larvae in 7 independent experiments. Mann-Whitney test, two tailed, P-value= 0.0002. GCamp6F-expressing (white) neutrophil being ejected from the cluster while undergoing apoptosis. Time in minute in relation to the first ejection. Quantification was performed by Morgane Boulch. Scale bar =10µm. Error bars indicate standard error of the mean.

I was able to detect dying neutrophils with the presence of interstitial PI. These neutrophils were distinguished by loss of GCamp6F signal followed by uptake of PI stain (Fig. 3.4, F). The number of dead cells increased as the inflammation build up during the first hour post wounding (Fig. 3.4, E). Importantly, only few neutrophils were dying in comparison to the overall number of neutrophils within the cluster. Additionally, more than half (58%) of the total number of neutrophils coming to the tissue damage site had already reached the wounded tissue when the first dead neutrophil was detected (Fig. 3.4, F, G).

Taken together, our data demonstrate that following a sterile wound injury, neutrophils migrate towards the injury and stop upon contact with necrotic tissue. When neutrophils engage with necrotic cells their intracellular calcium concentration rises. Despite maintaining a high intracellular calcium concentration, most of the neutrophils stopping at the site of injury were alive.

3.5 Actin dynamics in neutrophil clustering

Another important molecule in cell migration is actin. This major component of the cytoskeleton is important in cell migration, immune response and tissue repair (Abreu-Blanco et al., 2011; Cooper, 2000). To visualise actin dynamics, I used a transgenic zebrafish reporter line Tg(*lyz*:LifeActRuby) expressing LifeActRuby in neutrophil only (Riedl et al., 2008) (Fig. 3.5, A). LifeActRuby is a bioprobe for F-actin (Filamentous actin; this is the part of actin that composes actin microfilaments which are an essential part of the cytoskeleton as opposed to monomeric G-actin [globular G-actin]). It is composed of lifeact (17 amino acids of yeast Abp140) (Riedl

et al., 2008) fused to Ruby and detects all F-actin. Lifeact does not interfere with actin dynamics *in vivo* or *in vitro*.

I performed a laser wound on 3dpf Tg(*lyz*::LifeActRuby) larvae near the CHT (Fig. 3.5, B). During swarms, I observed actin polarisation in migrating neutrophils, with enrichment of actin at the leading edge of these cells and stable level of F-actin on the side and the tail. This is consistent with what has been previously described by Yoo *et al.* (Yoo et al., 2010) in neutrophil migrating towards a tail fin injury in similar Tg(*lyz*::LifeActRuby) zebrafish larvae. Interestingly, following neutrophil accumulation at the wound, I observed the complete relocation of F-actin within clustering neutrophils. In migrating neutrophils, it was possible to distinguish the edges of the neutrophil cells with the LifeActRuby but not in clustering neutrophil. F-actin gradually accumulated at the centre of the laser wound and disappeared from the rest of the cell. This accumulation of neutrophil's F-actin at the centre of the wound looked like a supracellular actin ring (Abreu-Blanco et al., 2011).

To conclude, I observed a distinctive F-actin distribution in neutrophils clustering at site of injury during swarms. This F-actin distribution pattern is reminiscent of actin rings observed in drosophila.

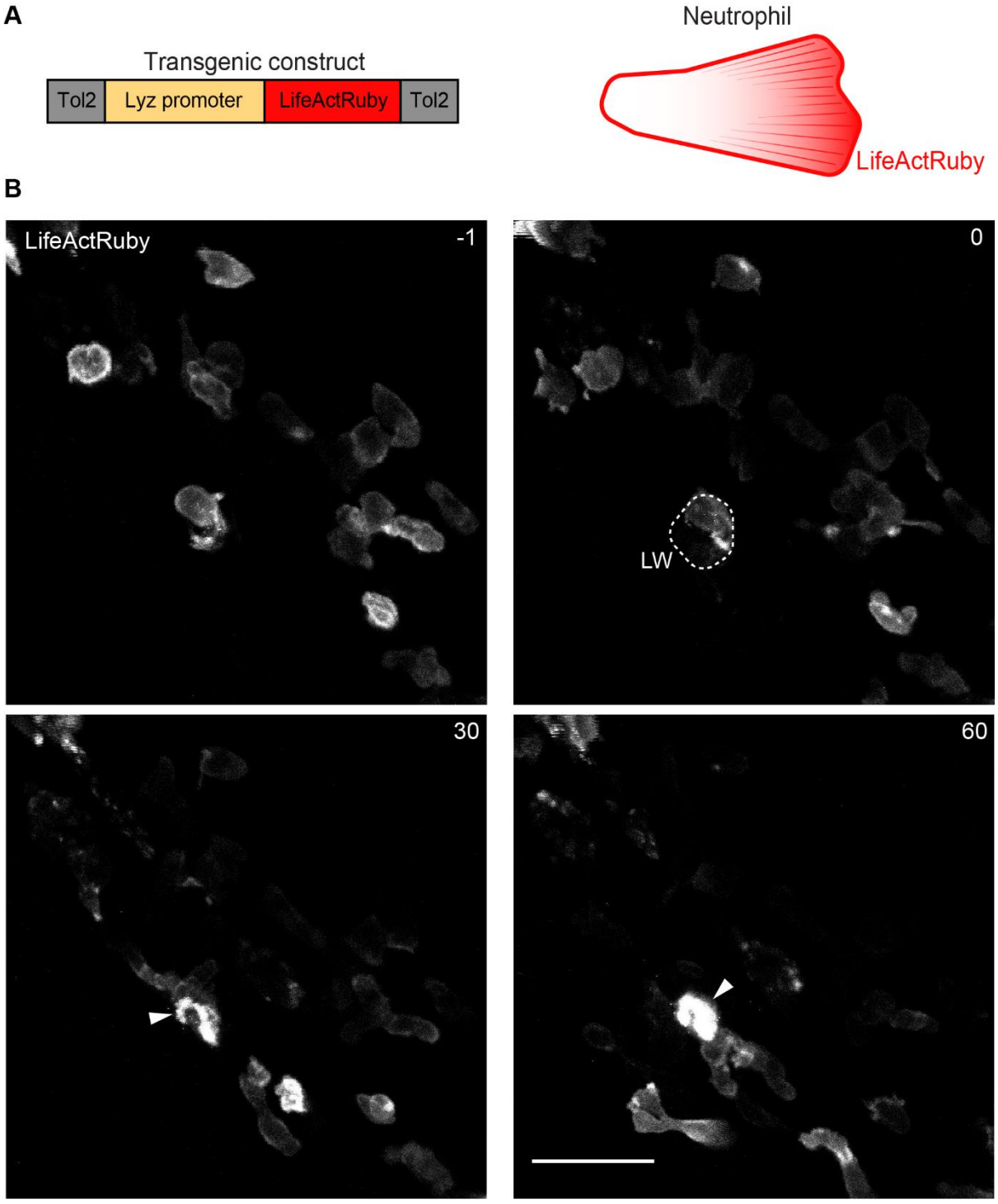


Figure 3.5. Actin dynamics during neutrophil swarming

A The LifeActRuby construct is expressed under the control of the lysozyme C promoter (Lyz). F-actin is visible on neutrophils expressing this construct. B Sequence of maximum intensity projection images of a LifeActRuby-expressing (white) neutrophils swarming towards a laser wound (LW, highlighted with dotted line) induced by a two-photon laser. The supracellular neutrophil actin ring is indicated with white arrows. Time related to the laser wound is indicated in minute; scale bar= 25µm.

3.6 Conclusion

In this chapter, I described a way to generate neutrophil swarms in response to sterile wound injuries in the zebrafish larva model. Building on this assay to further study the molecular dynamics of signals occurring during neutrophil swarms, I established a new transgenic zebrafish line stably expressing GCamp6F under the control of a neutrophil promoter. Using this calcium reporter, I showed that neutrophil swarming towards an injury exert an unusual calcium pattern upon stopping and clustering at the wound. This pattern is characterised by a sustained rise of intracellular calcium concentration. I further demonstrated that the elevation of intracellular calcium in neutrophils was triggered when neutrophils were entering in contact with necrotic epithelial tissue. Finally, I demonstrated that, in my model, neutrophil death was not required for the generation of neutrophil swarms. All in all, it appears that during swarming, neutrophils are in an unusual highly active state when clustering at the site of injury.

3.7 Discussion

First of all, it was important to establish an assay to trigger neutrophil swarms in zebrafish larvae. To allow for repetition of experiments to show statistically significant patterns in neutrophil migration behaviour and calcium dynamics I developed two assays to consistently trigger neutrophil swarms. The first assay consists in wounding the larva by performing a 2-photon laser injury. Laser injury has previously been used to elicit swarms in mice enriched with neutrophils by external pressure or cell transplantation (Lämmermann et al., 2013). The number of neutrophils recruited in my model is fewer than what was observed in the rodent model possibly because fewer neutrophils are present in 3dpf zebrafish larvae. The second assay consists in performing a mechanical wound with a scalpel. This has previously been performed on fin tail but usually triggering the migration of a dozen of neutrophils to the tip of the fin. Performing this mechanical wound near a tissue rich in neutrophils (CHT) allowed for the triggering of swarms. However, swarms elicited by mechanical injury had a different neutrophil behavioural migration pattern than swarms elicited by laser injury. The laser injury is convenient to follow the dynamics of neutrophils before, after and during the laser injury directly on the microscope as the injury is being performed. Additionally, the neutrophil behaviour in swarms triggered by laser wound injury was very consistent and neutrophils were quickly recruited to the injury. Using laser wounds, neutrophil migratory pattern characterised by the normalised radial speed was very similar to the one observed by Lämmermann and its colleagues in mice (Lämmermann et al., 2013). In the case of mechanically induced wounds, neutrophil recruitment was delayed and started only 45min post wounding. The migration

pattern of neutrophil from larva to larva. This is probably because mechanical wounds were performed manually and are therefore less consistent than laser wounds. The advantage of mechanical wounds however is the possibility to use a different microscope than the 2-photon such as the confocal spinning disc allowing higher resolution and fast imaging.

After establishing assays to trigger neutrophil swarms, I used GCamp6F, a genetically encoded calcium probe, to examine calcium dynamics in swarming neutrophils. As previously described in other studies, I observed a calcium wave upon tissue injury (Sieger et al., 2012) and polarisation of calcium distribution in cells, with accumulation of calcium at the leading edge of migrating cells (Beerman et al., 2015). An unexpected and important finding was the high, sustained calcium elevation in neutrophils stopping and clustering at the site of injury. This is the first time this is observed because no previous study of neutrophil swarms made use of a neutrophil-specific calcium probe. As calcium is important for many physiological processes, such as triggering the production of pro-inflammatory molecules, this observation has major implications for the role of neutrophils at the site of injury in the cluster (Dixit and Simon, 2012; Luo et al., 2003).

Calcium loading that exceeds the capacity of calcium regulatory mechanisms may trigger processes that lead to the formation of toxic products leading to cell death (Sattler and Tymianski, 2000). This phenomenon was shown in neurons and is called excitotoxicity. Notably, it was shown in neuronal culture stained with the calcium probe fura-2 that cell death was directly linked with the calcium concentration in cells. In this study, glutamate receptors (which are gated ion channels that facilitate calcium influx into cells) were stimulated by their agonist NMDA (*N*-methyl-D-aspartate) and AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) to stimulate calcium influx in neurons (Hyrc et al., 1997). Neutrophil in the cluster elicit a high sustain intracellular calcium concentration. To verify whether or not those neutrophils are alive, I used PI to monitor cell death during swarms. I observed very few neutrophil death events during swarms, and those events were occurring after most neutrophils were already recruited to the clustering site of injury. This would mean that neutrophil's death may not be absolutely essential for the recruitment of neutrophils to the site of injury. This appear inconsistent with two previous reports suggesting that neutrophil death might be necessary to trigger the recruitment of enough neutrophils to start a swarm (Lämmermann et al., 2013; Uderhardt et al., 2019). To clarify the impact of neutrophil's death on recruitment, precise laser ablation of single neutrophil could be performed using 2-photon laser ablation. The subsequent migration behaviour from surrounding neutrophils could be analysed. I also observed that dead neutrophils were mostly ejected from the cluster. this could be a mechanism involved in the resolution of inflammation, as apoptosis is considered itself a major means of resolution (Fox et al., 2010).

Using a reporter for F-actin, I observed a structure reminiscent of supracellular actin ring previously described in drosophila after laser wounding (Hannezo et al., 2015). Based on this observation I deducted that it was possible that a supracellular actin ring could exist in clustered neutrophils and play a role in stabilising these cells at the wound. Neutrophils could also be involved in the process of wound closure which would contradict the notion that neutrophils are delaying wound closure (Dovi et al., 2003).

Further experiments remain to be done in order to fully understand actin dynamics in neutrophil clusters. FRAP could be performed on LifeActRuby expressing cells to see the speed of actin ring generation during inflammation. Cytochalasin B is a drug that inhibits the addition of G-actin onto actin filament, embryos could be incubated with this drug to perturb actin and assess the impact on wound healing like what has been done to study actin ring in Xeopus oocytes wounds (Mandato and Bement, 2001).

4. LTB4 signalling dynamics in neutrophil swarming

4.1 Introduction

Neutrophils respond to myriads of chemoattractants during the immune response. Lämmermann *et al* showed, using a mouse model, that for neutrophil swarming one attractant was particularly essential, a lipid called LTB4 (leukotriene B4) (Lämmermann et al., 2013). Using LTB4 receptor knock-out in their model, they observed a severe inhibition of neutrophil swarming. LTB4 is a small leukotriene that is produced and sensed by neutrophils themselves. It is a product of arachidonic acid (AA) metabolism taking place at the nuclear membrane. Following cell activation and intracellular calcium elevation, cPLA2 (calcium-dependent PhosphoLipase A2) and 5-LO (5-Lipoxygenase) are recruited to the inner nuclear membrane (Fig. 4.1, A) (Brock et al., 2001; Iversen et al., 1994; Peters-Golden and Brock, 2003; Rådmark and Samuelsson, 2010; Yokomizo, 2011) from the interior of the nucleus. cPLA2 catalyses the release of AA from membrane phospholipids (Peters-Golden and Brock, 2003). Then 5-LO converts AA into LTA4, with the help of FLAP (5-LO activating protein), an integral nuclear membrane protein. LTA4 diffuses to the cytosol, where it is further metabolised into LTB4 by LTA4H (LTA4 hydrolase) (Brock et al., 2001; Iversen et al., 1994). LTA4 can also be processed into an anti-inflammatory molecule, lipoxin A4, however, neutrophils are geared to produce LTB4 (Kienle and Lämmermann, 2016; Serhan and Sheppard, 1990; Tobin et al., 2010).

The production dynamics of LTB4 by neutrophils during swarming are still not clear. *In vitro* work with human neutrophils showed that LTB4 was produced by neutrophils in response to fMLP stimulation (Afonso et al., 2012). In this article, the authors hypothesised that neutrophils may produce LTB4 as they migrate towards the primary attractant fMLP, amplifying the range of recruitment by relaying the signal from neutrophil to neutrophil ('signal relay' model). However, in this study, the *in vitro* under agarose assay used by the authors for studying neutrophil migration does not recapitulate neutrophil swarms and does not recreate the complexity of the *in vivo* environment (Nelson et al., 1975). Damage signal stemming from the wound are very diverse and including molecules such as histones, DNA, RNA or ATP released from dead cells, all of which can signal to neutrophils and impact their behaviour by binding PRR (Pattern Recognition Receptors) at the surface of the cells (Anders and Schaefer, 2014).

It is therefore not clear if the dynamics of LTB4 production during swarming *in vivo* actually rely on the signal relay mechanism suggested by Afonso *et al*. LTB4 signalling dynamics is likely to be a major determinant for controlling neutrophil swarms. By allowing the large recruitment of neutrophils, LTB4 plays a pivotal role in inflammation. Accumulation of neutrophils at site of injury can be beneficial for clearing pathogens but can also have some undesirable effects such as collateral damage to the tissue due to the release of proteolytic enzymes (Kolaczkowska and Kubes, 2013). Reducing neutrophil accumulation is therefore an important target for chronic inflammatory diseases, such as rheumatoid arthritis (Cecchi et al., 2018). Understanding LTB4 dynamics might help us to devise strategy to tune neutrophil accumulation to desirable levels.

As LTB4 is a small lipid molecule, it is so far not possible to observe it *in vivo*. Subcellular events associated with LTB4 signalling must be visualised instead. This is easier in zebrafish larvae than in mice, as the larvae are transparent and easier to image. Additionally, hundreds of zebrafish eggs can be obtained from one pair of fish allowing for many more repeats that in mice. Finally, in chapter 3, I demonstrated an assay to consistently trigger neutrophil swarms in zebrafish that unlike in mouse, do not require to pre-recruit neutrophils from the circulation and are quicker to execute (Lämmermann et al., 2013).

In the zebrafish larva model, neutrophils were also shown to use the LTB4 pathway during inflammatory responses. Indeed, it has been shown in zebrafish larvae that during *Mycobacterium marinum,* LTB4 played a major role for neutrophil recruitment to sites of infection (Tobin et al., 2010). The authors notably showed that LTB4 injection in the ear of the larva results in rapid neutrophil recruitment which can be inhibited by the LTB4 receptor blocker U75302. A similar experiment was performed by Yang et al, showed recruitment of zebrafish neutrophils after injection of LTB4 in the hindbrain (Yang et al., 2012). However, as neutrophil swarming in zebrafish has never been mechanistically studied, the link between LTB4 signalling and zebrafish neutrophil swarming has not yet been established.

In this chapter, I demonstrate a new biosensor to probe LTB4 biosynthesis. Using the assays, I presented in Chapter 3, along with this new tool, I describe for the first time the dynamics of LTB4 production in swarming neutrophils *in vivo*, challenging the concepts of the current hypothesis. Building on previous data, I show a link between the calcium patterns I observed in clustering neutrophils and LTB4 production dynamics.

4.2 The role of neutrophil-derived LTB4 in swarming is conserved in zebrafish

Neutrophil swarming appears to be conserved across species from human to zebrafish (Deng et al., 2013; Kienle and Lämmermann, 2016; Lämmermann et al., 2013). However, the link between LTB4 and neutrophil migration to inflammatory sites in zebrafish has yet to be demonstrated. In order to verify that the role of neutrophil LTB4 production is conserved in zebrafish, I genetically inhibited leukotriene A4 hydrolase, an enzyme that catalyses the conversion of LTA4 into LTB4 (Peters-Golden et al., 2005); the final step in LTB4 biosynthesis. For this I used a previously validated splice-blocking morpholino (Vincent et al., 2017) to suppress *lta4h* expression. I confirmed the presence of an alternative transcript in 3dpf larvae injected with the morpholino (Fig. 4.1, A). To observe a significant difference in neutrophil recruitment to sites of tissue damage, I performed tail fin wounds and measured neutrophil recruitment in larvae fixed after 3 hours. This allows for a high number of embryos to be assessed in comparison with live imaging.

In wild type (WT) larvae injected with *lta4h* MO the accumulation of neutrophils at the wound was severely diminished (Fig. 4.1, B, C). The phenotype observed with MO knockdown was rescued by neutrophil-specific transgenic expression of LTA4H. Specifically, I injected the *lta4h* MO in a transgenic zebrafish line I generated, Tg(*lyz:lta4h*-eGFP) (Fig. 4.1, D), expressing the fusion of LTA4H with eGFP in neutrophils only. Injection of *lta4h* MO did not suppress neutrophil accumulation in these transgenics (Fig. 4.1, D).

This confirmed that autocrine LTB4 signalling plays a role in zebrafish neutrophil accumulation at wounds, as observed in mammalian systems.

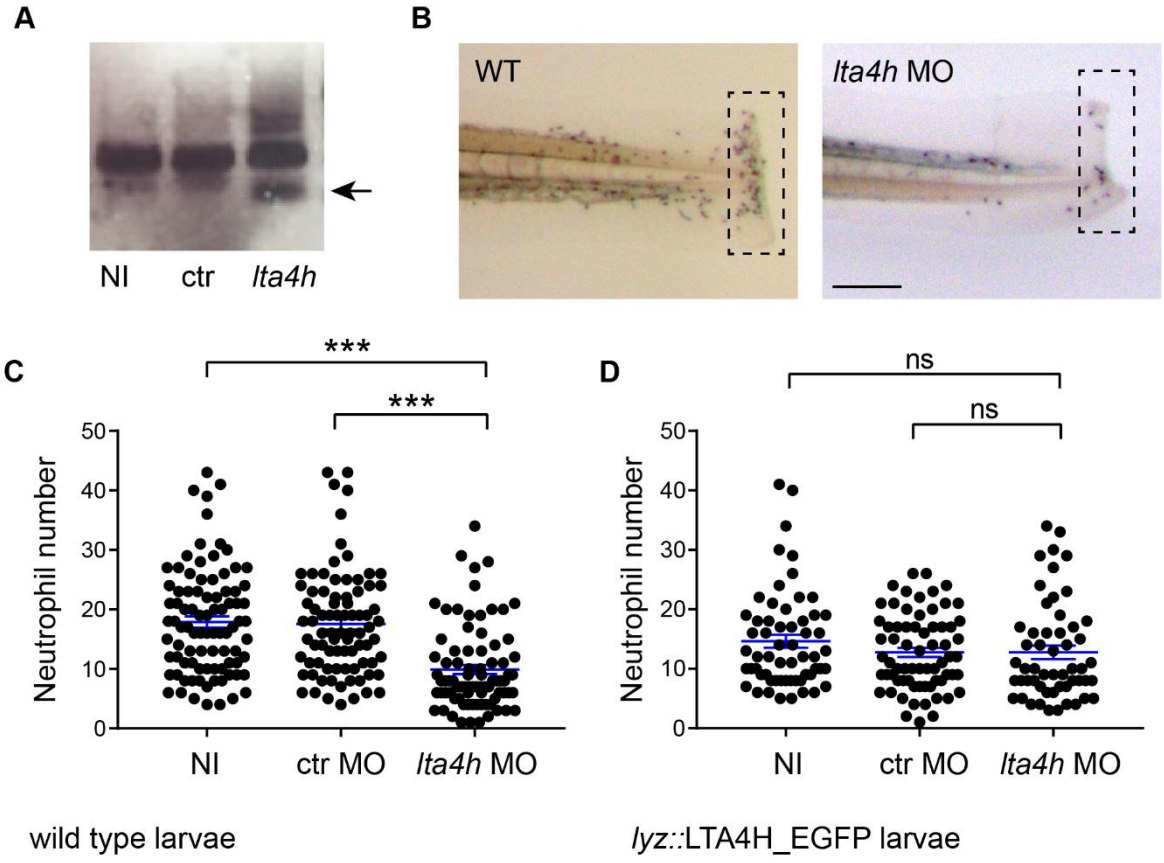


Figure 4.1. Role of neutrophil biosynthesis of LTB4 in zebrafish wounds.

A RT-PCR of *lta4h* from mRNA extracted from 3 dpf zebrafish. The arrow denotes the presence of an alternative transcript in larvae injected with a splice-blocking *lta4h* morpholino; NI = Non-Injected, ctr = Control, *lta4h* = injected with the splice-blocking *lta4h* morpholino. B Sudan black staining of neutrophils in WT and *lta4h* morpholino-injected 3dpf larvae. Embryos were injured with a scalpel and fixed 3h after wounding. The dotted lines represent the area in which neutrophils were counted; scale bar = 100µm. C, D Quantification of the total number of neutrophils recruited to control and morphant wounds. C 81-89 larvae per group pooled from 3 experiments. D 54-68 larvae per group pooled from 3 different experiments. p value <0.0001. Kruskal-Wallis test with Dunn's post-test.

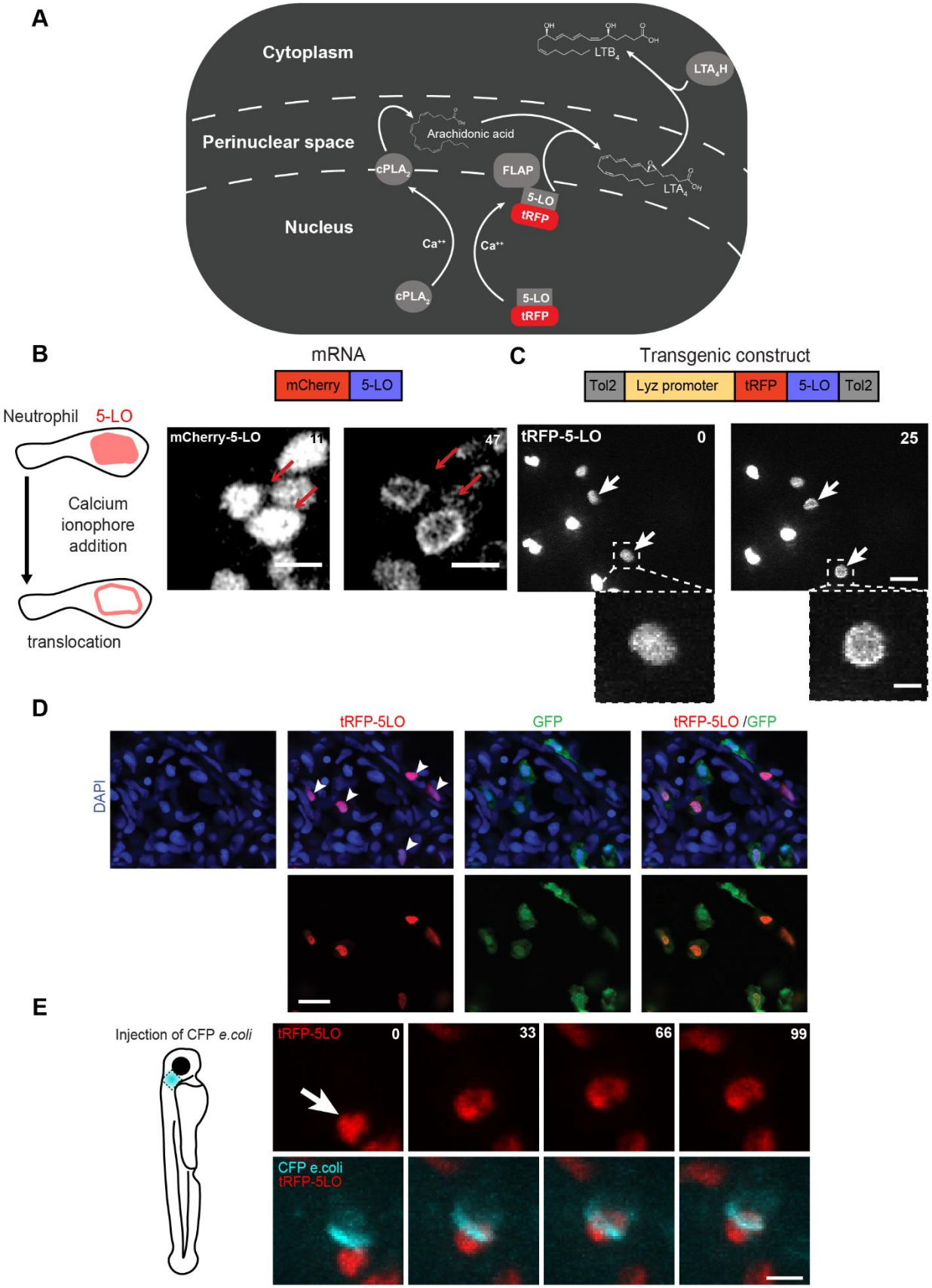

A
Cytoplasm
Perinuclear space
Nucleus
Arachidonic acid
cPLA2
FLAP
5-LO
tRFP
LTA4H
LTB4
LTA4
Ca++
Ca++
cPLA2
5-LO
tRFP
B
mRNA
mCherry
5-LO
Neutrophil
5-LO
Calcium ionophore addition
translocation
mCherry-5-LO
11
47
C
Transgenic construct
Tol2
Lyz promoter
tRFP
5-LO
Tol2
tRFP-5-LO
0
25
D
tRFP-5LO
GFP
tRFP-5LO /GFP
DAPI
E
Injection of CFP e.coli
tRFP-5LO
0
33
66
99
CFP e.coli
tRFP-5LO

Figure 4.2. A biosensor to follow LTB4 biosynthesis dynamics through 5-LO translocation.

A Schematic of LTB4 biosynthesis. cPLA2 (calcium-dependent phospholipase A2) and 5-LO (5-lipoxygenase) are recruited to the inner nuclear membrane and produce arachidonic acid (AA) and LTA4 respectively. LTA4 diffuses to the cytosol where it is further metabolised into LTB4 by LTA4 hydrolase. B Construct for ubiquitous transient mRNA expression of a fluorescent fusion of 5-LO with mCherry (top). Schematic of neutrophil with 5-LO nuclear translocation (left). Timelapse of 2dpf zebrafish injected with mRNA of the construct described above. Cells are shown translocating 5LO-mCherry after 50µM calcium ionophore A23187 addition. (time in relation to calcium ionophore addition is indicated in minutes) Scale bar= 5µm. C Constructs for transgenic expression of a fluorescent fusion of 5-LO with tRFP in neutrophils (top). Time-lapse images of neutrophils translocating 5-LO in 3dpf transgenic Tg(*lyz*:5LO-tRFP) zebrafish larvae taken on the spinning disc before and after incubation with calcium ionophore (bottom). Zoomed images of neutrophils showing 5LO-tRFP translocation (time in relation to calcium ionophore addition is indicated in minutes). Scale bars= 10 µm and 5 µm. D Whole-mount Immunostaining of a fixed 3dpf zebrafish larva. Imaging of cells in the Caudal Hematopoietic Tissue (CHT). Nuclei are stained with DAPI (blue). GFP (green) and 5LO-tRFP (red) are only expressed in neutrophils. GFP is localised in neutrophil nucleus. White arrows show 5LO-tRFP co-localising with DAPI indicating that 5LO-tRFP is localised in the neutrophil's nucleus. Scale bar= 10µm. E Schematic of a 3dpf zebrafish larvae injected in the interstitial tissue between the ear and the high with ECFP expressing *E. coli* (left). Image of a live 3dpf larva injected with CFP *E. coli* around the otic vesicle and the eye and expressing 5LO-tRFP in neutrophils. A translocation of 5LO-tRFP from the nucleus to the nuclear membrane is observed in a zebrafish neutrophil localised at the site of infection. Scale bar= 5µm. RNA injection and microscopy in B performed by Milka Sarris.

4.3 Probing for LTB4 biosynthesis in zebrafish neutrophils

As the key signal, LTB4 has never been directly monitored or manipulated *in situ*. I designed a strategy to visualise the activation of the biosynthesis of LTB4 *in vivo*. The critical steps in leukotriene synthesis occur at the nuclear envelope membrane (Luo et al., 2003). As mentioned in the introduction a major step for LTB4 production is the translocation of 5-LO from the nucleus to the inner nuclear membrane. Thus, to probe LTB4 biosynthesis in neutrophils I monitored the perinuclear recruitment of 5-LO (Fig. 4.2, A). Four genes coding for a protein similar to the human 5-LO are present in zebrafish (*ALOX2*, *ALOX3a*, *ALOX3b*, *ALOX3c*) (Peters-Golden and Brock, 2003). *ALOX2* is highly expressed during embryogenesis, shares the highest degree of amino acid conservation (74%) with the human ALOX5 and has been shown to exhibiting arachidonic acid 5S-lipoxygenase and leukotriene synthase activity (Peters-Golden and Brock, 2003). I therefore chose the *ALOX2* sequence to validate our approach, using a fluorescent fusion of this gene with mCherry (Fig. 4.2, B).

We injected mRNA of this construct to achieve transient, ubiquitous expression into zebrafish larvae. We observed a characteristic perinuclear distribution of the fluorescent fusion of the expressed construct after calcium influx induced with calcium ionophore A23187 treatment, as was described for human 5-LO (Luo et al., 2003) (Fig. 4.2, C). Global expression by mRNA injection is complex to analyse in detail because the signal is ubiquitous. Therefore, for further experimentations, I generated a stable transgenic line, expressing tRFP-5-LO under the control of a neutrophil-specific promoter (lysozyme C promoter; '*lyz*') (Lam et al., 2012, 2013; Peters-Golden and Brock, 2003). With the signal present only in neutrophils, it is easier to image and analyse quantitatively the translocation of 5-LO. Additionally, a stable transgenic fish gives more consistent results since there is minimal variation of transgene expression. For this transgenic, I used tRFP instead of mCherry because it possesses a higher brightness with 2-photon laser excitation at ~760 nm comparatively to mCherry (Peters-Golden and Brock, 2003).

In Tg(*lyz*:tRFP-5-LO) 3dpf zebrafish larvae, the location of tRFP-5-LO was consistent with what is observed in human cells, *in vitro*, as it was found to be soluble in the nucleus (Fig. 4.2, D) (Luo et al., 2003). To determine if the fusion protein translocated in neutrophils after an increase of intracellular calcium concentration, I incubated 3dpf zebrafish larvae with calcium ionophore A23187. After 25min I observed the translocation of tRFP-5-LO to its characteristic location at the nuclear membrane (Fig. 4.2, D). This confirmed the functionality of the construct and responsiveness to exogenous chemical stimuli.

5-LO translocation in neutrophils can also take place when cells are responding to an infection. To validate that this construct is translocated upon infection, I injected CFP-labelled *Escherichia coli* into the interstitial tissue between the otic vesicle and the eye of a 3dpf zebrafish larva (Fig. 4.2, E). This area contains neutrophils and allows quick visualisation of local neutrophils migrating towards a site of infection (see methods for details). Translocation of tRFP-5-LO to the nuclear membrane was observed in several neutrophils migrating towards the site of infection (Fig. 4.2, E). This indicated that the construct responds to the physiological stimuli of inflammation.

Altogether these data demonstrated a new functional tool to probe for LTB4 biosynthesis in neutrophils *in vivo* by visualising 5-LO translocation.

4.4 Activation of LTB4 synthesis pathway in clustering neutrophils

In chapter 3, I observed distinctive calcium dynamics in neutrophils clustering at the site of injury, characterised by high sustained intracellular calcium elevation. This prompted me to investigate if these calcium fluxes are consequential on LTB4 biosynthesis. To visualise 5-LO

translocation along with the calcium signals, I crossed our zebrafish line expressing 5-LO in neutrophils Tg(*lyz*:tRFP-5-LO) with Tg(*lyz:*GCamp6F) fish (Fig. 4.3, A). I monitored 5-LO translocation in neutrophils after acute laser wound, using spinning-disk microscopy in 3dpf larvae. I observed that tRFP-5-LO translocation occurred principally in neutrophils with a high calcium signal that were clustering at the wound focus and not in migrating cells (Fig. 4.3, B). Interestingly, I observed that swarms generated in Tg(*lyz*:tRFP-5-LO) larvae seemed to have a higher number of neutrophils recruited than swarms in Tg(*lyz:*GCamp6F) or Tg(*mpx:*GFP) larvae.

Because of the low resolution and low signal obtained by imaging tRFP-5-LO on the 2-photon, I realised that I could probably miss translocation events. I decided to either transfer the fish from the 2-photon to the spinning disc microscope after performing a laser wound or perform a mechanical wound (as described in chapter 3) and imaged the larva directly on the confocal spinning microscope in order to visualise tRFP-5-LO translocation events. I obtained higher image resolution and faster imaging using the confocal spinning disc microscope. I analysed results from mechanical wound separately. I discovered the same trend, in that 5-LO translocations were detected only among the clustering cells that exhibited whole-cell, high calcium fluxes (Fig. 4.3, C). We then quantified these dynamics by visually identifying 5-LO-translocating neutrophils and correlating this to their GCamp6F fluorescence level. To quantify GCamp6F I measured pixel intensity and normalised this to the average fluorescent level of other neutrophils in the same frame using a MATLAB script (see methods for more details). Neutrophils undergoing tRFP-5-LO translocation during laser induced swarms were within 20µm from the wound instead of 45 for the average non translocating neutrophils. In the case of the mechanical wound translocating neutrophils were on average within 10µm of the wound instead of 85µm for non-translocating neutrophils. This analysis confirmed that neutrophils translocating 5-LO are significantly closer to the wound and are displaying a significantly brighter GCamp6F fluorescence intensity in both laser and fin wound assays (Fig. 4.3, D and E). These results demonstrate that 5-LO translocation is specifically occurring in clustering cells with high intracellular calcium levels.

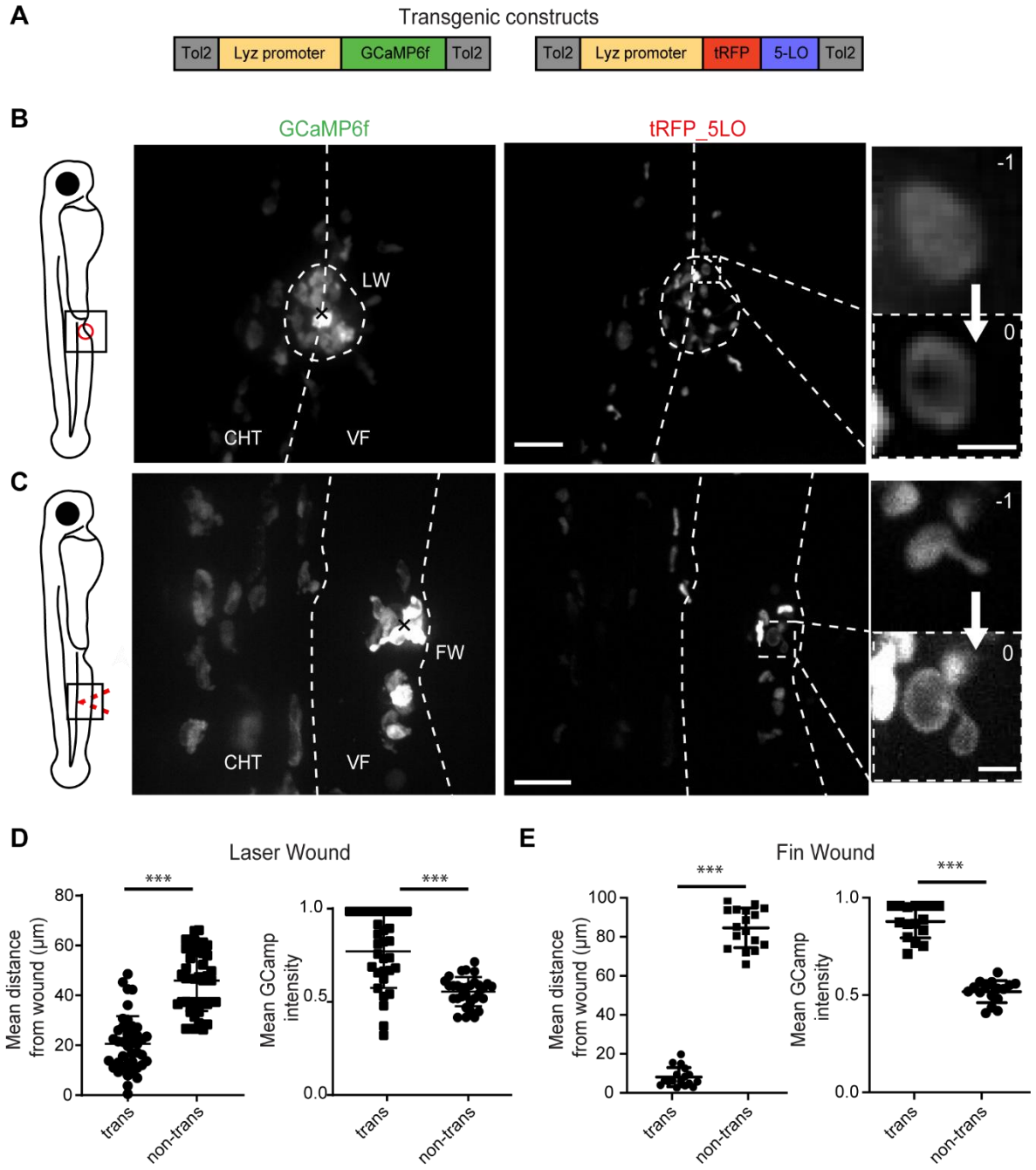


Figure 4.3. Activation of LTB4 biosynthesis is limited to clustering neutrophils.

A Stable transgenic line Tg(*lyz*:GCamp6F)xTg(*lyz*:tRFP-5LO) were crossed for subsequent experiments. B Maximum intensity spinning disk confocal time-lapse images of neutrophils in 3dpf double transgenic zebrafish larvae taken after two-photon laser wound in the ventral fin-CHT boundary C or after mechanical injury in the ventral fin. Zoomed images of neutrophils showing 5-LO translocation (time in relation to translocation indicated in minutes). Scale bars= 50 µm and 5 µm. D Quantification of mean distance from the wound center (indicated in c as red x) and normalised GCamp6F fluorescence intensity for 5-LO-translocating cells versus non-translocating cells in laser wounds D and fin wounds E. Data are from n=42 translocating and n=720 non-translocating cells (D left) and n=31 translocating and 289 non-translocating cells (d right) from 8 zebrafish larvae from 5 different experiments. Data are from n=17 translocating and n=634 non-translocating cells (E left) and n=16

translocating and 582 non-translocating cells (E right) from 5 zebrafish larvae from 3 different experiments. Two-tailed unpaired t test.

4.5 5-LO translocation in zebrafish neutrophils is initiated by intracellular calcium increase

To establish whether entry of extracellular calcium is sufficient to activate 5-LO translocation, I decided to artificially rise the neutrophil intracellular calcium concentration in neutrophils migrating towards a site of injury using a calcium ionophore. For this, I crossed Tg(*lyz*:tRFP-5-LO) and Tg(*lyz:*GCamp6F) and initiated neutrophil migration in the larva by performing a ventral fin wound. At 30-60 minutes post wounding, neutrophils started migrating towards the wound at which point a calcium ionophore was added in the bath in which the zebrafish was incubated (Fig. 4.4, A). Within 5 minutes following calcium ionophore addition, neutrophils experienced a prominent calcium flux that led to immediate arrest and concomitant translocation of 5-LO (Fig. 4.4, B). I quantified the percentage of 5-LO translocation overtime before and after calcium ionophore addition. I observed that 30min after calcium ionophore addition 90 to 100% of the neutrophils in the area imaged were translocating 5-LO. These results highlight the importance of calcium in neutrophil swarming and the correlation between high intracellular calcium, neutrophil stopping and 5-LO translocation.

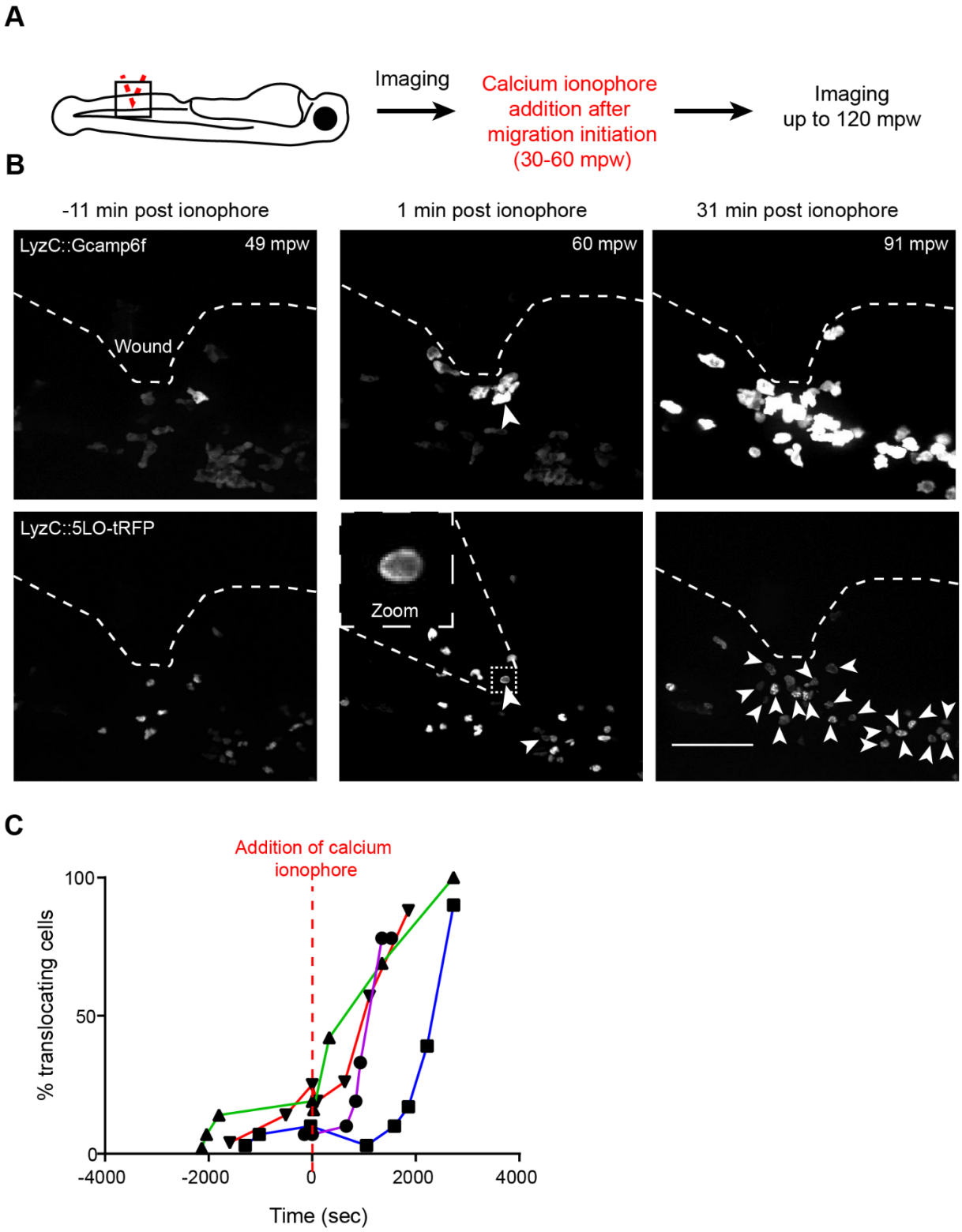


Figure 4.4. Extracellular calcium entry triggers 5-LO translocation in neutrophils *in vivo*.

A 3dpf zebrafish larvae Tg(*lyz*:GCamp6F) were wounded on the ventral fin (VF) and subsequently imaged on a spinning disc microscope for up to 120 minutes post wounding. 50μM calcium ionophore (A23187) was added after migration initiation, typically between 30- and 60-minutes post wounding (mpw) B The top images show confocal projections of the GCamp6F channel and the bottom images

show the 5-LO-tRFP channel at the indicated time points after wounding (the time relative to calcium ionophore is shown above the images). Arrows indicate translocation events. Scale bar = 50µm C Percentage of translocating cells over time. The time of calcium ionophore addition is set to zero. n=4 larvae from 2 different experiments.

4.6 Extracellular calcium is needed for neutrophil swarms

Calcium is known to play a role in neutrophil activation and signalling (Beerman et al., 2015; Dixit and Simon, 2012; Luo et al., 2003). Several pathways influence calcium dynamics in neutrophils (Putney, 2010; Shuttleworth et al., 2004). It was unclear which of these was at the origin of the fluxes observed neutrophils clustering at the wound focus. For this I decided to inhibit calcium signalling, using relevant inhibitors that have already been tested on zebrafish larvae (Sieger et al., 2012). I targeted various calcium pathways using chemical inhibitors. I used SKF (SKF 96365 hydrochloride), a broad inhibitor that blocks voltage-gated calcium and potassium channels (TRPC (Transient Receptor Potential Cation Channels) channel) and SOCE (Store-Operated Calcium Entry) (Beech, 2013). Interestingly, after performing a laser wound near the CHT, neutrophils in Tg(*lyz*::GCamp6F) larvae incubated with 20µM of SKF were still able to flux calcium and stop at the wound, although the recruitment seemed diminished (Fig. 4.5, A). Incubating larvae in 100 µM of another calcium pathway inhibitor, ML9 (Myosin Light chain) which inhibits SOCE (Store-Operated Calcium Entry) did not make any major difference in the calcium pattern elicited by neutrophils (Fig. 4.5, B). In both examples, the initial tissue calcium wave (a phenomenon that might contribute to the early stage of cell migration in response to an injury (Sieger et al., 2012)) was still observed.

To ask whether extracellular calcium entry is required for the calcium fluxes observed in clustering neutrophils, I used a calcium chelator EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid). Ventral fin wounds were performed on fish incubated in a bath of calcium free embryo medium with 1mM EGTA. The fish was subsequently mounted in 1% agarose and imaged on the spinning disc while being incubated in the bath of this calcium free medium supplemented with EGTA. Here, I observed a diminution of neutrophils recruited to the wound and less prominent GCamp6F increases of fluorescent in both migrating cells and clustering cells. Interestingly, I still observed the initial tissue calcium wave. The tissue calcium wave was only inhibited after injecting 10nL of a 1mM EGTA solution in the zebrafish circulation *via* the duct of cuvier (Fig. 4.5, C). This also completely inhibited neutrophil migration.

To conclude, extracellular calcium is required for neutrophil migration and stopping at the wound. Inhibitors of SOCE and TRPC pathways of calcium signalling did not show any notable

effect on the calcium fluxes of clustering neutrophils, suggesting other pathways of extracellular calcium entry to play a role.

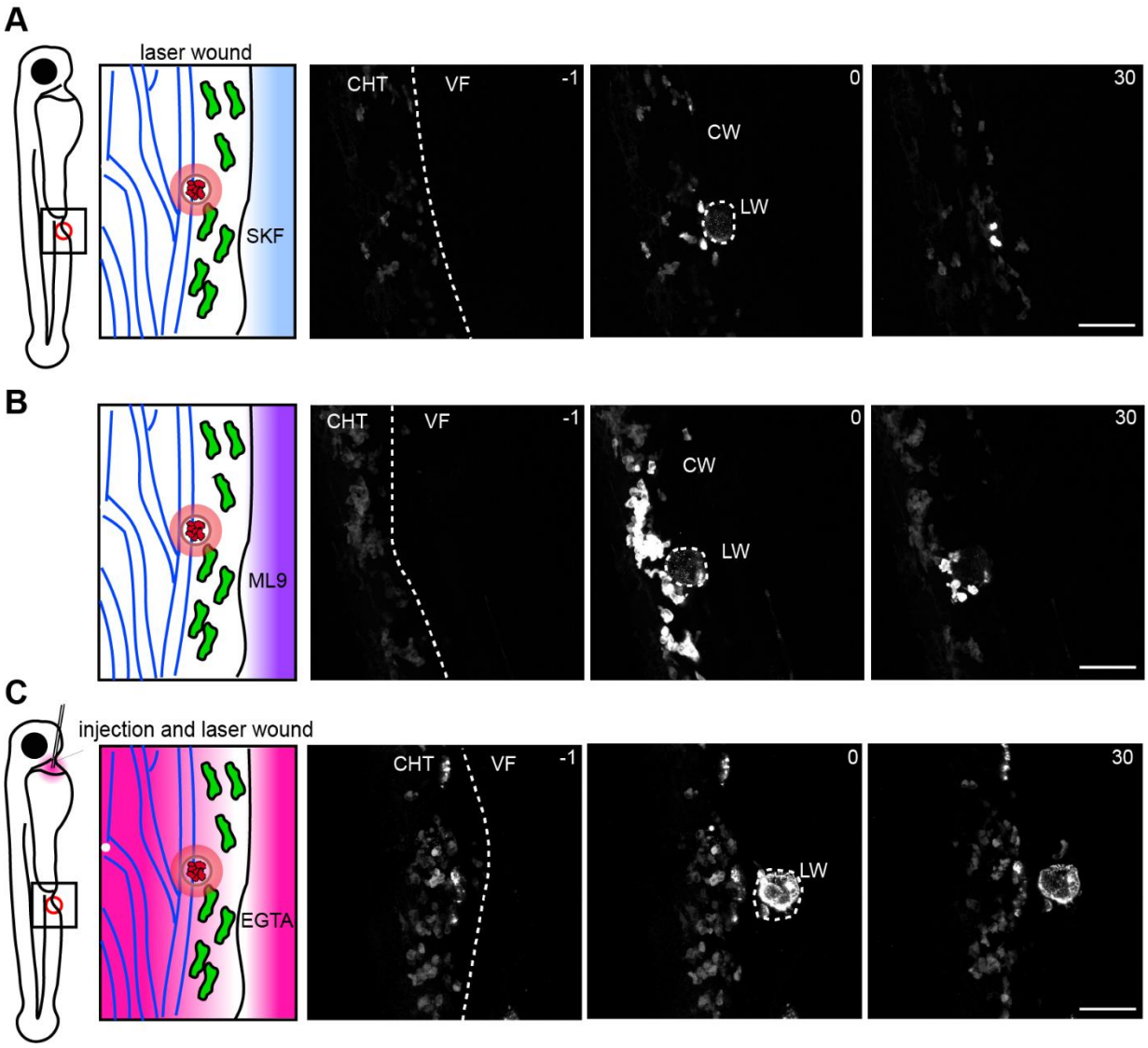


Figure 4.5. Calcium inhibition impairs neutrophil recruitment.

A Schematic of two-photon laser wounding performed on 3dpf larvae in the continuous presence of 100µM ML9 (Myosin Light Chain inhibitor) in the embryo medium (left). Maximum intensity 2-Photon time-lapse images of neutrophils showing calcium wave upon laser injury (CW) and neutrophil recruitment (right). B Schematic of two-photon laser wounding performed on 3dpf larvae in the continuous presence of 20µM SKF (SKF 96365 hydrochloride) In the embryo medium (left). Maximum intensity 2-Photon time-lapse images of neutrophils showing a moderate CW upon laser injury and low neutrophil recruitment (right). C Schematic of an injection in duct of cuvier of 10nL of 100µM EGTA followed by two-photon laser wounding performed on 3dpf larvae in the continuous presence of 1mM EGTA in the calcium-depleted embryo medium (left). Maximum intensity 2-Photon time-lapse images of neutrophils shows no CW upon laser injury and no neutrophil recruitment (right). All scale bars =50µm. Time in minutes, relative to laser wound injury.

4.7 Conclusions

In this chapter, I used a newly generated stable transgenic line Tg(*lyz*:5LO-tRFP) to probe for the biosynthesis of LTB4. I discovered that neutrophils synthesise LTB4 specifically upon clustering at the focus of injury. These observations correlated with the findings highlighted in chapter 3 showing that “activated” neutrophils with elevated intracellular calcium concentration were found in the cluster at the site of injury. By dual visualisation of 5-LO and GCamp6F dynamics, I demonstrated that 5LO translocation occurs specifically in the dense neutrophil cluster at the wound core. Using inhibitors of calcium pathways and calcium chelator I identified extracellular calcium as the source of calcium used in the clustering cells. All in all, I demonstrated a new paradigm challenging the current hypothesis of LTB4 signal relay, showing that the biosynthesis of this lipid occurs in static clustering cells at the site of injury *in vivo*.

4.8 Discussion

Here I confirmed that LTB4 was important for neutrophil recruitment at sites of injury in zebrafish larva. This is consistent with what has been shown in the mouse model (Lämmermann et al., 2013) and illustrates the conservation of the mechanism of neutrophil swarming in the different species and validates the use of zebrafish for further mechanistic studies. To further show the importance of LTB4 for neutrophil swarming in zebrafish it could be interesting to use the laser wound assay and analysis of neutrophil directionality that I describe in chapter 3 in conjunction with *lta4h* knockdown.

I used new tools to image the dynamics of events leading to LTB4 production. For this I design and genetically engineered a new reporter, following a key event to produce LTB4, the translocation of 5-LO to the nuclear membrane. A similar tool has been design in the past to follow cPLA2 translocation in zebrafish epithelial cells after injury (Enyedi et al., 2013). I generated stable transgenic zebrafish line expressing this construct in neutrophils only. The tool I generated is functional and I was able to observe 5-LO translocation in neutrophils in 3dpf zebrafish larvae One limitation of this probe is that it requires high resolution in order to visualise the translocation event. Indeed, neutrophil nuclei are thin and multilobed, which makes them more difficult to image than epithelial cells nuclei. In order to have a resolution high enough and a fast-enough imaging, those larvae had to be imaged on a spinning microscope. Another limitation of this probe is that it gives us an indication of LTB4 biosynthesis but not an indication of LTB4 release.

Interestingly, swarms generated in Tg(*lyz*:tRFP-5-LO) larvae seemed to have a higher number of neutrophils recruited than swarms in Tg(*lyz:*GCamp6F) or Tg(*mpx:*GFP) larvae. This could be a potential advantage to study swarms as small differences observed upon genetic or chemical inhibition of pathways might be more obvious in this context. However, if Tg(*lyz*:tRFP-5-LO) is acting as a gain of function, it also means that the swarms are enhanced and are not exactly like the one observed in WT physiological conditions. To verify if neutrophil recruitment during swarms is indeed amplified by Tg(*lyz*:tRFP-5-LO), directionality of cells swarming in those different conditions and the number of neutrophils recruited could be quantified and we could assess whether, by being overexpressed, 5-LO is increasing the number of neutrophils recruited and the overall magnitude of swarms. This could be occurring as a result of an increase in LTB4 production.

By visualising 5-LO translocation during swarming, I determined that LTB4 production was limited to static clustering neutrophils. These results contradict the current signal-relay hypothesis postulating that neutrophils produce LTB4 while migrating towards the injury (Afonso et al., 2012; Majumdar et al., 2016). As previously mentioned in the introduction of this chapter, the signal-relay hypothesis is based on *in vitro* with human neutrophils stimulated with fMLP only and migrating in an under-agarose assay. This assay, does not replicate the *in vivo* complexity of the wound environment in which a myriad of signals can be recognise by cells (Anders and Schaefer, 2014). Additionally, Afonso *et al.* did not have any means to precisely assess when and by what neutrophils LTB4 was secreted. In my study, using the tRFP-5-LO probe I was able to determine, precisely with a 30sec resolution (the temporal resolution of my time-lapse imaging), when LTB4 biosynthesis is taking place in neutrophils during swarms in physiological conditions. In the signal-relay theory, it is hypothesised that a gradient of LTB4 emerges gradually and “strengthen and stabilise” when enough neutrophils are migrating towards the site of injury (Afonso et al., 2012). In my case, I hypothesise that a LTB4 gradient source is being generated at the wound focus by clustering neutrophils. This gradient seems to appear when an undetermined threshold quantity of neutrophils is recruited in the cluster.

Finally, I used a set of candidate calcium signalling inhibitors, in order to identify the calcium pathways leading to calcium elevation in neutrophils at the cluster. Surprisingly using SKF, which inhibits the calcium channels TRPC reported to play a role in neutrophils (Beerman et al., 2015), I did not see a significant difference than in control wounded fish. Similarly, I did not observe a major difference in the calcium pattern elicited by neutrophils after using ML9 to inhibit store operated calcium entry. Injection of EGTA, however, did completely inhibit neutrophil migration and swarms, indicating that extracellular calcium entry is important for movement of neutrophils *in vivo*. The impact of EGTA on neutrophil migration, has never been studied. However, it was shown in neutrophils *in vitro* that EGTA only partially inhibited the

response to fMLP. In this study, the authors assessed the neutrophil response by measuring the release of lysozyme (Smolen et al., 1981). It is possible that external calcium inhibited by EGTA plays an important role in neutrophil migration. All processes dependent on external calcium might be inhibited in presence of EGTA in all cell type. It is possible that some of these processes taking place in other cell types and which would have trigger initial neutrophil migration might have been inhibited by EGTA. Complementary to those inhibitors, it could be interesting to use 2-APB. This drug inhibits store operated calcium release at high concentration. It is an antagonist of IP3 receptor, and it also blocks TRP (Transient receptor potential) channels. It has already been used in zebrafish to inhibit cytosolic calcium in muscle cells of 24hpf embryos (Wu et al., 2015).

5. Polarisation of LTB4 signalling during neutrophil swarms.

5.1 Introduction

Escalation of neutrophil migration into swarms was shown to be largely dependent on production of LTB4 by neutrophils (Lämmermann et al., 2013). Our work highlighted in Chapter 4 shows that zebrafish neutrophil swarming is also strongly associated with LTB4 signalling. According to the current model depicting neutrophil swarming, individual neutrophils secrete LTB4 while migrating along a gradient of primary attractants towards the site of inflammation (Afonso et al., 2012). This was first hypothesised in an *in vitro* study, where the authors postulated that LTB4, acting as a signal relay molecule, is secreted from neutrophils as they are migrating towards the primary attractant fMLP (N-ForMylmethionine-Leucyl-Phenylalanine) (Afonso et al., 2012). In this study, the authors used neutrophils knocked down for the fMLP receptor FPR1 (Formyl Peptide Receptor 1) and showed that these neutrophils could migrate directionally towards a source of fMLP by sensing LTB4 produced by WT neutrophils. However, for cells to migrate directionally while producing LTB4, a chemoattractant they can sense, LTB4 secretion and sensing would need to be polarised, otherwise cells would be misguided. Interestingly, it has been shown, in human neutrophils, *ex vivo*, that LTB4 production and secretion were polarised (Majumdar et al., 2016). In this study, the authors stimulated neutrophils with fMLP and used a combination of electron microscopy, cellular fractionation and fluorescent imaging to show that LTB4-producing enzymes, 5-LO and LTA4H were located towards the trailing edge of neutrophils. Additionally, they showed that these enzymes along with LTB4 are packed in exosomes and signals to cells further away, providing ground for the current hypothesis of the signal relay (Majumdar et al., 2016). The authors suggest that LTB4 is reinforcing the directional migration of neutrophils already migrating towards a fMLP gradient and that LTB4 acts in an autocrine and paracrine manner. However, this appears to be counter intuitive for a cell to sense an attractant produced at its rear and migrate towards another source of attractant located at its front. For the cells to migrate towards the front source, sensing of LTB4 may need to be polarised, with LTB4 receptors located at the front of the cells. Polarisation of chemoattractant receptor towards the leading edge of lymphocytes has already been reported (Nieto et al.,

1997). In this case, chemokine receptors CCR2 and CCR5 were redistributed to the leading edge of T lymphocyte migrating towards a source of chemokine *in vitro*.

Despite increasing interest in neutrophil swarming, polarisation of LTB4 production or sensing *in vivo* has not yet been demonstrated and the signal relay hypothesis has not been established *in vivo* either. In this chapter, I investigate whether enzymes and receptor-associated with LTB4 are polarised during neutrophil swarming in my model of zebrafish larvae. For this, I generated new fluorescent reporter probes for LTA4H and LTB4 receptor. I subsequently used the sterile injury assay I previously described in chapter 3 to analyse the polarisation of LTB4 signalling.

5.2 Polarisation of LTB4 receptor

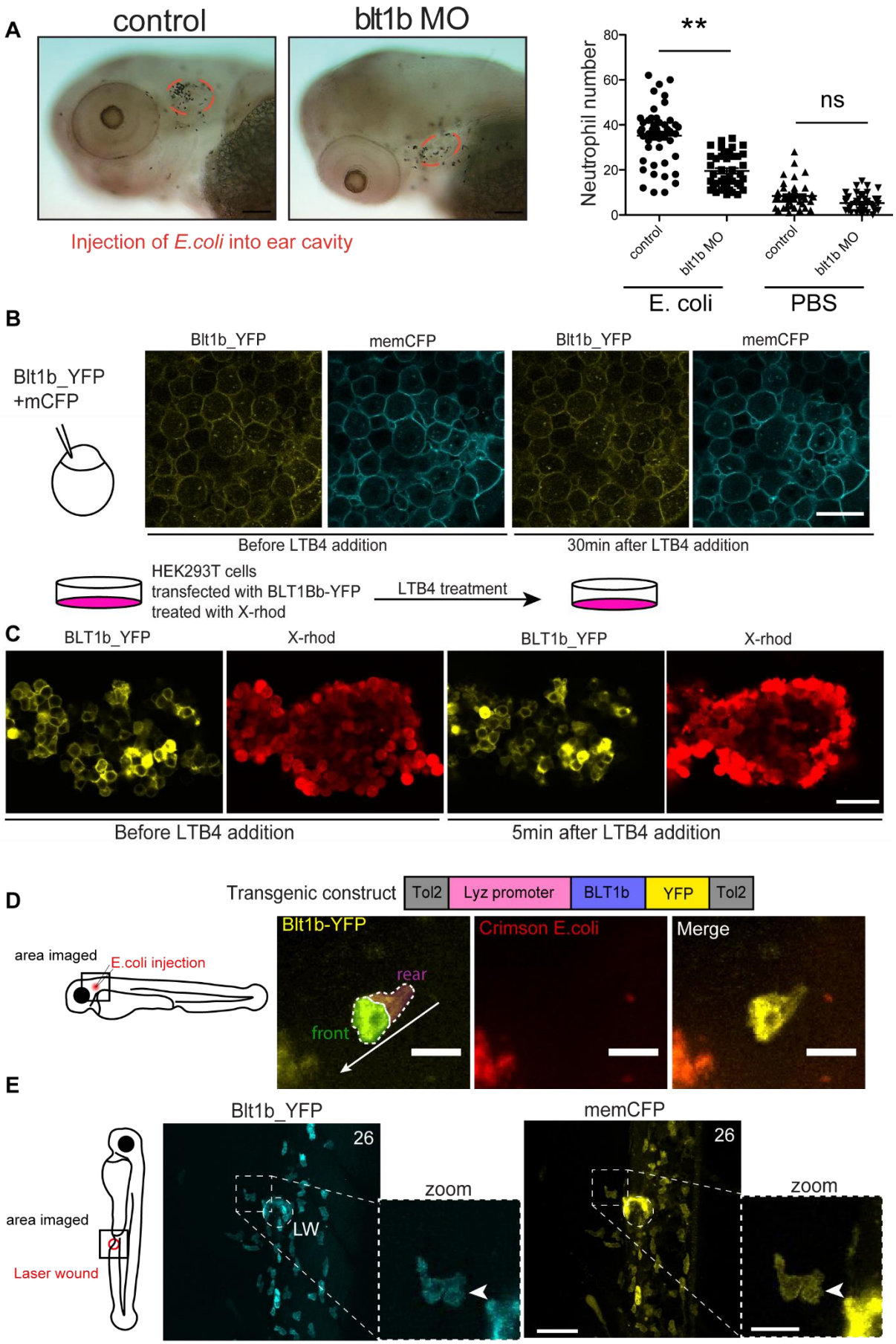
LTB4 signals through two receptors in humans, Blt1 (high affinity) and Blt2 (low affinity), each of which appears to have two homologues in zebrafish. Three homologues (*blt1, blt2a* and *blt2b*) were reported by others (Okuno et al., 2015), while our lab found one additional gene (*blt1b*) to be expressed by zebrafish neutrophils in a microarray analysis of their transcriptome (unpublished). Previous work has been done in the lab to verify the implication of Blt1b for LTB4 signalling in zebrafish larvae. A morpholino was used to KD (knockdown) *blt1b*. WT and *blt1b* KD 3dpf larvae were injected with non-pathogenic *Escherichia coli* in the area near the ear in the head. After Sudan black staining of multiple larvae following bacterial injection, neutrophils accumulating at the site of infection were quantified (Fig. 5.1, A). Neutrophil recruitment to sites of bacterial infection was severely reduced when *blt1b* expression was inhibited. PBS was injected as a control and it was determined that *blt1b* morpholino was not altering the number of neutrophils normally present in the ear in absence of *Escherichia coli*. This confirmed the major role of this receptor for neutrophil trafficking *in vivo*.

To investigate polarisation of LTB4 signalling at the receptor level, I designed a fluorescent fusion of the BLT1b receptor with YFP. GPCRs (G Protein Coupled Receptor) such as BLT1b may internalise after ligand binding, and surface receptor levels can be used as readout for ligand sensing. The lab and others (Venkiteswaran et al., 2013) validated this approach for chemokine receptors. I similarly tried this approach for BLT1b in the presence or absence of LTB4 (Fig. 5.1, B). I injected embryos with the construct, Blt1b-YFP RNA and mounted 6hpf embryo to visualise internalisation of the fusion receptor. The membrane marker mCFP and Blt1b-YFP were colocalised with and without addition of LTB4 showing no internalisation of the receptor. To further investigate the functionality of this construct, I performed *in vitro* experiments. When a ligand binds a GPCR such as BLT1b, an intracellular signalling cascade leading to calcium elevation is triggered (Tuteja, 2009). I generated and transfected a pCS2+

DNA plasmid containing BLT1b-YFP in HEK 293T cells and subsequently incubated the cells with X-rhod, a chemical calcium probe which fluorescent intensity increases upon intracellular calcium elevation. In cells expressing the fluorescent fusion of the receptor, a peak of fluorescence corresponding to a sharp increase of intracellular calcium concentration was observed 5 min after LTB4 addition to the medium. In contrast, no calcium elevation was observed in control cells transfected with a mock construct after exposure to LTB4 (Fig. 5.1, C). In conclusion, I confirmed that the receptor function of BLT1b is conserved in this fluorescent fusion with YFP by showing that calcium signalling was being triggered upon addition of LTB4.

I subsequently generated a stable transgenic line expressing this receptor under the control of a neutrophil specific promoter Tg(*lyz*:Blt1b-YFP). I tested this stable transgenic line by injected red fluorescent *Escherichia coli* (E2-crimson) in 3dpf Tg(*lyz*:Blt1b-YFP) larvae (Fig. 5.1, D). The fluorescent receptor was observed at the neutrophil's membrane and was polarised towards the leading edge of neutrophils migrating towards the site of infection (Fig. 5.1, D). To clearly identify polarisation of the receptor BLT1b in neutrophils, I crossed a zebrafish line expressing a membrane CFP marker Tg(*lyz*:mCFP) with Tg(*lyz*:Blt1b-YFP). This allows me to compare the fluorescence level at the membrane and therefore, the accumulation of the receptor. I went on to perform a laser wound in the CHT of these larvae and observed that BLT1b-YFP fluorescence was visibly brighter than the control membrane marker mCFP at the leading edge of neutrophils. This suggests that BLT1b accumulates at the leading edge of cells migrating towards the laser wound (Fig. 5.1, E).

In conclusion, I demonstrated that the fluorescent fusion of BLT1b and YFP was functional using stable transgenic larvae expressing this construct in neutrophils and obtained preliminary evidence suggesting that this receptor is redistributed of at the leading edge of neutrophils during recruitment to inflammatory sites.


A
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memCFP
Before LTB4 addition
30min after LTB4 addition
HEK293T cells
transfected with BLT1Bb-YFP
treated with X-rhod
LTB4 treatment
C
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BLT1b_YFP
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Lyz promoter
BLT1b
YFP
Tol2
area imaged
E.coli injection
Blt1b-YFP
Crimson E.coli
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Blt1b_YFP
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Figure 5.1. BLT1b_YFP, a fluorescent probe for LTB4 receptor.

A 3dpf larvae treated or not with *blt1b* morpholino (MO) were injected with bacteria. Neutrophil accumulation within the red bordered area was assessed at 6 hours post infection after sudan black staining. Quantification of neutrophil numbers is shown on the right. Scale bar=100µm. B 100pg of BLT1b_YFP mRNA with 100pg of mCFP mRNA was injected in a one cell stage embryo. Laser scanning confocal slices of gastrulating embryos showing expression and distribution of BLT1b_YFP and mCFP. LTB4 was added to the medium of gastrulating embryo during imaging. LTB4 receptor is shown in the yellow channel. Control membrane marker is shown in the blue channel. Scale bar=50µm. C HEK239T cells were transfected with a PCS2+ plasmid containing BLT1b_YFP. Cells were then incubated with X-Rhod for 30min and subsequently imaged with laser scanning confocal microscope. LTB4 was added during the imaging. Scale bar =50µm. D The BLT1b_YFP construct is expressed under the control of the lysozyme C promoter (Lyz). 3dpf Tg(*lyz*:Blt1b_YFP) larvae were injected with non-pathogenic E. coli. Following bacterial injection in the head, larvae were imaged with a confocal spinning disc. The white arrow indicates the direction of the cell, the front and rear of the cell are also indicated. Scale bar =10µm. E Maximum intensity Z projection of neutrophils in a Tg(*lyz*:Blt1b_YFP) X Tg(*lyz*:mCFP) larva. Images show neutrophils (expressing mCFP on the left and BLT1b_YFP on the right) within the caudal hematopoietic tissue (CHT) migrating towards the laser wound damage (LW). White arrows indicate the polarisation of BLT1b_YFP to the front of the neutrophil. Scale bar = 50µm and 10µm for the zoom pictures.

5.3 Polarisation of LTB4 production

The polarisation of LTB4 production is a central element of the current LTB4 signalling hypothesis in neutrophil swarming. LTA4H is involved in the last step of LTB4 production and its polarisation was reported in human neutrophils *in vitro* (Majumdar et al., 2016). To test LTA4H polarisation in neutrophils, *in vivo*, I generated a transgenic zebrafish line expressing LTA4H-EGFP Tg(*lyz*:LTA4H-EGFP) in neutrophils. I tested the construct in response to red fluorescent *Escherichia coli* (Crimson) injected in a 3dpf larva expressing LTA4H-EGFP (Fig. 5.2, A). Using confocal imaging, I only observed uniform expression of the construct in the cytoplasm. I also studied the distribution of LTA4H-EGFP during neutrophil swarming. For this, I used 3dpf larvae from Tg(*lyz*:LTA4H-EGFP) and performed a laser wound in the ventral fin near the CHT. Here, again, the expression of the construct was uniform showing no specific polarisation for the LTA4H enzyme *in vivo*. (Fig. 5.2, B).

To conclude, I generated a new reporter line for LTA4H, an enzyme that is crucial for LTB4 biosynthesis. Using this new transgenic reporter line and the previously generated reporter Tg(*lyz*:tRFP-5-LO), I demonstrated that those enzymes are not polarised during neutrophil swarming in zebrafish larvae.

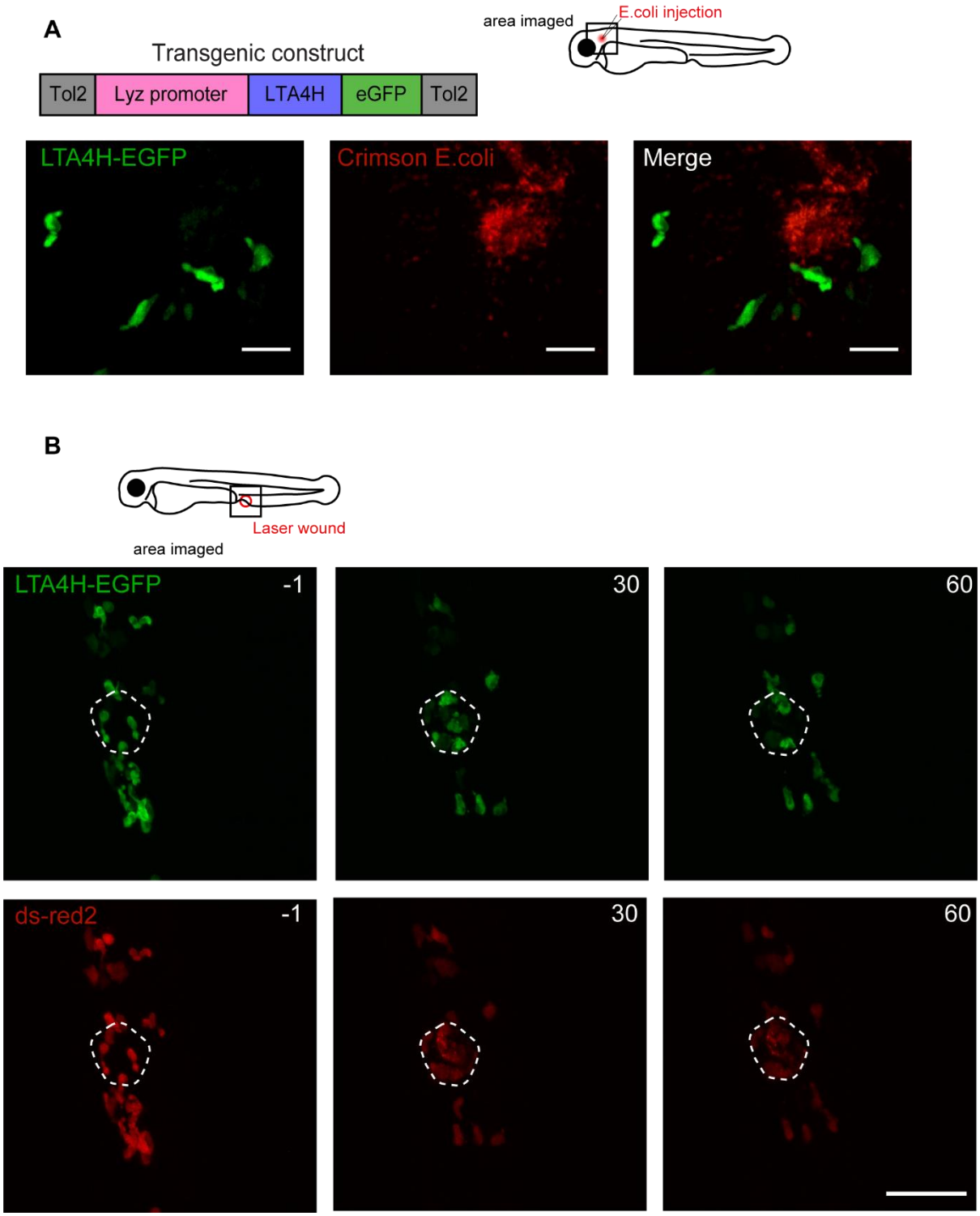


Figure 5.2. A fluorescent probe for LTA4H.

A The LTA4H_EGFP construct is expressed under the control of the lysozyme C promoter (Lyz). 3dpf Tg(*lyz*:LTA4H_EGFP) larvae were injected with non-pathogenic E. coli. Following injection of bacteria in the head, larvae were imaged with a confocal spinning disc. Scale bar =10µm. B Maximum intensity Z projection of neutrophils in a Tg(*lyz*:LTA4H_EGFP) X Tg(*lyz*:ds-red2) larva. Images show neutrophils expressing ds-red2 and LTA4H_EGFP within the caudal hematopoietic tissue (CHT) migrating towards the laser wound damage (LW). Scale bar = 50µm.

5.4 Conclusion

In this chapter, I generated transgenic zebrafish lines expressing fluorescent reporters to study the polarisation of LTB4 signalling. I first studied the polarisation of LTB4 receptor with a fluorescent fusion of BLT1b with YFP. I obtained preliminary evidence suggesting redistribution of this receptor on the leading edge of neutrophil migrating towards an injury. To investigate the distribution of LTB4-producing enzymes, I generated a new fluorescent probe, LTA4H-EGFP in addition to the previously generated Tg(*lyz*:tRFP-5-LO) line. I did not observe a specific polarisation of the redistribution of these constructs during neutrophil swarming.

5.5 Discussion

In this chapter I studied the polarisation of the distribution of LTB4-producing enzymes and one of the LTB4 receptors BLT1b. This receptor of LTB4 was used because it was found to be expressed by neutrophils in zebrafish larvae and plays an important role for neutrophil accumulation during *Escherichia coli* infection. I generated new fluorescent probes to track LTA4H and BLT1b. I first tested the fluorescent fusion of BLT1b in early stage zebrafish embryos and noticed that upon LTB4 binding, the fluorescent fusion of the receptor was not internalised. These results could have been explained by cell-type dependent differences in receptor trafficking. Previous studies in immortalised cells, namely COS-7 and HEK293T cells, showed that the internalisation of human BLT1 required GRK2 (G-Protein-Coupled Receptor Kinase 2), which may result in differential cell-type specific internalisation of LTB4, through differences in GRK2 expression levels.

However, I also did not observe Blt1b internalisation in neutrophils and this was consistent with a parallel study that reported that BLT1 failed to internalise upon LTB4 stimulation (Subramanian et al., 2018). Moreover, I confirmed the functionality of BLT1b-EGFP in HEK293T cells *in vitro* and showed that upon LTB4 addition, cells transfected with BLT1b-YFP elicited calcium signalling, a typical event happening downstream of ligand binding. These pieces of evidence suggest that zebrafish BLT1b does not internalise in response to endogenous ligand *in vivo*.

I obtained preliminary evidence for the redistribution of BLT1b-EGFP to the leading edge of the neutrophils migrating towards an infection or a sterile injury. This is in line with previously observed polarisation of the distribution of GPCRs in lymphocyte migrating towards a chemoattractant source (Nieto et al., 1997). In order to validate this conclusion, further analysis is required. The distribution pattern of BLT1b has to be quantified in relation to a control membrane marker in the presence or absence of LTB4 wound challenge or in the

presence or absence of LTB4 signalling inhibitors. This would confirm the specificity of the relocalisation. Additional experiments could be performed to block the redistribution of BLT1b and assess the importance of this on neutrophil migration. For this I could use the pertussis toxin that is known to block GPCR receptor redistribution at the membrane (del Pozo et al., 1995). The pertussis toxin could be either injected in the embryos or expressed in zebrafish under the control of a neutrophil specific promoter.

To study the distribution of LTB4 producing enzymes during neutrophil swarming, I generated another zebrafish line expressing a fluorescent reporter of LTA4H, LTA4H-EGFP. LTA4H metabolises LTA4 into LTB4 and is therefore crucial for LTB4 production. Using transgenic Tg(*lyz*:LTA4H-EGFP) larvae , I did not observe any apparent redistribution of this enzyme in neutrophils during cell migration to sites of infection or sterile injury. Additionally, in chapter 4, using Tg(*lyz*:tRFP-5-LO) reporter larva, I observed that 5-LO, a key enzyme for LTB4 production was not redistributed outside the nucleus during neutrophil swarms. The results I obtained in the zebrafish larvae are contradicting previous observations in human neutrophils *in vitro*. Indeed, in a study by Majumdar *et al*, 5-LO and LTA4H were observed being redistributed towards the rear of neutrophils migrating towards a source of fMLP and also packed in exosomes (Majumdar et al., 2016). This contradiction could be explained by the fact that human neutrophils might behave differently to zebrafish neutrophils. However, I think the major difference between my system and theirs is the environment surrounding the neutrophils. Neutrophils behave differently *in vitro* than *in vivo*, it has notably been shown that the complex 3D *in vivo* environment was playing a major role on their migration (Lämmermann et al., 2008). In their study, the authors do not recapitulate a neutrophil swarm *in vitro* and fMLP is the only signalling molecule present in their system which does not reflect the broad range of signalling molecules that are present during an infection or injury *in vivo*. It would be interesting to isolate zebrafish neutrophils and test if they would behave differently *in vitro*. This could be achieved by extracting neutrophils from the adult zebrafish whole kidney marrow that contains enough neutrophils for performing *in vitro* studies (Peters-Golden and Brock, 2003). Similar to what has been done in human neutrophils in vitro, zebrafish neutrophils could be stimulated with fMLP. However, here we would also be able to track LTB4 biosynthesis and calcium dynamics, enzyme and receptor distribution by extracting neutrophils from the stable transgenic lines I previously described.

It would also have been interesting to verify if traces of the enzymes could be found in exosomes, but this is not possible using our current live imaging settings. For this I would have to perform immunostaining of fixed zebrafish larvae to detect CD63, a protein associated with exosome and determine if it co-localises with 5-LO and LTA4H. Alternatively, electron microscopy could be performed to visualise exosomes and colocalise the enzyme using immunogold labelling (Calzia et al., 2018).

6. The role of connexins in neutrophil swarming to sites of inflammation

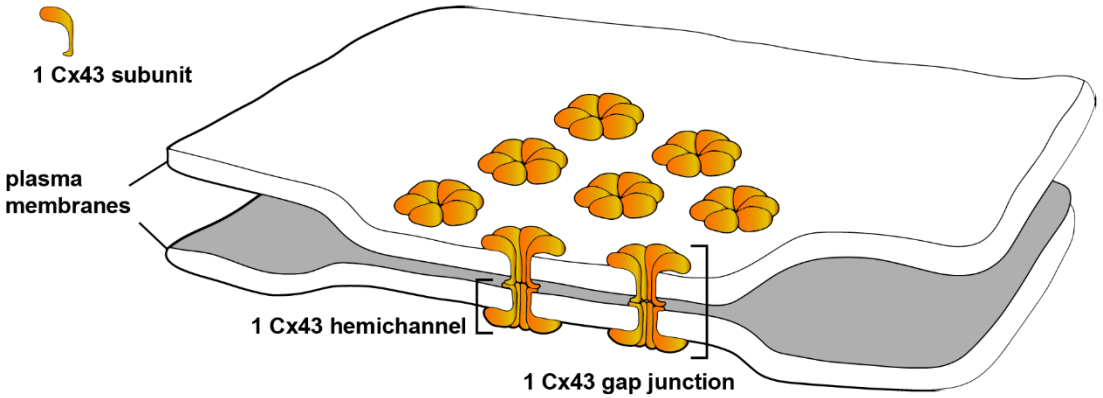
6.1 Introduction

In Chapter 3 and 4, I established that during neutrophil swarming, high calcium elevation followed by activation of LTB4 biosynthesis occurs in neutrophils clustering at the site of tissue injury. These molecular events lead to the generation of a centralised gradient of LTB4 stemming from clustering neutrophils and trigger swarms. However, it was still unclear, at this stage, what were the molecular mechanisms leading to calcium elevation in clustering neutrophils.

In vitro evidence has shown that molecules associated with cell death can trigger neutrophil activation. Neutrophils were found accumulating following necrotic cell transplantation in mouse (Iyer et al., 2009). In this study, the authors showed that mitochondria from necrotic cells alone can trigger leukocyte recruitment. After subcellular fractionation of the necrotic cells and treatment with ATPases, the authors identified ATP (Adenosine TriPhosphate) released by mitochondria from damage cells as the molecule required for leukocyte accumulation (Iyer et al., 2009). In a recent study, human neutrophils loaded with a calcium probe (Fluo-4), *in vitro*, showed rapid calcium elevation upon exposure to ATP (Wang et al., 2017b). The authors showed that this calcium influx was due to the opening of ATP-dependent gated ion channel P2X1, using NF279, a P2X1 inhibitor. P2X1 is known to have a high permeability to calcium ion (North, 2002). Interestingly, 'activated' neutrophils can release ATP. This was shown with high-performance liquid chromatography and luminometric ATP on isolated neutrophils stimulated with fMLP, *in vitro* (Eltzschig Holger K. et al., 2006). In the same study, following a drug screening on isolated neutrophils, the authors discovered that ATP was released from neutrophils through Cx43 (Connexin43) hemichannels (Box 6.1).

Box 6.1 Connexin hemichannels and gap junctions.

Connexin hemichannels or connexons are composed of 6 connexin proteins and are present on the cytoplasmic membrane. Connexins are expressed by a wide range of cells including leukocytes and are named according to their molecular weight; for example, Cx43 (Connexin



43) is 43kDa. Calcium and other signalling molecules under 1 kDa including ATP can transit through these hemichannels. Hemichannel-mediated intercellular communications are critical for several physiological processes, including electric propagation in the heart and neurons, embryonic development, cell proliferation and death, and the immune response (Gleisner et al., 2017). Cx43 is the most widely distributed and predominant connexin member in the immune system (Neijssen et al., 2007); it is notably expressed in neutrophils and macrophages.

Further studies showed that ATP released through Cx43 hemichannels was triggering a stop signal in migrating neutrophils. This was demonstrated in human neutrophils migrating towards the source of an fMLP gradient, *in vitro*, and using gap19 to selectively inhibit Cx43 hemichannels (Wang et al., 2017b).

In vivo data also showed that Cx43 plays a role in neutrophil trafficking. Using a mouse model of cutaneous wound healing, it was shown that Cx43 genetic inhibition was drastically reducing the number of neutrophils recruited to the wound (Qiu et al., 2003). This study demonstrates an important role for Cx43 in neutrophil recruitment but fails to explain the underlying molecular mechanisms leading to this recruitment.

In this chapter, I bring pieces of the puzzle together by making the link between connexin mediated inter-neutrophil communication and neutrophil swarming. For this, I used the wound assay I previously described in chapter 3 with chemical and genetic inhibitors of Cx43. Finally, I developed a new wound infection assay to understand the function of neutrophil swarming in protecting open wound against pathogens.

6.2 The death alarm signal "ATP" triggers neutrophil stopping and is required for intracluster calcium fluxes

I previously observed in Chapter 3, figure 3.3, that upon reaching the necrotic tissue, neutrophils underwent a sustained calcium flux. After analysis, we found that the interaction of neutrophils with the necrotic tissue correlates with the increase of intracellular calcium and neutrophil stopping. I observed a similar rise of intracellular calcium in neutrophils coming into contact with clustering neutrophils with high calcium levels (Fig. 6.1). These observations led me to think that rapid juxtracrine signalling was taking place between those cells and that ATP signalling might play a role.

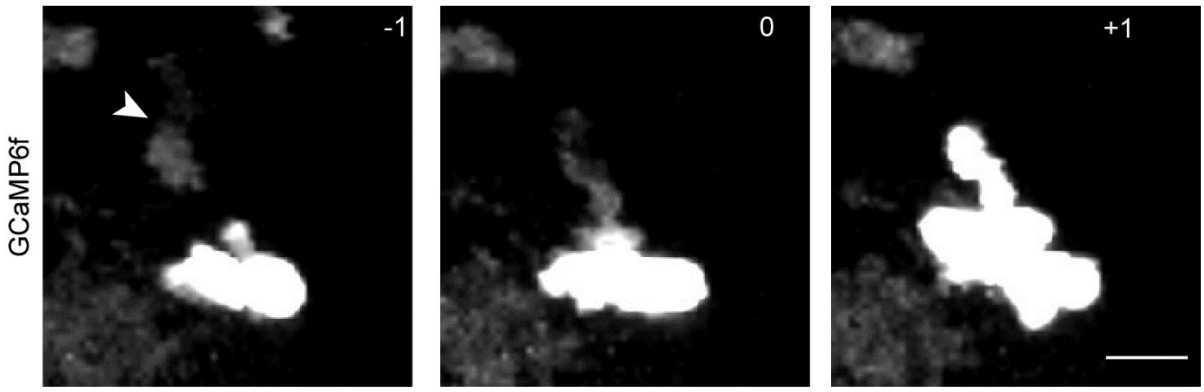


Figure 6.1. Calcium fluxes are triggered upon contact with neutrophils with ongoing fluxes.

Time lapse images of a dim GCamp6F^{+} neutrophil (arrow) contacting a bright GCamp6F neutrophil. Time in minutes is indicated relative to cell-cell contact. Scale bar= 10 μm.

To test whether ATP/P2X1 signalling was responsible for the rise of intracellular calcium in neutrophils, *in vivo*, I incubated Tg(*lyz*:tRFP-5LO) x Tg(*lyz*:GCamp6F) 3dpf larvae with NF279 a receptor antagonist of P2X1. I then performed mechanical wounds on these larvae. Neutrophils responding to the injury elicited a completely different signalling pattern in presence of the drug in comparison to the control (Fig. 6.2, A, B). We quantified the fluorescence intensity of GCamp6F in neutrophils at the wound in comparison to the average GCamp6F fluorescence intensity in all neutrophils. After analysis, we found that GCamp6F intensity in the cluster was significantly lower in presence of NF279 (Fig. 6.2, C). I also quantified the percentage of 5-LO translocation in neutrophils reaching the fin. There were significantly less 5-LO translocations in neutrophils when the larvae were exposed to NF279 (Fig. 6.2, D). These data demonstrate that the coordinated rise of intracellular calcium leading to LTB4 production in neutrophils at the cluster is dependent on ATP signalling through the gated ion channel P2X1. ATP diffusing to the extracellular space through hemichannels

formed by Cx43 at the surface of neutrophils would bind P2X1 which would in turn let calcium diffuse inside the cells.

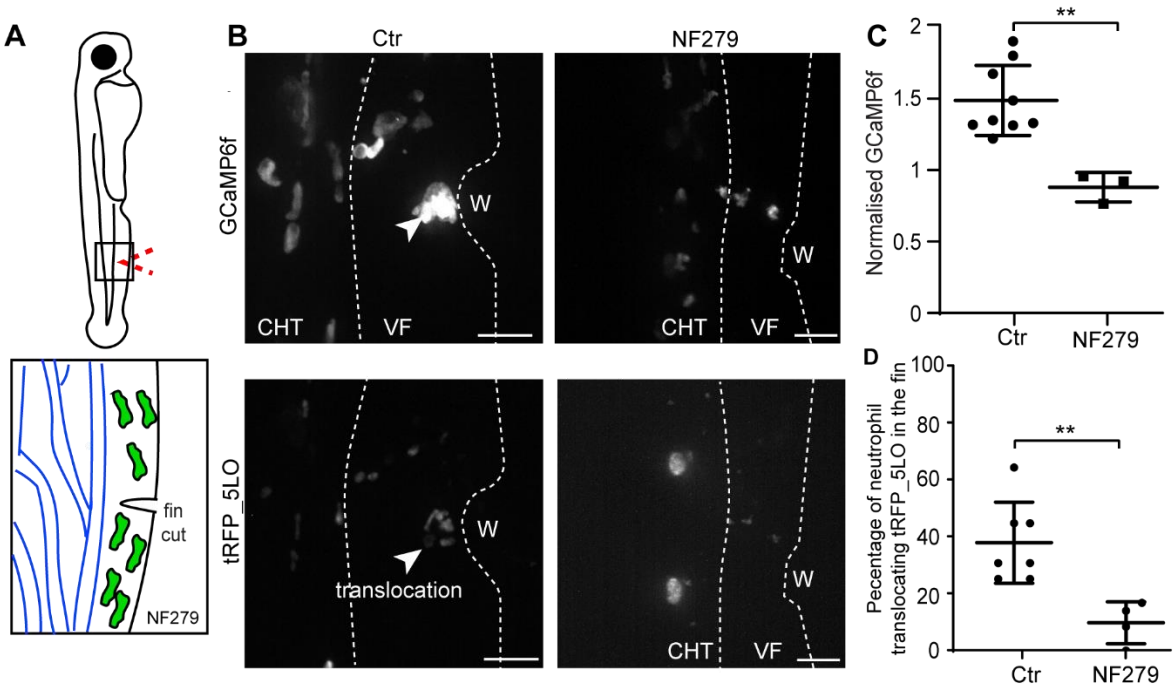


Figure 6.2. Extracellular calcium entry triggers 5-LO translocation and arrest in neutrophils in vivo, 5-LO translocation in neutrophils at mechanical fin wounds in the presence or absence of NF279.

A Schematic of ventral fin wounding in the presence of NF279. B Maximum intensity projection images of neutrophils in Tg(*lyz*:GCamp6F) x Tg(*lyz*:tRFP-5LO) zebrafish larvae 120 minutes after ventral fin wound (VF) in the presence (right) or absence (left) of 10μM NF279. Scale bar = 25μm. C Mean normalised GCamp6F intensity in larvae with and without NF279. NF279-treated larvae: n= 3 larvae; control larvae; n=9 larvae. Mann-Whitney test. D Number of translocating neutrophils per larva over two hours of imaging starting 10 min post-wound. Data are means of n=7 control larvae and n=4 NF279-treatd larvae. Mann-Whitney test, two tailed, p-value= 0.0030. Quantification was performed by Antonios Georgantzoglou.

6.3 Cell to cell transfer of ATP through connexins hemichannels

I then examined whether Cx43-mediated release of ATP by neutrophils might be linked to the intracluster calcium propagation of neutrophil swarms. To investigate the possible role of Cx43 in neutrophil swarming, I incubated 3dpf Tg(*lyz*:GCamp6F) x Tg(*lyz*:tRFP-5LO) larvae with an inhibitor of connexin channel activity: Carbenoxolone (CBX) (MILLS and MASSEY, 1998). CBX treatment appeared to inhibit neutrophil calcium elevation and cell motility behaviour in response to laser wound injury (Fig. 6.3, A). To analyse calcium elevation in neutrophils at the wound, we quantified GCamp6F fluorescence intensity in neutrophils at the site of injury in comparison to the average GCamp6F fluorescence intensity in all neutrophils. After analysis,

we found that CBX treatment led to a significant reduction of intracellular calcium in neutrophils at the wound (Fig. 6.3, B). To quantify the coordination of neutrophil directional migration we quantified the radial speed of neutrophils over time (Lämmermann et al., 2013). As mentioned in chapter 3, when cells move in synchrony in the same direction, the amplitude of radial speed of the population is high. Here we found that that CBX treatment led to a significant reduction in the amplitude of the wave of neutrophil migration, consistent with a deficiency in coordination of cell migration (Fig. 6.3, C).

To genetically corroborate this finding, I investigated connexin expression in zebrafish larval neutrophils and found two connexin genes to be expressed, Cx43 and Cx43.4 (Fig. 6.4, A and B). In my first attempt to inhibit Cx43 expression, I found that CRISPR and Cas9 knockout led to early zebrafish embryonic death, at 1 dpf, before the onset of neutrophil development. This is consistent with previous report showing embryonic lethality of Cx43 null mutations observed in mice (Lo et al., 1999). I thus changed my strategy and used a combination of Cx43 translation morpholinos to knockdown the two homologs of Cx43 in zebrafish (Hoptak-Solga et al., 2008). The combination of these two morpholinos resulted in a phenotype characterised by reduced retina size, a developmental phenotype consistent with previous studies using hypomorphic mutations of Cx43 in zebrafish (Hoptak-Solga et al., 2008) (Fig. 6.4, C).

I performed laser wounds on 3dpf Tg(*lyz*:GCamp6F) x Tg(*lyz*:tRFP-5LO) larvae injected with the combination of Cx43 morpholinos. As observed in CBX treated larvae, calcium elevation was diminished, and neutrophils did not migrate in a coordinated manner. We used the same analytic method as the one used with CBX-treated larvae to analyse neutrophil coordination and calcium elevation at the wound. We showed that *cx43* knockdown significantly reduced normalised GCamp6F fluorescence and normalised radial speed in neutrophils at the wound (Fig. 6.3, B and C). This is consistent with the results obtained with CBX and confirms the major role of Cx43 for calcium signalling at the cluster during neutrophil swarming.

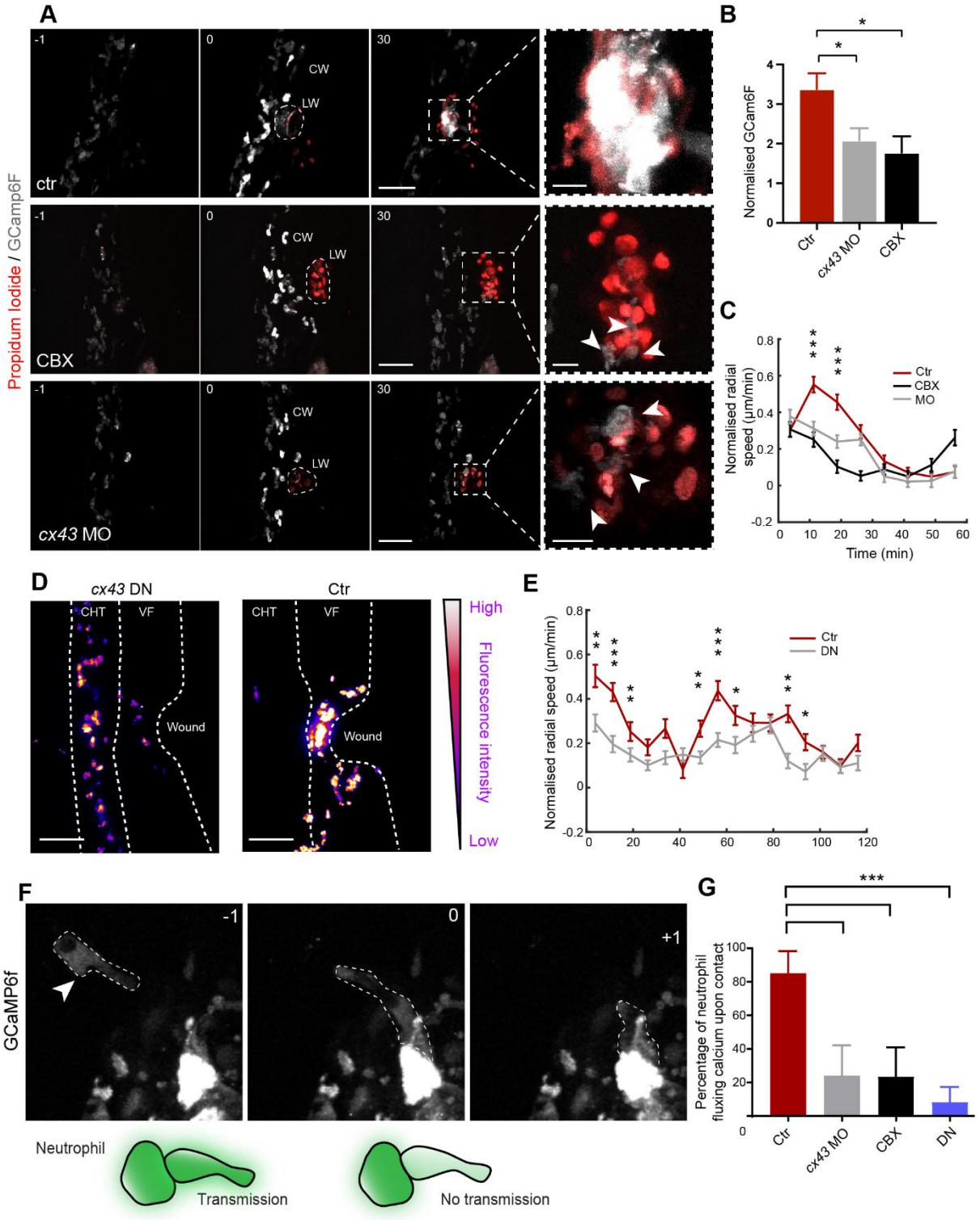


Figure 6.3. Cx43 is required for neutrophil calcium fluxes and aggregation.

A Neutrophils in Tg(*lyz*:GCamp6F) larvae in the presence of PI, without treatment (ctr), with 50 µM CBX or with morpholinos against *cx43*/*cx43.4* (*cx43* MO). Scale bars = 50µm and 10µm. CW; calcium wave. Time after laser wound (LW) is shown in minutes. B Normalised GCamp6F levels in control, *cx43* MO-treated and CBX-treated larvae. n=8 control, n=7 *cx43* MO-treated, n=5 CBX-treated. One-way ANOVA with Dunnet's post-test. C Normalised radial speed-time plots of neutrophils. Each line shows pooled

cell data from multiple larvae binned every 7.5 min. Pooled cell data from control (n=12), CBX-treated (n=8) or MO-injected larvae from x-y experiments. Mann-Whitney test results between ctr and MO shown. D Neutrophils in Tg(*lyz*:GCamp6F)xTg(*lyz*:*cx43dn*-T2A-mCherry) zebrafish larvae, positive (*cx43* DN) or negative for the transgene (Ctr), 2h after mechanical fin wound. GCamp6F channel is shown, colour-coded for intensity. E Normalised radial speed-time plots of neutrophils. Data pooled from n=11 *cx43DN* transgenics and n=8 control siblings. Mann-Whitney test. F Time lapse images of a dim GCamp6F^{+} neutrophil (arrow) contacting a bright GCamp6F^{+} neutrophil in a CBX treated larvae. Time in minutes is indicated relative to cell-cell contact. scale bar = 15µm. G Quantification of the percentage of neutrophil-neutrophil contact resulting in calcium fluxes or not, n= 15 control, 10 *Cx43* morphants, 6 CBX treated and 10 dominant negative larvae. One-way ANOVA, Tukey's multiple comparisons test. Quantification was performed by Antonios Georgantzoglou.

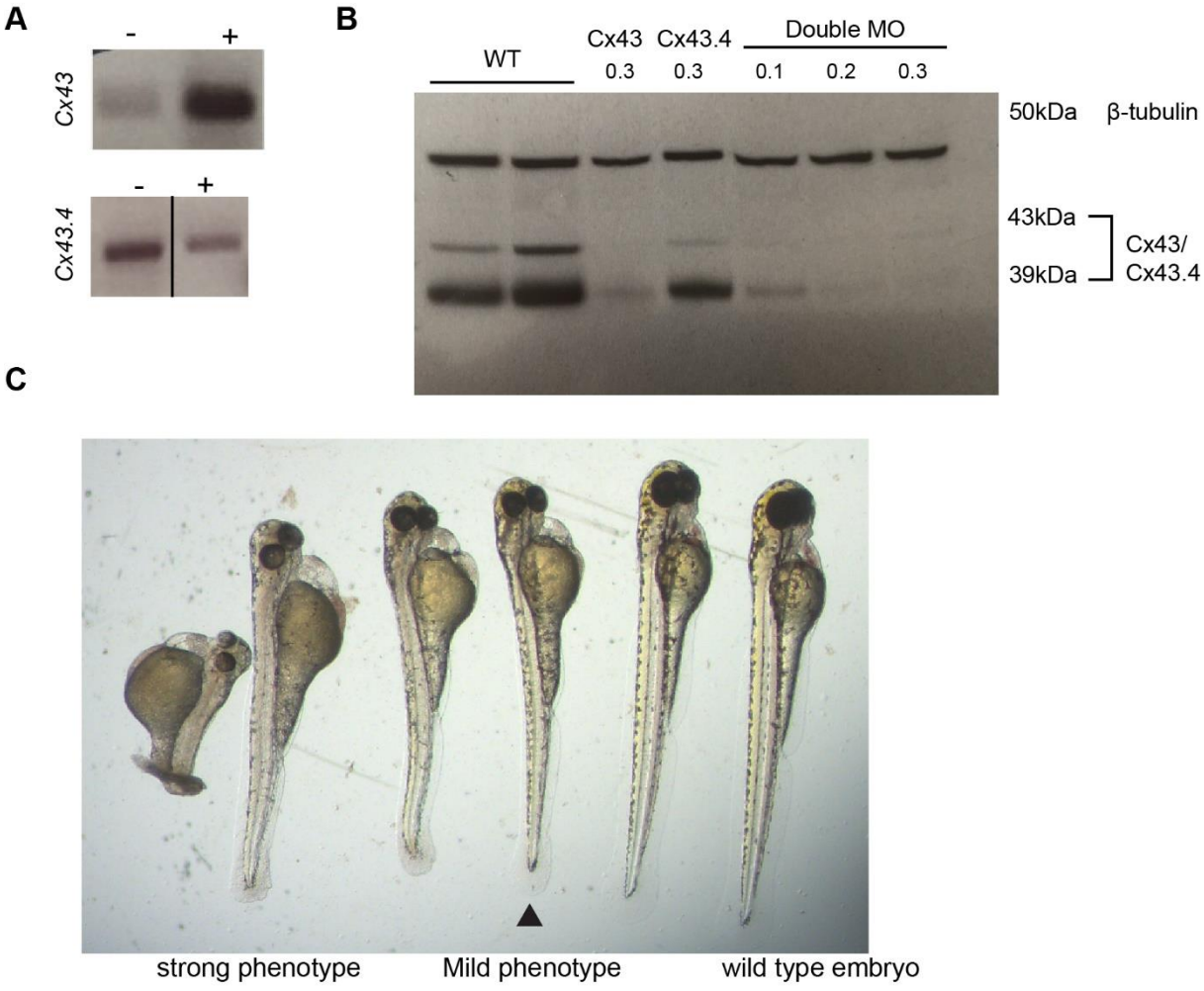


Figure 6.4. Neutrophil Cx43 expression and knockdown in zebrafish larvae.

A RT-PCR for Cx43 and Cx43.4 expression from cDNA samples of FACS-sorted GFP^{+} (+) or GFP^{-} cells (-) from Tg(*mpx*:GFP) 4.5 dpf larvae. B Western Blot showing the expression of Cx43 and Cx43.4 in 3dpf larvae in WT and morphant zebrafish larvae. Amount injected for each morpholino in pmol is shown. C Image of different phenotypes of 3dpf zebrafish larvae injected with cx43/cx3.4 combination

MOs. The black arrow indicates the mildest phenotype with detectable difference in eye size that was selected for subsequent experiments.

Previous studies show that Cx43 hemichannels play a major role in the release of ATP from neutrophils themselves (Eltzschig Holger K. et al., 2006; Wang et al., 2017b). To determine if the role played by Cx43 was neutrophil specific, I generated a stable transgenic zebrafish line whereby neutrophils express a dominant-negative version of Cx43 Tg(*lyz:cx43DN*-T2A-mCherry) (Oyamada et al., 2002). The dominant negative modification consists in a deletion of eight amino acid residues from the internal loop of Cx43 at positions 130-137, inducing allosteric modification of Cx43 protein and resulting in channel occlusion (Krutovskikh et al., 1998). Using the same methodology used for analysing neutrophil swarming with morpholino knockdown and CBX chemical inhibition, we analysed the response of these neutrophils to a mechanical wound. In the dominant negative mutant (Cx43DN), neutrophils showed reduced whole-cell calcium fluxes, clustering at the wound core and less coordinated motility (Fig. 6.3, D and E). This was consistent with the results obtained in Cx43 morphants and CBX treated larvae, demonstrating a major role for Cx43 in neutrophils. To further characterise the effect of neutrophil specific Cx43 inhibition, I quantified the difference in the number of neutrophils recruited to tail wounds in *cx43* morphants, Cx43DN and WT embryos. For this, I performed tail fin wounds followed by Sudan black staining of neutrophils on 3dpf zebrafish larvae. After quantification, I showed that inhibition of neutrophil Cx43 significantly reduced neutrophil accumulation to a similar degree as global Cx43 inhibition (Fig. 6.5). To interrogate a link between Cx43 and P2X1 signalling in neutrophil swarming *in vivo*, I performed a similar tail fin wound followed by Sudan black staining of neutrophils. I found that Cx43 inhibition did not cause further reduction in neutrophil accumulation in NF279-treated larvae (Fig. 6.6). This indicated that Cx43 and P2X1 act in the same pathway, providing indirect evidence for hemichannel-based relay of ATP signalling.

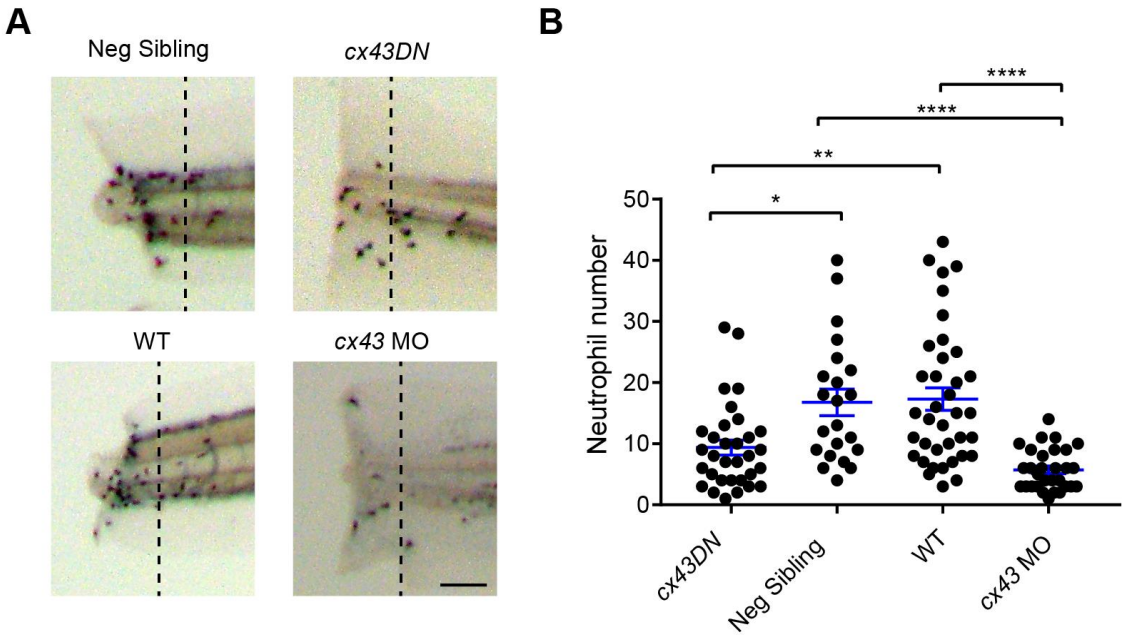


Figure 6.5. Cx43 inhibition leads to reduced neutrophil accumulation at mechanical fin wounds.

A Sudan black staining of neutrophils in transgenic Tg(*lyz:cx43DN*-T2A-mCherry) larvae (*cx43DN*) and their negatively screened siblings (sibling) WT larvae, larvae injected with (*cx43/cx43.4 combination of* morpholinos (*cx43* MO). 3 dpf larvae were amputated with a scalpel and fixed 3h after wounding. The dotted lines represent the area in which neutrophils were counted. Scale bar = 50µm. B Quantification of the total number of neutrophils recruited to wounds in the different conditions pooled from three independent experiments. *dn cx43,* n= 32 larvae; negative siblings, n=22 larvae; wt, n=37 larvae; *cx43* MO, n= 30 larvae. Kruskal-Wallis multiple comparisons test with Dunn's post-test.

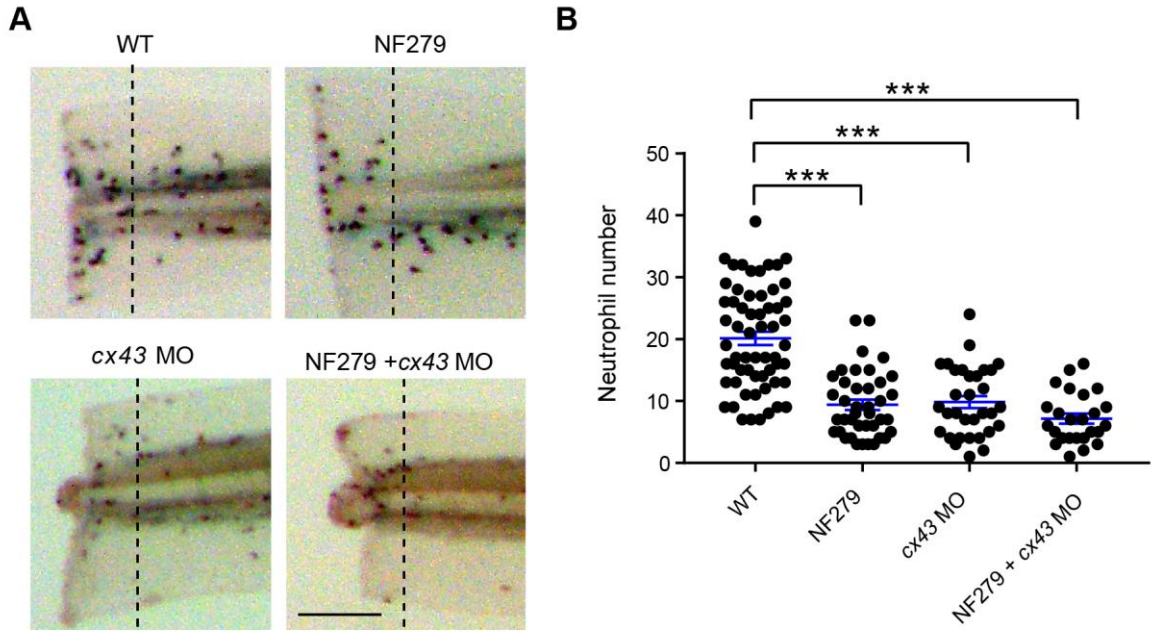


Figure 6.6. Absence of additive effect of Cx43 inhibition on neutrophil migration over P2X1 inhibition.

A Sudan black staining of neutrophils in wild type larvae injected or not with *cx43/cx43.4 combination of* morpholinos (*cx43* MO) and treated or not with 10μM NF279. 3 dpf larvae were amputated with a scalpel and fixed 3h after wounding. Scale bar =100μm. B Quantification of neutrophil number at wounds in the different conditions. Data pooled from two independent experiments. One-way ANOVA with Tukey's multiple comparison test. WT (non-injected and not treated with drug), n=60 larvae, NF279, n=40, cx43 MO n=32, cx43 MO with NF279 n=25.

Importantly, using inhibitors of Cx43 or dominant-negative Cx43, I did not observe contact-dependent propagation of calcium fluxes (Fig. 6.3, F). The percentage of neutrophils fluxing calcium upon contact with other neutrophils was significantly reduced upon genetic or chemical inhibition of Cx43 (Fig. 6.3, G). Altogether, these data demonstrate an important role for neutrophil Cx43 in coordinating neutrophil calcium signalling, LTB4 synthesis and swarming.

6.4 Connexin proteins are important for neutrophil-mediated immunity against opportunistic pathogens

The function of neutrophil swarming during wound defence against external pathogen is unknown. To address if lack of neutrophil accumulation via Cx43 inhibition affects wound defence against external pathogen, I established a new wound infection model in zebrafish

larvae. For this, I used *Pseudomonas aeruginosa*, one of the most common pathogens found in chronic wounds in humans (Gjødsbøl et al., 2006). *Pseudomonas aeruginosa* is a rod-shaped gram-negative opportunistic bacterium that causes diseases in a broad range of organisms including human, mouse, plants and zebrafish. This bacterium is a serious concern in hospitals as it causes a range of severe and chronic nosocomial infections on immunocompromised patients (Trautmann et al., 2005). *Pseudomonas aeruginosa* is prevalent on burn wound infections and is in most cases multidrug resistant (Hsueh et al., 1998). In zebrafish larvae, *Pseudomonas aeruginosa* infection can result in the death of the embryo. Clatworthy *et al* showed that myeloid cells play an important role for *Pseudomonas aeruginosa* clearance in 2-3dpf zebrafish larvae (Clatworthy et al., 2009). In this study, the knockdown of PU.1, a key transcription factor regulating the fate of myeloid cells, resulted in the complete depletion of neutrophils and macrophages and was associated with increased zebrafish larvae deaths upon *Pseudomonas aeruginosa* infection. In another study, using 3dpf zebrafish embryo, neutrophil swarms were observed following *Pseudomonas aeruginosa* infection near the ear (Deng et al., 2013). These evidences suggest a role for neutrophils or macrophages in controlling *Pseudomonas aeruginosa* infection in zebrafish. I therefore hypothesized that Cx43 might support wound defence from *Pseudomonas aeruginosa* infection, through supporting neutrophil accumulation and aggregation.

For this I developed a new model for wound infection by bacteria (previous infection models bypassed bacterial invasion and administered bacteria by local injection of a bacterial inoculum). This consisted in incubating 3dpf larvae that had been mechanically wounded in a medium containing PAO1 *Pseudomonas aeruginosa*, the most commonly used strain for research on this pathogen (Klockgether et al., 2010) (Fig 6.7, A). I first determined which concentration of bacteria will be sufficient to trigger around 50% zebrafish death at 18 hours post infection (hpf). For this, I used increasing concentrations of bacteria in the bath in which the larvae are incubated. This led to a dose-dependent decrease in survival, which was not observed in non-wounded larvae confirming the functionality of this wound infection model (Fig 6.7, B and C). Following these results, I decided to use an intermediate infection dose of bacteria causing around 50% mortality at 18 hpf for further experimentation. Using this model, I then interrogated the role of neutrophil accumulation at sites of injury for wound defence against *Pseudomonas aeruginosa*. For this, I inhibited *cx43* with previously described morpholinos to inhibit neutrophil accumulation at wounds. I found that *cx43* morphant larvae showed significantly reduced survival and increased bacterial burden after infection (Fig 6.7, D and E). This demonstrated that by controlling neutrophil accumulation, Cx43 restricts wound colonisation by pathogenic bacteria.

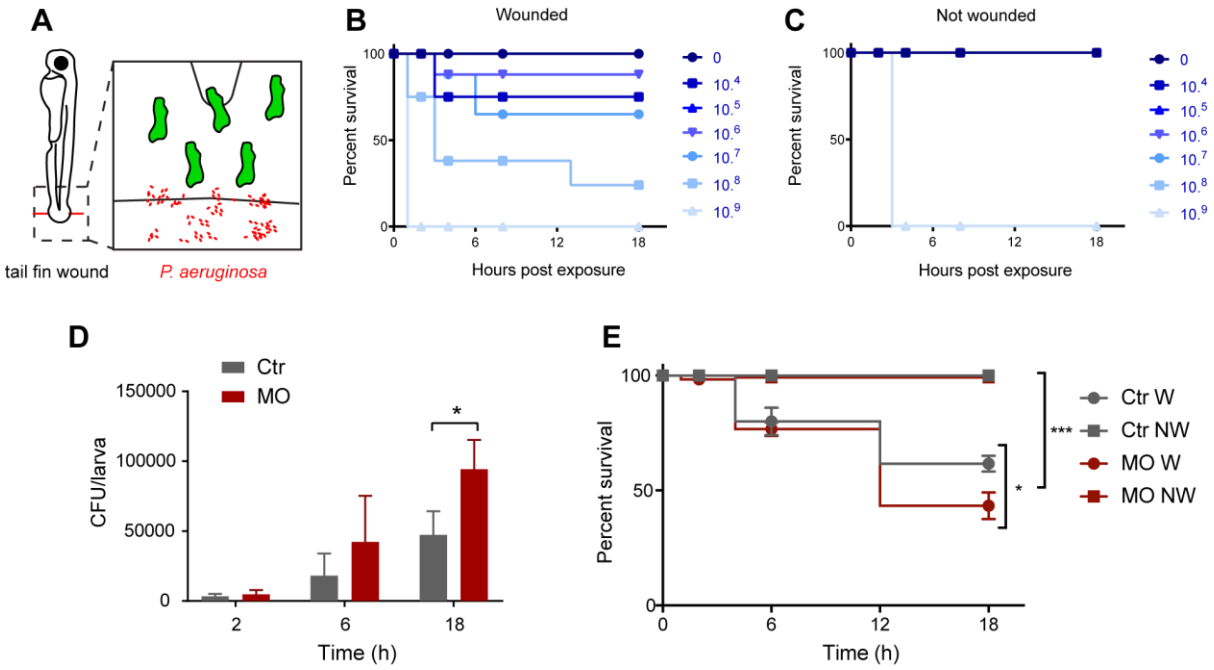


Figure 6.7. Cx43 is required for neutrophil immunity.

A 3 dpf larvae were injured at the tail fin with a scalpel in the presence of titrating doses of *P. aeruginosa* in the bath. B Concentration-dependent effects on survival in wounded larvae are shown. C Same titration in non-wounded larvae. Injury-independent death is observed only at doses less above 10^9 CFU/ml. Data represent one experiment with 20 larvae per condition. D. Colony forming units (CFUs) per larva in control non-injected larvae or *cx43* MO-injected larvae. Mann-Whitney test. n=4 experiments, with 5 larvae per group. E. Survival over time in control/MO-treated larvae, wounded or not wounded in the presence of PAO. n=4 experiments, with 20 larvae per group. One-way ANOVA, Tukey's multiple comparisons test.

6.5 Conclusion

In this chapter, I showed that high calcium elevation and 5-LO translocation in clustering neutrophils during swarms is the consequence of ATP signalling. My results suggest that ATP is released through Cx43 hemichannels from clustering neutrophils and signals through P2X1 a gated ion channel. ATP triggers the opening of P2X1 channels, leading to intracellular calcium intake in neutrophils. In turn, calcium elevation triggers the activation of LTB4 biosynthesis pathways, eventually leading to neutrophil accumulation at sites of injury. Finally, taking advantage of the capacity to supress neutrophil swarms by inhibiting Cx43, I demonstrated the importance of neutrophil aggregation at sites of injury for defending the wound against bacterial colonisation.

6.6 Discussion

In this chapter, I investigated the molecular mechanisms underlying calcium fluxes observed in clustering neutrophils in chapter 3. My evidence supports an important role for ATP in neutrophil calcium elevation, stop signal and LTB4 production *in vivo*. My evidence further suggests, that ATP is being released not only passively from dead cells (Iyer et al., 2009) but also actively from neutrophils themselves (Eltzschig Holger K. et al., 2006; Wang et al., 2017b). Though *in vitro* evidence existed on the capacity of ATP release by connexins, the *in vivo* relevance of this has so far remained unclear. The pronounced effects of Cx43 inhibition on neutrophil signalling and calcium fluxes that I discovered illustrate that active release of ATP form neutrophils and amplification of damage signalling is crucial for *in vivo* responses.

Cx43 has not only channel-dependent but also channel-independent functions. Such functions include in the regulation of gene transcription, differentiation, proliferation and cell adhesion (Kotini et al., 2018; Ribeiro-Rodrigues et al., 2017). CBX and Cx43DN act on connexin channel activity, suggesting a channel-dependent function in this context. However, it remained unclear whether connexins mediate gap-junction coupling or hemichannel-based signal transmission in neutrophils. Interestingly, the PI I used previously to detect dead cells is transferable from cell to cell through gap junctions and uptake of PI can also be used to measure hemichannel activity (Elfgang et al., 1995). However, I did not observe PI uptake in live neutrophils, suggesting either absence of hemichannel activity or that the level of transport is below our detection limit. Thus, my results do not directly prove hemichannel activity. However, the inhibition of P2X1 did not cause further reduction in neutrophil accumulation over the Cx43 inhibition. This supports the idea that Cx43 supports swarming, at least in part, through a link with ATP signalling.

My results show that zebrafish motility arrest is coupled with calcium elevation and LTB4 biosynthesis activation. Furthermore, I show that Cx43 is required to coordinate those molecular events. This is consistent with the role of Cx43 in human neutrophils stopping, *in vitro*, which was shown to depend on MLC (Myosin Light Chain) activation (Wang et al., 2017b). Specifically, MLC is phosphorylated and therefore activated upon calcium influx due to ATP signalling in neutrophils responding to LPS. The phosphorylated MLC triggers the contraction of myosin II inhibiting the posterior retraction/formation of the cellular cytoskeleton needed for neutrophils to navigate in dense tissue. Consequentially, the phosphorylation of MLC triggered a stop signal in migrating neutrophils. This mechanism could account for my observation of zebrafish neutrophils stopping at wounds after calcium elevation *in vivo*. This could be tested by inhibition of MLC kinase using appropriate inhibitors (Wang et al., 2017b). Integrin activation by calcium could also play a role in stopping. Using mice deficient for Talin,

a protein that link integrin to the cytoskeleton, it was also shown that integrin plays a major role for neutrophil adhesion during swarms. In this study, laser wounds performed in ears of mice transplanted with talin-deficient neutrophils showed a significant decrease of neutrophil swarming at the wound in comparison to the WT control (Lämmermann et al., 2013). A possible combination of integrin binding and contraction of myosin II upon calcium elevation in neutrophils could be at the origin of neutrophil stopping at the site of injury.

All in all, these findings reveal that that connexin-mediated amplification and neutrophil-neutrophil juxtacrine propagation of ATP via Cx43 hemichannels in clustering cells coordinates LTB4 biosynthesis to generate a gradient source that is critical for the swarming response. These results shed light on a molecular pathway that provides a compelling mechanistic explanation for so far unexplained reports of anti-inflammatory effect of Cx43 inhibition in mice wounds and infection. In particular, a report by Qiu *et al*, showed a significant decrease in neutrophils recruited at wounds in mice knockdowned for *cx43* using antisense oligodeoxynucleotide (Qiu et al., 2003). Another report by Sarieddine *et al* showed reduced neutrophils recruitment to mice lungs infected with *Pseudomonas aeruginosa* when Cx43 hemichannels are blocked by gap26 (Sarieddine et al., 2009). The mechanism I describe relies on collective reinforcement of ATP signalling, this means that a threshold number of neutrophils need to reach the damage site to generate a pro-inflammatory source of attractant strong enough for swarm to emerge. This is in line with what has been observed in neutrophil swarming *ex vivo* using a microscale array zymosan-particle cluster. In this study, the authors showed that a critical number of neutrophil was necessary for generating swarms. (Reátegui et al., 2017).

Finally, I studied the effect of neutrophil accumulation on wounds colonised by bacterial pathogens. For this, I developed a new wound contamination assay using *Pseudomonas aeruginosa* and found a link between Cx43-mediated aggregation of neutrophils and wound defence from bacterial infection. These results are in line with another study in zebrafish larvae showing that recruitment of neutrophils from an injury preceding the infection is important to protect the organism against *Pseudomonas aeruginosa* (Huang and Niethammer, 2018). In this case, the authors performed an injury in the zebrafish ear before injection of bacteria in this locus and found that damage cues from the injury were important for neutrophil accumulation and subsequent defence of the infected area. The intercellular communication dynamics described in this chapter provide a new explanation of how LTB4 chemoattractant gradients are generated by neutrophils to provide an effective immune defence.

Chapter 7. An optogenetic strategy to control LTB4 biosynthesis

7.1 Introduction

LTB4 is the key attractant in neutrophil swarming (Lämmermann et al., 2013). However, many other attractants impact neutrophil migration *in vivo*. The myriad of attractants available to neutrophils make the signalling dynamics very complex. It is therefore difficult to precisely dissect the function of LTB4 dynamics *in vivo*. For example, it is unclear how many cells need to produce LTB4 for effective swarm initiation and when the biosynthesis of this lipid has to take place to have effects in neutrophil migration and function. To further dissect LTB4 signalling dynamics and its role, I developed a tool to manipulate the production of LTB4 in neutrophils. LTB4 is a product of arachidonic acid metabolism which takes place at the inner nuclear membrane. A limiting step in the production of LTB4 is the translocation of the enzyme 5-LO (5-lipoxygenase) from the nucleus to the inner membrane of the nucleus (Luo et al., 2003) to produce LTA4 from arachidonic acid. The final step of LTB4 synthesis is the hydrolysis of LTA4 by LTA4H to generate LTB4. LTA4 is also a precursor of anti-inflammatory lipoxin LXA4 (Pettitt et al., 1991). Lipoxins are related to leukotrienes and are synthesised via common intermediates and pathways. However, unlike leukotrienes, lipoxins lead to the resolution of the inflammation and initiate tissue repair (Basil and Levy, 2016). 12- or 15-lipoxygenase can directly generate LXA4 from LTA4. However, it is known that in presence of LTA4H the product of LTA4 is skewed towards the production of pro-inflammatory LTB4. More specifically, it has been shown that *lta4h* expression in human and zebrafish was directly correlated to high level of LTB4 (Tobin et al., 2010). I reasoned that if the translocation of 5-LO is sufficient to trigger LTB4 production then I could manipulate this translocation with light using an optogenetic construct.

Optogenetics is a term that describes the use of light to control cellular functions. Optogenetics was first used in 1971, in this case nerve cells in the mollusk *Aplysia californica* were stimulated will blue (488nm) light and “caused changes through some mechanism other than damage” (Fork, 1971). More recently, optogenetics has also been used track cells, notably neutrophils in zebrafish with photoconvertible probe such as dendra2 which switch from red

to green fluorescence upon UV (405nm) exposure (Peters-Golden and Brock, 2003). Neutrophil reverse migration was notably discovered *in vivo* using this probe in zebrafish larva (Peters-Golden and Brock, 2003). One optogenetic approach for manipulation of cell signalling consists in controlling protein-protein interaction with light. Such a system has been used by our lab to control the release of chemoattractant *in vitro* and *in vivo*. It uses UVR8, a protein that cluster in the endoplasmic reticulum and that is not able to traffic to the Golgi (Chen et al., 2013a). However, upon UV exposure, the clustered UVR8 molecules are release and can traffic. Our lab achieved the control of CXCL2 release using a fusion of UVR8 with this chemokine linked to a furin site that is cleaved outside of the Endoplasmic reticulum. It was shown in both mice and zebrafish transplanted with cells expressing this construct that neutrophils were recruited to the transplanted cells releasing CXCL2 upon UV exposure (Sarris et al., 2016). The control of protein-protein interactions using optogenetics has also been demonstrated in transgenic zebrafish expressing an optogenetics system to control the apical polarity of Pard3 (Buckley et al., 2016). This system is based on *Arabidopsis thaliana* Phytochrome B (Peters-Golden and Brock, 2003). A limitation of the PHYB system is that it requires a co-factor (phycocyanobilin) only found in plants (Buckley et al., 2016). Another system from *Arabidopsis thaliana* is LOV (Light Oxygen Voltage), which does not require a cofactor, but has slow kinetics of dimerization, requiring tens of minutes to trigger protein-protein interactions (Yazawa et al., 2009). For my study, I used the light sensitive CRY2 (cryptochrome 2) system which requires no external chromophore and triggers protein interactions in a sub-second time scale. This system is derived from *Arabidopsis thaliana* (Taslimi et al., 2014), in which context CRY2 mediates photoperiodic control of flowering (Liu et al., 2008). CRY2 is composed of a N-terminal PHR (Photolyase-Homologous Region) and a chromophore-binding region. PHR binds FAD (Flavin Adenine Dinucleotide), a chromophore which is present in all organisms. CRY2 interacts with CIB1 (CRY-Interacting bHLH 1) in less than a second after blue light (488 nm) stimulation. This interaction between CRY2 and CIB1 is reversible within minutes. Shorter versions of CRY2 and CIB1 have been developed to improve the expression of CRY2 and CIB1 in vertebrate system which was reported as very low. In my study, I used CRY2PHR, a shorter version of CRY2 containing only the PHR domain and a truncated version of CIB1 referred to as CIBN. These shorter versions of CIB1 and CRY2 were identified and tested for their functionality by Kennedy *et al* in yeast two-hybrid experiments (Kennedy et al., 2010). The authors showed that CRY2PHR and CIBN were much better expressed and their basal activity was higher than the original full-length proteins. In this chapter, I demonstrated a new optogenetic tool composed of two constructs to control LTB4 production in time and space using light. I first tested and controlled the translocation of CRY2PHR-mCherry-5LO from the lumen to the nuclear membrane in immortalised cells. I then verified that LTB4 was produced as a result of CRY2PHR-mCherry-5LO translocation in

immortalised cells expressing the optogenetic tool. Following *in vitro* verification of this tool, I tested the functionality of these two constructs *in vivo* in zebrafish embryos using confocal microscopy.

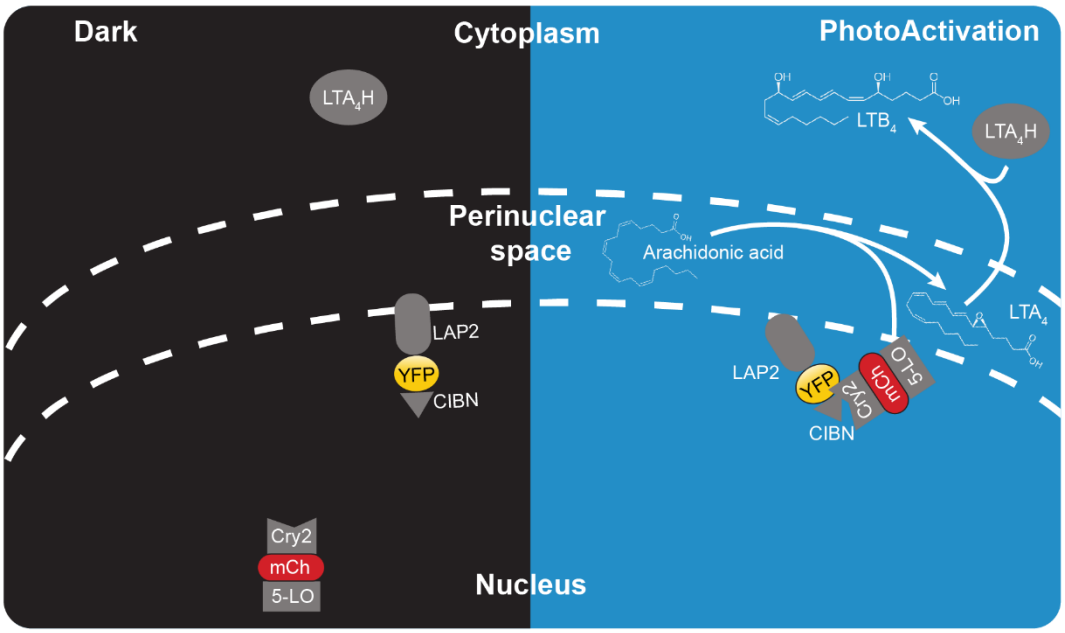


Figure 7.1. Optogenetic strategy to control LTB4 production with light.

Strategy for triggering the production of LTB4 by light using the cryptochrome system from *Arabidopsis thaliana*. The two domains composing this system are CRY2PHR and CIBN. They bind each other's upon blue light exposure. After blue light exposure the fusion of 5-LO with CRY2PHR and mCherry is translocated to the nuclear membrane where it binds the fusion of LAP2β which YFP and CIBN. At this location 5-LO produce LTA4 from arachidonic acid. LTA4 is subsequently turned into LTB4 by the enzyme LTA4H.

7.2 Light induced translocation of 5-LO in vitro

I engineered a fusion of CRY2PHR with 5-LO and the fluorescent protein mCherry. I fused the other optogenetic domain, CIBN, to YFP and LAP2β, an integral membrane protein that acts as a protein anchor and recruits the CRY2PHR-fusion to the nuclear membrane (Finlan et al., 2008) (Fig. 7.1). To test light-mediated 5-LO translocation, I co-transfected CRY2PHR-mCherry-5LO and CIBN-YFP-LAP2β in HEK 293T cells. Using the 488nm laser of the confocal microscope in single cells, CRY2PHR-mCherry-5-LO translocated to the inner nuclear membrane and co-localised with CIBN-YFP-LAP2β precisely in illuminated cells (Fig. 7.2, A). Using a blue LED (Light-Emitting Diode) as a light source I was able to simultaneously stimulate the optogenetic proteins in a large number of cells (Fig. 7.2, B). In addition, I was able to maintain CRY2PHR-mCherry-5-LO at the nuclear membrane by keeping cells

illuminated for more than one hour, whereas after a 30-sec pulse of illumination, translocation was recovered in the lumen within 20minutes (Fig. 7.2, B).

In conclusion, I demonstrated that CRY2PHR-mCherry-5LO can be translocated from the lumen to the nuclear membrane in the presence of CIBN-YFP-LAP2β using 488nm laser or LED light. I further demonstrated that the translocation can be precisely manipulated in space and in time.

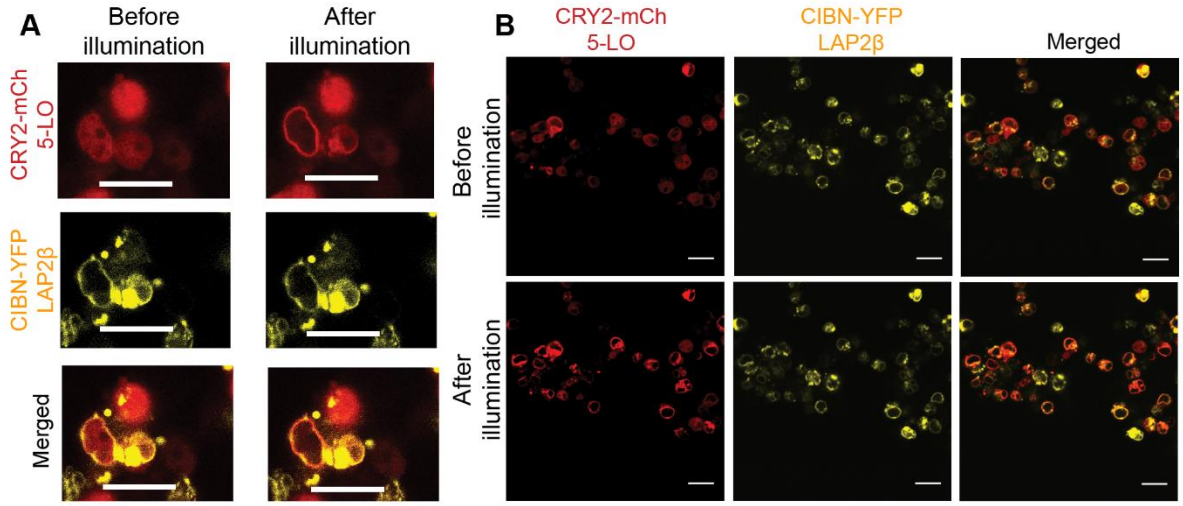


Figure 7.2. Controlling 5-LO translocation with light in vitro.

A HEK 293T cells expressing CRY2PHR-mCherry-5-LO (red) and CIBN-YFP-LAP2β (yellow). Illumination of a single cell with 488nm laser for 1second at 25 μW. After photoactivation CRY2-mCherry-5-LO co-localise with CIBN-YFP-LAP2β at the nuclear membrane. B Global illumination of HEK 293T cells expressing CRY2PHR-mCherry-5-LO and CIBN-YFP-LAP2β with a LED light at 4mW. Upon LED exposure CRY2PHR-mCherry-5-LO is translocated to the nuclear membrane, colocalising with CIBN-YFP-LAP2β (C). Scale bar= 25μm

7.3 Light-induced production of LTB4 in vitro

After achieving spatial and temporal control of CRY2PHR-mCherry-5-LO translocation to the nuclear membrane, I set out to determine if this translocation resulted in LTB4 production. For this I used NIH3T3 cells which endogenously express LTA4H (Brock et al., 2005). I first verified that successful temporal and spatial control of CRY2PHR-mCherry-5-LO translocation was achievable in these cells. I then developed an assay to measure LTB4 content in NIH3T3 supernatant, using ELISA-based detection in supernatants of treated cells (Fig. 7.3, A). As a positive control, to determine the level of LTB4 production achievable in this *in vitro* setting, cells were transfected with tRFP-5LO or CRY2PHR-mCherry-5-LO (alone) and incubated with A23187, a calcium ionophore, to induce the translocation of the construct and subsequent LTB4 production. LTB4 presence was detected which confirmed that 5-LO translocation was

enough to trigger LTB4 production and could be detected with ELISA. To test the production of LTB4 with the optogenetic tool, I co-transfected the cells with the two constructs and photo-treated them with blue LED light. With the optogenetic tool, I detected a similar level of LTB4 production as with ionophore addition (Fig. 7.3, B). Conversely, in cells co-transfected with both optogenetic constructs and left in the dark or with cells transfected with CRY2PHR-mCherry-5-LO alone and photo-treated with LED light, LTB4 level measured were markedly lower (Fig. 7.3, B).

To conclude, I achieved LTB4 production in immortalised cells expressing LTA4H by controlling 5-LO translocation to the nuclear membrane with light.

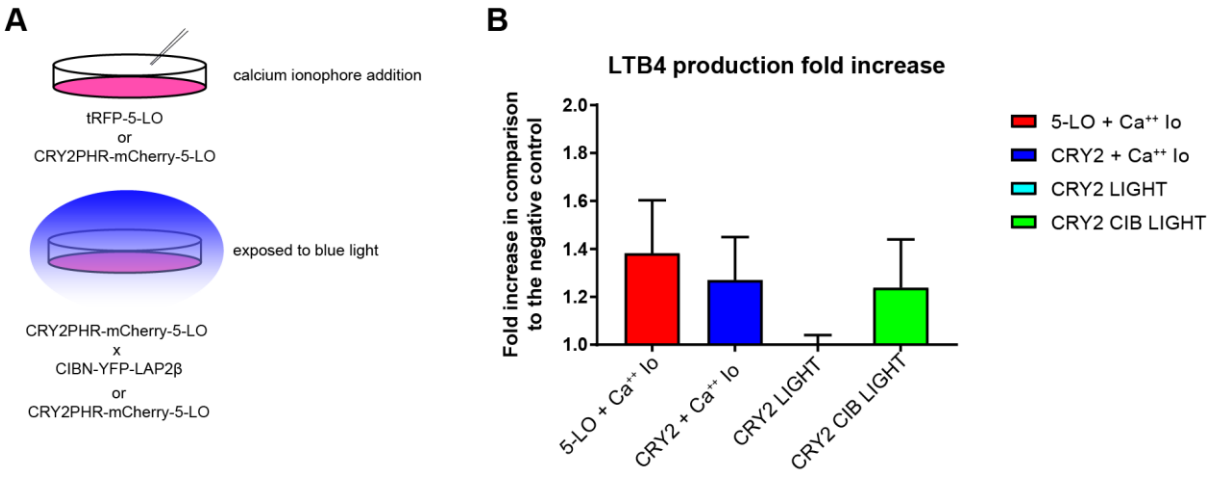


Figure 7.3. Optogenetic 5-LO translocation leads to the production of LTB4 in vitro.

A Schematic representation of the different conditions in B. NIH3T3 cells were transfected with tRFP-5-LO or CRY2-mCherry-5-LO in presence of calcium ionophore A23187 as a positive control, respectively 5-LO + calcium ionophore and CRY2 + calcium ionophore. CRY2-mCherry-5-LO transfected in NIH3T3 exposed to blue light LED without addition of calcium ionophore was used as a negative control. Production of LTB4 upon optogenetic translocation of 5-LO was tested by transfecting CRY2PHR-mCherry-5-LO along CIBN-YFP-LAP2β in presence or blue LED light (CRY2 CIB light). B Fold increase of LTB4 production in comparison to the negative control for the different conditions described in A, tested with ELISA; n=4 different experiments.

7. 4 Light-induced translocation of 5-LO in vivo

After validating the optogenetic tool in immortalised human cells, I set out to test the two constructs in zebrafish larvae *in vivo*. Because of the photosensitivity of our tool, the risk of "leaky activation" of the construct by ambient light is high. Therefore, my strategy was to generate two different stable transgenic zebrafish lines expressing each construct under a neutrophil specific promoter. By crossing those two lines, I would obtain larvae expressing both constructs in neutrophils, which could be temporarily kept in the dark to avoid leaky

activation (Fig. 7.4, A). I verified neutrophil-specific expression by crossing the Tg(lyz:CIBN-YFP-LAP2β) fish with Tg(*lyz*:dsred2) fish. I found fluorescent molecules from both transgenic lines to be co-expressed in neutrophils (data not shown). However, in order to successfully generate Tg(l*yz*:CRY2PHR-mCherry-5-LO) using the TOL2 insertion strategy, the CRY2PHR had to be optimised. Indeed, the original CRY2PHR-mCherry-5-LO construct used in the *in vitro* experiment, was not expressed in neutrophils. I therefore generated a version of CRY2PHR with optimised codons for expression in zebrafish. With this new codon-optimised construct, I obtained expression of CRY2PHR-mCherry-5-LO in zebrafish neutrophils after TOL2 transgenesis. Tg(*lyz*:CRY2PHR-mCherry-5-LO) embryos with positive expression of CRY2PHR-mCherry-5-LO were selected for generating the future stable transgenic line.

To test the optogenetic tool in zebrafish embryos while the transgenic line Tg(*lyz*:CRY2PHR-mCherry-5-LO) was reaching maturity, I performed transient TOL2 transgenesis on Tg(*lyz*:CIBN-YFP-LAP2β) eggs to insert CRY2PHR-mCherry-5-LO DNA. As a result, I obtained sub-optimal expression of CRY2PHR-mCherry-5-LO characterised by low mCherry fluorescent signal in few neutrophils. Nonetheless, I observed neutrophils co-expressing both constructs. I used confocal microscopy to image these neutrophils and performed photo-treatment with a 488nm laser. I observed the translocation of CRY2PHR-mCherry-5-LO in a neutrophil co-expressing both constructs after light treatment (Fig. 7.4, B).

To verify if pro-inflammatory LTB4 is produced *in vivo* as a result of CRY2PHR-mCherry-5-LO translocation, I investigated the behaviour of neutrophils in response to photo-treatment. For this, I needed more neutrophils co-expressing both optogenetic constructs in my embryos. I used the established Tg(*lyz*:CIBN-YFP-LAP2β) and transiently injected CRY2PHR-mCherry-5-LO RNA in the larvae. As a result, I observed a higher number of double positive neutrophils than with the previous approach. Using this method, I obtained 3dpf larvae ubiquitously expressing CRY2PHR-mCherry-5-LO but with a neutrophil specific expression of CIBN-YFP-LAP2β. I performed a ventral fin wound on a larva and subsequently imaged the recruitment of neutrophils swarming towards the site of injury. Using the 488nm laser, I activated the optogenetic tool in neutrophils located in an area beyond the injury. Due to the dim expression of the fluorescent molecules associated to the constructs expressed in the sample, I was not able to verify CRY2PHR-mCherry-5-LO translocation. However, a few minutes after the photo-treatment, neutrophils diverted from their original target (the site of injury) to migrate towards the light-treated neutrophils. This suggested that LTB4 might have been produced as a result of photoactivation of the optogenetic constructs leading to the attraction of neutrophils diverting from their original target (Fig. 7.4, C).

To conclude, I generated two stable transgenic zebrafish lines, each expressing one part of the optogenetic tool to control LTB4 production. Despite initial low expression of CRY2PHR-

mCherry-5-LO, I achieved the translocation of this protein fusion in larval neutrophils. I obtained promising preliminary data suggesting that the optogenetic tool is functional *in vivo*.

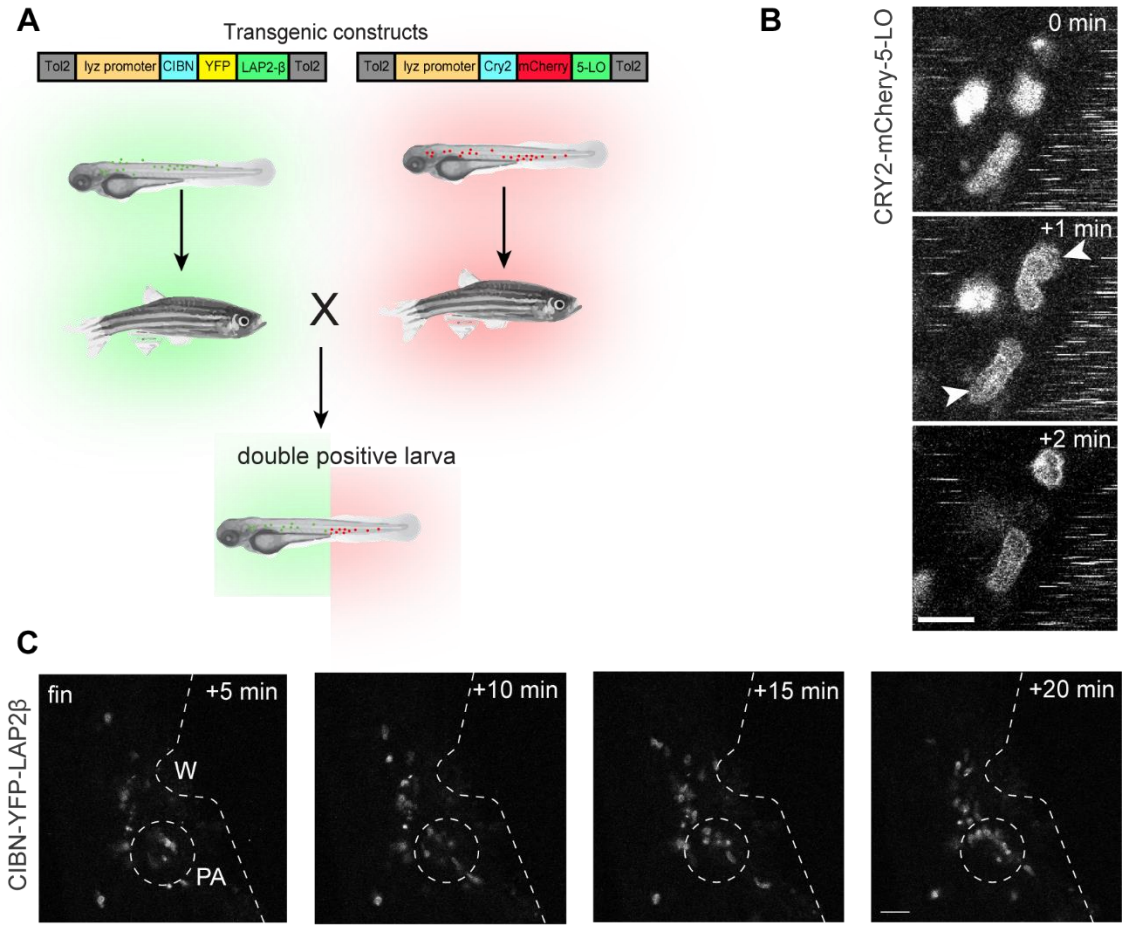


Figure 7.4. Controlling 5-LO translocation with light in vivo.

A Schematic representation of the strategy to obtain transgenic zebrafish larvae expressing both part of the construct in neutrophils. F0 WT fish are injected with Tol2 RNA and DNA of one of the constructs flanked with Tol2 sites. Positive F0 fish are then screened for expression of the fluorescent construct in neutrophil and grown. Positive adult F0 can then be crossed together to obtain double positive larvae. To improve neutrophil expression of each construct in those transgenic line F1 and F2 will subsequently be grown and screen as described in the methods. B Zebrafish larva expressing CRY2PHR-mCherry-5-LO and CIBN-YFP-LAP2β under the control of the lysozyme promoter was imaged on the confocal microscope. The whole area imaged was exposed to blue light with the 488nm laser, time is relative to blue light exposure. Translocation events are highlighted with white arrows. Scale bar=10µm. C Tg(*lyz*:CRY2PHR-mCherry-5-LO) injected with RNA, 3dpf larva was wounded on the ventral fin (W) and subsequently imaged on a confocal microscope. 30min post wounding, neutrophils were exposed to blue laser light (488nm) in the area shown with a dotted white circle. Time is relative to light exposure. Scale bar = 20µm.

7.5 Conclusion

In this chapter I described the design and generation of an optogenetic tool to trigger LTB4 biosynthesis. More specifically, this optogenetic tool triggers the translocation of the enzyme 5-LO, an important event leading to LTB4 biosynthesis. I first achieved translocation of CRY2PHR-tRFP-5-LO in immortalised cells *in vitro*. I then demonstrated that triggering the translocation of 5-LO *in vitro* was enough to produce LTB4. Following these results, I obtained successful 5-LO translocation using this optogenetic tool *in vivo*. Preliminary data obtained *in vivo* suggest that LTB4 is produced as a result of CRY2PHR-tRFP-5-LO translocation, but more work remains to be accomplished to support this conclusion.

7.6 Discussion

I generated a construct using the CRY2 CIBN optogenetic system to trigger protein-protein interactions between 5-LO and LAP2β. When expressed in immortalised cells, as expected, CIBN-YFP-LAP2β which is composed of an integral membrane protein was present on the nuclear membrane of HEK293T cells (Finlan et al., 2008). CRY2PHR-mCherry-5-LO as expected was expressed in the lumen of the nucleus. Blue light exposure triggered the translocation of CRY2PHR-mCherry-5-LO from the nucleus to the nuclear membrane in immortalised cells. The translocation following photo-treatment was very fast, however, it took 20 min for CRY2PHR-mCherry-5-LO to recover its original position in the nucleus. The phytochrome B system allows faster reversibility of protein-protein interaction but an external chromophore is needed for this approach and it would have to be delivered to neutrophils using an invasive technique (Buckley et al., 2016).

After achieving CRY2PHR-mCherry-5-LO translocation, I demonstrated that LTB4 could be produced with my optogenetic approach in immortalised cells expressing LTA4H (NIH3T3). This reveals that in presence of LTA4H, translocation of 5-LO alone is the limiting factor to produce LTB4.

To test the optogenetic tool *in vivo*, I generated stable zebrafish transgenic lines expressing one of the two constructs each. However, after performing TOL2 insertion of *lyz*:CRY2PHR-mCherry-5-LO, I did not observe any expression of this construct in neutrophils. This is surprising because I previously performed a transgenesis with a similar construct: *lyz*:tRFP-5-LO which showed excellent expression in neutrophils after transgenesis. Additionally, a previous report shows that the expression of CRY2 in zebrafish embryos was achieved with mRNA injection of CRY2 in zebrafish eggs (Liu et al., 2012). However, the authors only used

early stage embryos (6hpf) in their experiments. In my case, codon optimisation of CRY2PHR was necessary for the construct to be expressed in neutrophils. It therefore seems that neutrophil specific expression of CRY2PHR requires the optimisation of the codons of this gene.

To test the functionality of this optogenetic tool *in vivo*, I observed the behaviour of neutrophils in response to the photo-activation of the constructs. For this, I injected the mRNA of CRY2PHR-mCherry-5-LO in Tg(*lyz*:CIBN-YFP-LAP2β) eggs. I used the resulting 3dpf embryos and performed a mechanical wound on their ventral fin to recruit neutrophils. While neutrophils were migrating towards the site of injury, I diverted their migration by photo-activating the optogenetic tool in cells away from the wound. This observation is promising but I cannot exclude that the 488 photo-treatment could be at the origin of the neutrophil diversion, recruiting neutrophils by inflicting light damage. A negative control in similar conditions but with neutrophils expressing only CRY2PHR-mCherry-5-LO remains to be obtained. To verify that LTB4 is produced by zebrafish neutrophils as a result of optogenetic activation, I could quantify LTB4 concentration in zebrafish larvae. LTB4 level contained in zebrafish larvae are too low to be analysed by ELISA. The analysis of LTB4 quantity in zebrafish larvae samples could be achieved by performing High-Pressure Liquid Chromatography which has higher sensitivity (Colas et al., 2019).

Once validated, this promising new tool will be used to further understand LTB4 signalling dynamics *in vivo*. Specific patterns of light could be applied to activate LTB4 biosynthesis and determine whether this is sufficient to generate neutrophil swarms. By forcing neutrophils to generate LTB4, the recruitment of neutrophils to wounds or sites of infection could be increased. This will be useful to further assess how neutrophil swarms affect infection clearance. Enhancing the magnitude of the neutrophil recruitment to sterile injury could also provide insights on how neutrophils affect tissue integrity and wound regeneration.

The utility of this optogenetic method could be expanded to produce other inflammatory metabolites. As shown in zebrafish larvae by Tobin *et al*., the genetic or chemical inhibition of LTA4H skew the production of LTB4 towards the production of LXA4 (Lipoxin A4) an anti-inflammatory molecule (Tobin et al., 2010). In this case, the product of 5-LO translocation is used to produce anti-inflammatory LXA4. The optogenetic tool could therefore be tested to generate LXA4 in larvae incubated in bestatin (chemical inhibitor LTA4H). In this condition, this tool would be used to decrease the amplitude of swarms or create anti-inflammatory conditions. This will be useful to investigate the role of neutrophil in various physiological processes such as tissue regeneration or wound defence.

8. Discussion

8.1. Aim and summary of results

The overall aim of this project was to determine the fundamental cellular and molecular mechanisms of neutrophil swarming *in vivo*. Knowing that LTB4 is the key chemoattractant required to provoke neutrophil swarming (Lämmermann et al., 2013), I investigated the molecular and cellular triggers of LTB4 biosynthesis in neutrophils. I observed a specific calcium pattern in neutrophils clustering at sites of injury. This pattern was characterised by a prominent elevation of intracellular calcium which was relatively long-lived and led to a cellular mass which sustained net calcium levels at a high level in comparison to migrating cells. I further established that LTB4 production is taking place in these high calcium-fluxing neutrophils. I then set out to interrogate the molecular and cellular pathways underlying calcium elevation and subsequent LTB4 production. Using a set of chemical and genetic inhibitors I showed that ATP binding to gated ion channels P2X1 was at the origin of the calcium intake previously observed in clustering neutrophils. I demonstrated that P2X1 and Cx43 (Connexins 43) act in the same pathway, supporting the idea that Cx43 hemichannels conduct ATP signalling in the cluster. I concluded that damage sensing through ATP was amplified by Cx43 hemichannels in an autocrine and juxtacrine manner, leading to the production of a source of LTB4 in clustering neutrophils. Another important aspect of my project was to determine the physiological relevance of swarming in the context of a bacterial contamination of the wound. For this, I developed a new model using the bacterium, *Pseudomonas aeruginosa* to colonise zebrafish wounds. With this set up, I demonstrated the crucial role of neutrophil accumulation for protection against external pathogens. Finally, I developed an optogenetic tool to further dissect LTB4 signalling. This tool consists in manipulating a key molecular event associated with the eicosanoid pathway to trigger LTB4 production. I validated this tool *in vitro* and obtained promising preliminary results *in vivo*.

8.2. General discussion and outlooks

8.2.1. Establishing the conditions for neutrophil swarming in zebrafish

Neutrophil swarming was first observed by Chtanova and her colleagues in 2008 in response to Toxoplasma gondii in mouse lymph nodes (Chtanova et al., 2008). Soon after this discovery, it was demonstrated that LTB4 was the key signalling molecule responsible for neutrophil swarming *in vivo* (Afonso et al., 2012; Lämmermann et al., 2013). However, the

dynamics of LTB4 signalling remained unclear. LTB4 is a small lipid and is difficult to track through direct labelling. In this study, in order to follow LTB4 dynamics *in vivo* I tracked events associated with its production and sensing. With this approach I was able to provide insights into the molecular mechanisms and cellular triggers leading to neutrophil swarming *in vivo*.

The first step was to generate an assay to trigger neutrophil swarms in zebrafish larvae. Indeed, so far, there was no assay reporting consistent generation of neutrophil swarms in zebrafish. In Chapter 3, I demonstrated two assays to trigger swarms *in vivo*. Because of the self-amplifying nature of the swarming behaviour in neutrophils, a threshold number of neutrophils at the wound was needed in order to obtain stable neutrophil swarms. This was shown in neutrophils responding to sterile injury in mice (Park et al., 2018). In this model, a threshold of 10 neutrophils accumulating at the wound during the first 20 minutes post wounding was critical for further stable aggregation. I determined that an ideal tissue to consistently recruit enough neutrophils to injuries was the CHT (Caudal Haematopoietic Tissue) as it is rich in neutrophils. I developed two assays to generate swarms in zebrafish larvae. One in which I used a 2-photon laser to induce damage precisely near the CHT and one in which I mechanically wounded the fish with a scalpel on the ventral fin near the CHT. To verify the effectiveness of the assay I used Tg(*mpx*:GFP) embryos that express GFP in neutrophils. I monitored the recruitment of neutrophils to the site of injury using either time lapse confocal microscopy or 2-photon (in the case of laser wounding) or laser microscopy imaging (following laser wounding or mechanical wounding). To precisely identify and characterise the coordinated migration that neutrophils elicit during swarms, Antonios Georgantzoglou developed a method to analyse the directionality of migrating neutrophils based on the one used by Lämmermann *et al* to study swarms in mice (Lämmermann et al., 2013). For this, he analysed the radial speed of neutrophils with respect to the centre of the site of injury. Specifically, they found that the radial speed calculated, is the rate of change of the distance between neutrophils and the site of injury. The radial speed is the product of the chemotactic index (the cosine of the directional angle between the cell and the site of injury) and the speed of the neutrophils. Antonios pooled and normalised data from multiple embryos by dividing instantaneous speed values of individual neutrophils by the mean instantaneous speed value of neutrophils in the corresponding embryo. The data that we obtained with the laser wound experiment was consistent with the data obtained by Lämmermann *et al* in their laser wound mouse model of neutrophil swarming. The migratory behaviour of neutrophils recruited to a mechanical injury was different to the behaviour observed in neutrophils recruited to a laser injury. In the case of the mechanical injury, cell recruitment to the wound was delayed and multiple small waves of neutrophils were observed to be recruited overtime whereas only one major continuous wave of recruitment was observed with the laser wound assay. In both assays, however, neutrophils ended up forming a static cluster at the site of

injury. I concluded that both mechanical and laser wounding techniques were effective for triggering neutrophil swarms. I used these assays to study neutrophil swarms in zebrafish larvae. However, I focused more on the 2-photon laser injury because it would elicit more consistent swarms and radial speed profiles, the neutrophils would reach the wound in a shorter duration and time lapse imaging could be taken before, during and after the injury. However, a limitation with the 2-photon microscope is the imaging speed, resolution and the number of dyes used as notably red fluorescent molecules such as mCherry are hard to visualise with 2-photon microscopy (Peters-Golden and Brock, 2003). Therefore, in chapter4, to visualise subcellular events (5-LO translocation) after a laser injury, I instead transferred embryos wounded under the 2-Photon microscope to the Spinning disc confocal microscope for imaging. However, this manoeuvre was complicated because embryos must be embedded in agarose for wounding under an upright 2-Photon microscope and then be re-embedded for imaging on an inverted spinning disc confocal microscope. This had to be done in a limited amount of time as laser injury triggers quick recruitment of neutrophils to the wound. In the future, this might not be a limitation, with the development in the CAIC (Cambridge Advance Imaging Centre) of a microscope combining 2-photon irradiation and confocal spinning disk imaging.

Another important step before starting to delve into the molecular mechanisms underlying neutrophil swarming was to verify that as reported in mice (Lämmermann et al., 2013), LTB4 was the key chemoattractant to trigger swarms in zebrafish. For this I used *lta4h* MO in chapter 4 and *blt1b* MO in chapter 5 to respectively knock out the production and the sensing of LTB4. This demonstrated that neutrophil recruitment to inflammatory sites was dependent on LTB4 signalling and on production of this mediator by neutrophils.

Altogether these datasets provided a basis for exploring the molecular mechanisms of neutrophil swarming in the zebrafish model.

8.2.2. The calcium dynamics underlying neutrophil swarming

LTB4 production requires the calcium-dependent translocation of two enzymes, 5-LO (5-Lypoxygenase) and PLA2 (Phospholipase A2), to membrane compartments where lipid metabolism takes place (Dixit and Simon, 2012; Luo et al., 2003). It was shown in a laser wound model of *Drosophila melanogaster* that calcium is the earliest signal in the wound inflammatory response (Razzell et al., 2013). In their study the authors show that calcium inhibition with EGTA reduced the inflammation demonstrating an important role for calcium in tissue injury. Prior to my study, intracellular calcium dynamics had only been observed in zebrafish epithelial cells (Enyedi et al., 2013) and neutrophils migrating in a solitary manner (Beerman et al.,2015), but not in swarming neutrophils. I hypothesised that observing the

calcium dynamics taking place in neutrophils during swarms would generate important insights on the sequential dynamics of molecular events happening during swarms.

To this end, in chapter 3, I described how I generated and used genetically modified fish expressing a calcium probe (GCamp6F) in neutrophils. With this transgenic line, I observed very distinctive calcium patterns elicited by neutrophils during swarms. Most neutrophils fluxing calcium at the cluster were alive and considering their atypical high intracellular calcium level, I hypothesised that important calcium-dependent biochemical processes might be taking place in these neutrophils. Depletion of extracellular calcium induced by injection of the calcium chelator EGTA in zebrafish embryos led to the complete inhibition of calcium signals in neutrophils and the total abrogation of neutrophil migration upon tissue injury. This was not expected as it has been previously reported that depletion of calcium using EGTA on human neutrophils *in vitro* had no effect on their chemotaxis towards a source of fMLP (Wang et al., 2017b). In chapter 4, to further understand the distinctive neutrophil calcium dynamics during swarms, I used inhibitors of specific calcium pathways. I inhibited TRP channels and voltage-gated calcium channels using SKF (SKF 96365 hydrochloride) (Harteneck et al., 2011). I did not observe a significant difference in the calcium and migratory pattern of neutrophils exposed to SKF during laser induced swarms, except perhaps a diminution in neutrophil recruitment to the site of injury which could mean that TRP channels and voltage-gated calcium channels might play a role in neutrophil swarming. This experiment would need to be repeated and analysed to assess the effect of SKF on neutrophil swarms. This analysis would consist in comparing the relative GCamp6F fluorescence intensity at the wound in WT vs SKF-treated embryo and to determine if a significant change is observed in neutrophil behaviour by quantifying the normal radial speed of neutrophils migrating towards the site of injury.

I also used ML-9 to inhibit SOCE (Store Operated Calcium Entry), but again, I did not observe an apparent difference in neutrophil's calcium dynamics and cellular behaviour during swarms. More repetitions of these experiments and quantifications need to be done in order to conclude on the role of store-operated calcium channels for neutrophil influx during swarms. Specifically, they would need to be analysed to assess a change in neutrophil migratory behaviour through their normalised radial speed and to assess the calcium level in neutrophils based GCamp6F fluorescence.

This might reinforce the idea that calcium plays a major role in forcing the production of pro-inflammatory molecules that leads to secondary recruitment. Unfortunately, this event has not been quantified and more repeats of the experiments would be required to conclude on this effect.

8.2.3. The attractant dynamics underlying neutrophil swarming

No previous study has determined the time and place of the production of LTB4 during neutrophil swarming *in vivo*. However, it was hypothesised, based on *in vitro* data, that LTB4 was produced in neutrophils while they were still in motion and migrating towards a primary attractant such as fMLP towards the site of injury (Afonso et al., 2012). In this hypothesis, neutrophils would relay the LTB4 signal from neutrophil to neutrophil while migrating towards the site of injury. LTB4 would be produced and released at the rear of the neutrophils migrating and signalling to neutrophils further away. Unlike what is postulated in this signal-relay hypothesis, I only observed 5-LO translocation in static neutrophils clustering at the wound with high intracellular calcium levels. I only observed LTB4 production in clustering neutrophils and not in migrating neutrophils. Calcium levels in clustering neutrophils are comparable with what I observed in neutrophils after stimulation with a calcium ionophore. In the latter case, calcium rise induced by the ionophore was triggering 5-LO translocation in every neutrophil. This confirms that high calcium is a requirement for 5-LO translocation.

Based on these observations and subsequent calcium and 5-LO analysis during neutrophil swarms, I determined that clustering neutrophils generate a centralised gradient source of LTB4 when they cluster to the site of injury during swarms rather than relaying LTB4 signals while migrating to the site of injury. This discovery is important because it demonstrates that the amplification of neutrophil signal is dependent on the initial recruitment of pioneer neutrophils to the wound. These neutrophils will subsequently amplify LTB4 signal and attract further cells creating a positive feedback loop with newly recruited cells further amplifying the signal. As shown in Chapter 3, the size of the cluster is directly correlated with GCamp6F fluorescence. The amplitude of the neutrophil response therefore seems to be directly correlated with the number of clustering neutrophils at the site of inflammation. This allows scaling of the immune response to the degree of tissue damage suffered. If the initial damage is minimal, few neutrophils will be recruited, and the signal will not be amplified. However, if the initial damage is substantial and triggers the recruitment of enough neutrophils, then the response can escalate into swarms. This is therefore an important mechanism needed to balance the trade-off between rapid defence and excess inflammation. It would be interesting to validate this finding in a mouse model of sterile injury like the one used by Lämmermann *et al* (Lämmermann et al., 2013). tRFP-5-LO zebrafish neutrophils could be isolated from the whole kidney marrow of transgenic fish Tg(*lyz*:tRFP-5-LO) and transplanted in a mouse ear. Live microscopy imaging of the mouse ear could then be performed to analyse and quantify 5-LO translocation in this setting. Alternatively, mouse ear tissues could be fixed at different timepoint after the injury and immunostained for 5-LO. Those samples could be imaged on confocal microscope and 5-LO translocating event could subsequently be quantified.

8.2.4. LTB4 receptor signalling and polarisation during neutrophil swarming

To probe for neutrophils sensing LTB4, I tested the possibility of Blt1b internalisation as readout. However, despite being functional the receptor reporter was not internalised. This was consistent with the absence of internalisation of BLT1 in human neutrophil-like cells *in vitro* in a parallel study published in 2018 (Subramanian et al., 2018). The authors notably concluded that "BLT1 is largely resistant to endocytosis under physiological conditions". This is based on *in vitro* analysis of internalisation of BLT1-GFP expressed in neutrophil-like cells stimulated with chemicals inducing LTB4 production, fNLFNYK and C5a. This meant that internalisation could not be used as readout and raises questions about the dynamics of LTB4 signalling. This sustained plasma membrane residence of the receptor could also have an effect on neutrophil behaviour. Indeed, internalisation of GPCR triggered via its phosphorylation participate in the desensitisation of the receptor (Kelly et al., 2008). Receptor desensitisation is the loss of response of the receptor following a prolonged or repeated administration of an agonist. If the receptor is not desensitised and maintained at the surface this might increase its sensitivity (Borregaard et al., 2007). We could therefore hypothesise that if other GPCRs (G Protein Coupled Receptor) are desensitised and LTB4 is not it could mean that neutrophils are primarily following cues of LTB4 over other chemoattractants.

GPCRs may be polarised at the leading edge of lymphocytes migrating towards attractant sources. Notably, it was shown that chemokine receptors CCR2 and CCR5 were redistributed to the leading edge of T lymphocyte migrating towards a source of chemokines *in vitro* (Nieto et al., 1997). In chapter 5, I obtained preliminary evidence that suggests that Blt1b distribution may be polarised at the leading edge of neutrophils. Polarisation of Blt1b distribution might act as a sensor mechanism to increase responsiveness to LTB4 and lead neutrophil motion along the LTB4 gradient during neutrophil swarms.

Further quantitative analysis in relation to a membrane marker remains to be done to confirm this conclusion. For this, it could be interesting to determine when LTB4 receptors start being redistributed, (and by which neutrophils) in order to determine when and where LTB4 is sensed by migrating neutrophils. I could also use pertussis toxin (del Pozo et al., 1995), an inhibitor of receptor trafficking, in order to see the impact of receptor redistribution on neutrophil migration. For this I will have to either inject the toxin in the larva or generate stable transgenic fish expressing it under the control of a neutrophil specific promoter because the toxin is a protein and therefore won't diffuse freely in the bath in which the zebrafish is incubated.

It has been shown in human neutrophils *in vitro* that LTB4 producing enzymes, 5-LO and LTA4H are located at the rear of neutrophils migrating toward a source of fMLP (Majumdar et al., 2016), however this was not during a neutrophil swarm. Additionally, the authors visualised

that exosomes were released by neutrophils using electron microscopy. They purified the exosomes and found that they were containing 5-LO, LTA4H and LTB4. It is difficult to observe exosomes in larvae *in vivo*, although this might be possible in fixed zebrafish larvae samples stained with antibody binding exosomes marker such as CD-63 or by using electron microscopy on fixed zebrafish larvae (Calzia et al., 2018). In my system, I did not observe vesicular localisation of 5-LO and LTA4H or polarisation of these enzymes at the rear of the cells which means that what is described by Majumdar *et al* might not be required for neutrophil swarming. However, their discovery might have other implications in the human system. This polarisation of LTB4 signal could notably be used for long range guidance of other leukocytes like the neutrophil trails enriched in chemokines left by neutrophils in blood vessels and the trachea in an influenza-infection model of mouse trachea (Lim et al., 2015).

8.2.5. The molecular mechanisms underlying the generation of a centralised LTB4 source during neutrophil swarming

My data using the calcium and 5-LO reporters suggested that a centralised gradient source of LTB4 is produced by high calcium neutrophils clustering at sites of injury. However, it was still unknown what were the molecular mechanisms underlying the rise of intracellular calcium in neutrophils that leads to the subsequent production of LTB4. One hypothesis is that neutrophil death could be the cellular trigger required for subsequent neutrophil swarms. Previous studies (Lämmermann et al., 2013; Uderhardt et al., 2019), suggested that neutrophil death could be the cause of the initial wave of neutrophils recruited during swarms. This was suggested by the observation that neutrophils quickly accumulated at the site of laser injury following neutrophil death. (Lämmermann et al., 2013; Uderhardt et al., 2019). It remained unclear whether this was essential for swarming as additional non dying neutrophils could be seen at the wound focus in some instances (Uderhardt et al., 2019). A systematic analysis to correlate swarm incidence with neutrophil death, as opposed to tissue death, is still lacking. In Chapter 4, I used propidium iodide to stain for dead cells during neutrophil swarms in zebrafish larvae. Surprisingly, after quantifying and analysing neutrophil death, the majority of neutrophils fluxing calcium were alive. Some neutrophils died after the major wave of neutrophil migration to the site of injury had happened. Therefore, my experiments did not suggest neutrophil death/necrosis was essential for neutrophil swarming. It is possible that neutrophil death plays a role in enhancing neutrophil migration and swarming by the release of DAMPs such as ATP or cytokines and other pro-inflammatory molecules that would be released by dead neutrophils. But it remains unclear whether neutrophil death is functionally different to tissue death in triggering swarming. To clarify this, one could specifically target neutrophils with the 2 photon lasers and subsequently analyse the behaviour of the

surrounding neutrophils and systematically compare this with the behaviour of neutrophils in response to death of other cells.

I Identified a role for the ATP receptor P2X1 and Cx43 (Connexin 43) in the intra-cluster neutrophil calcium fluxes. ATP signalling to the gated ion channel P2X1 could be coming from dead cells and/or neutrophils themselves. Indeed, it was shown that neutrophils can release ATP using high-performance liquid chromatography and luminometric ATP on isolated neutrophils stimulated with fMLP, *in vitro* (Eltzschig Holger K. et al., 2006). In the same study, following a drug screening on isolated neutrophils, the authors discovered that ATP was released from neutrophils through Cx43 (Connexin43) hemichannels. Furthermore in another study on human neutrophils *in vitro*, Cx43 dependent release of ATP was shown to trigger neutrophil arrest and calcium influx by signalling via P2X1, which is in accordance with my observations (Wang et al., 2017b). The release of ATP is therefore likely to be Cx43 hemichannel-dependent. Cx43 also have channel-independent functions, one of which is adhesion and it could therefore be argued that this function could play a role in neutrophil aggregation to sites of injury, similarly to what have been shown for integrins in a model of mouse sterile injury (Lämmermann et al., 2013). However, with carbenoxolone inhibition and the dominant-negative mutant I generated, I only inhibited Cx43 channel function and not Cx43 adhesion function, it is therefore unlikely that Cx43 adhesion is responsible for the phenotype I observed.

Cx43 is continuously phosphorylated in resting cells. Upon dephosphorylation by MAP Kinase, Cx43 opens the channel to release ATP (Riquelme et al., 2015). It could be interesting to stain zebrafish larvae with Cx43 and Phospho-Cx43 antibodies after performing a laser injury to identify which neutrophils have phosphorylated connexins and have their channels effectively open. Additionally, I could perform a similar experiment as Wang *et al*, by stimulating isolated zebrafish neutrophils *in vitro* with a DAMP such as fMLP and look at the effect on Cx43 phosphorylation by performing a western blot on stimulated cells and probing for phosphorylated Cx43 (Wang et al., 2017b). This will indicate whether or not Cx43 channels are activated upon DAMP sensing. To further verify the channel activity of Cx43 I could use PI (propidium iodide), a drug I previously used to detect dead cells. This vital stain can be transferred from cell to cell through gap junctions (Elfgang et al., 1995). However, in zebrafish larva, I was not able to visualise PI uptake by neutrophils clustering at sites of injury during swarms. This might be because the level of transport between neutrophils is below our detection limit. Instead, to investigate Cx43 channel activity, I could electroporate (De Vuyst et al., 2008) isolated neutrophils *in vitro* to load them with a tracer dye called Trojan-LAMP (Guo et al., 2008). Trojan LAMP is used to trace the channel activity of gap-junctions. In normal conditions, this molecule cannot be transferred through gap-junction as it is composed of a fluorescent coumarin linked to a 40kDa dextran sugar. However, upon UV light exposure, the

coumarin fluorophore is released from the dextran sugar, becomes fluorescent and can freely diffuse through hemichannels and gap junction (Guo et al., 2008). I could mix isolated neutrophils from adult zebrafish loaded with trojan-LAMP with neutrophils not loaded and stimulate those cells *in vitro* with fMLP and/or LTB4 and subsequently expose them to UV to release the coumarin from the dextran. I could then observe if the trojan-LAMP diffuses from loaded cells or not to control cells through hemichannels/gap junction. Hemichannel/gap junction activity could also be inhibited chemically with CBX, or alternatively neutrophils could be isolated from Tg(*lyz:cx43DN*-T2A-mCherry) fish to verify that the release of coumarin is Cx43 dependent.

8.2.6. The role of neutrophil swarming in defending the wound against bacterial infection

The role of neutrophils in response to an injury is controversial. Some lines of evidence suggest the main role of neutrophils is to protect the wound against possible external pathogens during injuries. Patients with Felty's syndrome, which is characterised by neutropenia, are more likely to develop bacterial infections, particularly *Staphylococci* and *Streptococci* (Sienknecht et al., 1977). Interestingly, a case study described how chronic wound infection in these patients could be treated by drugs stimulating neutrophil production (Fohlman et al., 1994). This suggests that neutrophils play an important role for wound defence against pathogen. However, other studies argue that the presence of neutrophils might be detrimental for wound healing. One such study shows that in a mouse injury model, wound closure was accelerated in mice which had been depleted for neutrophils using an anti-neutrophil serum (Dovi et al., 2003). In another mouse study, PU.1 knock out mice were used to study the effect of neutrophils and macrophages in wound healing (Martin et al., 2003). PU.1 is a transcription factor essential for both neutrophil and macrophage maturation. PU.1 deficient mice are born with no functional macrophages and neutrophils. Interestingly wounds performed in PU.1 mice showed increase angiogenesis and faster wound repair than in WT mice. The benefit of neutrophil recruitment in tissue injury might be the result of an evolutionary compromise. On one hand, neutrophil accumulation might delay wound healing but on the other hand it prevents infection which might be more important than quicker wound repair.
To study the role of neutrophils in wound defence against external pathogens in zebrafish, I developed a wound contamination assay using *Pseudomonas aeruginosa* that I describe in chapter 6. Using this assay, I found that Cx43 plays a beneficial role in wound defence from *Pseudomonas aeruginosa* colonisation. This provides a possible link between swarming and host defence given the specific role of Cx43 in swarming. However, it is also possible that Cx43 might affect host defence through swarming-independent effects. An interesting complementary experiment would be to perform a laser injury on a larva incubated in a bath

of *Pseudomonas aeruginosa* to dissect the subsequent neutrophils and bacterial dynamics. The effect of neutrophil swarming could then directly be visualised and assessed by inhibiting Cx43.

In addition to host defence, it would be interesting to investigate if the regeneration process is delayed or accelerated when Cx43 is inhibited. This could be done by performing mechanical wounds on embryos and imaging the regeneration over time. Quantification of the fin area and length overtime can be performed on *Fiji* software to follow wound regeneration (Lisse et al., 2015).

8.2.7. An optogenetic tool to control neutrophil aggregation

To further understand the role of neutrophil aggregation in wound regeneration and wound defence against external pathogens, I designed a new methodology to manipulate the coordinated migration of neutrophils *in vivo*. In chapter 7, I show that I achieved precise control of the translocation of CRY2PHR-mCherry-5-LO in HEK293T cells by blue light. I next showed in NIH3T3 cells endogenously expressing LTA4H (Brock et al., 2005) that LTB4 was produced as the result of the activation of this optogenetic construct. Finally, I managed to trigger translocation of CRY2PHR-mCherry-5-LO in neutrophils *in vivo* and obtained promising results. The results indicated that neutrophils which express both optogenetic constructs initially migrated towards a site of injury but could be diverted from their original destination upon blue light stimulation of neutrophils located beyond the site of injury. However, it is possible that the diversion of neutrophil migration towards these illuminated cells could be the result of laser phototoxicity at the alternative site, induced by the photoactivation itself. A negative control experiment using larvae which express only one of the two constructs is therefore needed to determine if the recruitment was due to laser damage or optogenetic activation. Further experiments will be required to validate this tool *in vivo*. Analysis of LTB4 levels after global illumination of larvae expressing both optogenetic constructs could be performed and compared to the levels of LTB4 in a negative control expressing only one part of the optogenetic construct. For detecting such low amount of LTB4 produced by neutrophils in the larvae, multiple larvae samples will have to be pooled together and high-Pressure Liquid Chromatography will be performed to precisely analyse LTB4 levels. Our laboratory is planning to conduct this research in collaboration with Dr. Jesmond Dalli who is an expert and has successfully performed LTB4 detection from zebrafish larvae (Colas et al., 2019). This assay might also allow better characterisation of neutrophil swarms by detecting other inflammatory mediators that might be produced.

Once validated, this tool may prove useful in the study the importance of the dynamics of LTB4 production in neutrophil swarming, tissue physiology and host defence. LTB4 production could

be triggered in the absence of any inflammation to assess whether LTB4 alone is sufficient to generate neutrophil swarms. Neutrophils could also be diverted from their presupposed migratory route to an alternative secondary site. The impact of this diversion on wound defence or regeneration could be assessed. This tool could also be used to increase the recruitment of neutrophils to wounds and assess the resulting physiological effect on wound repair, neutrophil death and defence against pathogens.

As shown in zebrafish larvae by Tobin *et al*., genetic or chemical inhibition of LTA4H can skew metabolism of LTA4 towards the production of LXA4 (Lipoxin A4) an anti-inflammatory molecule (Tobin et al., 2010) through either 12- or 15-lipoxygenase activity. The optogenetic tool I introduced could therefore be amended and utilised to generate LXA4 in larvae incubated in bestatin (chemical inhibitor LTA4H). In this condition, this tool would be used to decrease the amplitude of swarms or create anti-inflammatory conditions. This would increase the versatility of this tool and may prove beneficial in the interrogation of neutrophil behaviour in various physiological processes such as tissue regeneration or wound defence.

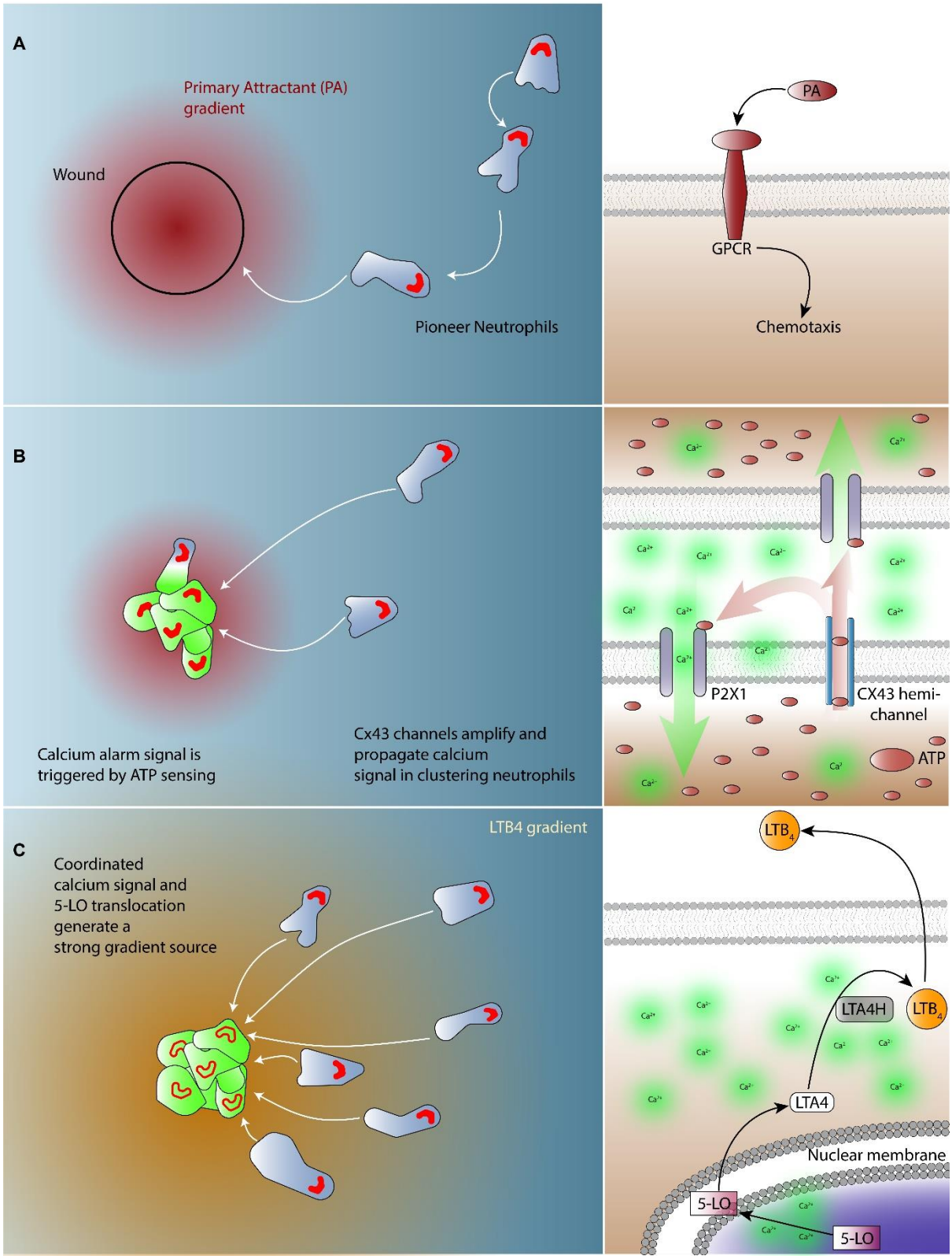


Figure 8.1. Model for initiation of neutrophil swarms by connexin-dependent calcium signals.

A Pioneer neutrophils are attracted by primary attractants/cues (DAMPs) to the wound site. The distribution of 5-LO and levels of calcium are indicated in red and green respectively. B The primary attractant gradient may decay but pioneer neutrophils are triggered to release ATP through Cx43

channels, amplifying damage sensing in an autocrine and juxtacrine manner. This prolongs the half-life of damage signals and 'buys time' for the formation of an effective multicellular LTB4 gradient. Threshold levels of ATP lead to opening of P2X1 channels and a rise of intracellular calcium that leads to neutrophil arrest and 5-LO translocation, ultimately activating LTA4/LTB4 synthesis and further clustering. Note that the intracellular concentration of ATP is high compared to extracellular levels, and the opposite gradient applies in the case of calcium. C Coordinated activation of LTA4/LTB4 synthesis in the cluster builds a powerful and stable LTB4 gradient source that triggers a coordinated wave of migration.

8.2.8. Future questions on termination of neutrophil swarming

The current understanding of the molecular mechanisms leading to neutrophil swarming states that LTB4 and integrins are needed for neutrophil swarms (Lämmermann et al., 2013). My study of neutrophil swarming in zebrafish larvae confirms the role of LTB4 for neutrophil swarming and reveals that the production of LTB4 relies on Cx43 hemichannel-mediated purinergic juxtacrine signalling in clustering neutrophils (Fig. 8.1). Theoretically, with more neutrophils recruited, more LTB4 is produced providing a positive-feedback loop that drives neutrophil recruitment. A question that remains is: how can this self-perpetuating process be turned off?

It is known that the resolution of the inflammation is dependent on the biosynthesis of anti-inflammatory and pro-resolution lipid mediators, such as lipoxins, resolvins and protectins (Serhan et al., 2008). COX-2 (Cyclooxygenase 2) is an enzyme present in neutrophils which catalyses the production of LXA4 (Lipoxin A4), an anti-inflammatory molecule and PGE2 (Prostaglandin E_2) a pro-resolution molecule. In a study by Bruce Levy *et al*, a sequential production of pro-inflammatory and then anti-inflammatory molecules during the inflammation process was observed (Levy et al., 2001). For this, the authors used a mouse model of inflammation in the dorsal air pouch. In this model, inflammation is mainly driven by neutrophils and resolves spontaneously. They injected a pro-inflammatory molecule TNF-α to mediate the inflammation. 60min after the inflammation, the level of LTB4 increased by 180%. Maximal neutrophil accumulation was detected 2 hours 30min after the inflammation and this correlated with maximal level of PGE_2. When levels of LTB4 started dropping, the authors detected a 750% increase of LXA4, 4 hours after TNF-α injection. At this point, neutrophils number diminished until complete resolution 24 hours post injection. The authors then show using RT-PCR that in human neutrophils exposed to PGE_2, *ex vivo*, 15-LO (15-lipoxygenase) was upregulated. 15-LO is an important enzyme that is capable of initiating lipoxin biosynthesis as well as converting the Leukotriene intermediate LTA4 into LXA4 (Haeggström and Serhan, 1999). Based on these results, they suggested that PGE_2 which is generated early during

inflammation induces the subsequent production of LXA4 that lead to the resolution of the inflammation.

Interestingly, a recent research article shows that PGE2 regulates neutrophil migration and LXA4 production in zebrafish larvae after a tail fin injury (Loynes et al., 2018). In this study, the authors suggested that the gene *alox12* in zebrafish functions as *alox-15* in humans. They incubated zebrafish larvae in a bath of E3 supplemented with PGE2, this resulted in the upregulation of alox12, measured by RT PCR. They also observed the reverse migration of neutrophils which had accumulated at a tail fin injury upon exposure to PGE2. For this they used a zebrafish line expressing the protein Kaede. Kaede undergoes photo conversion from green to red fluorescence under UV exposure (Ando et al., 2002). After photoconverting neutrophils, they determined that, in their model, most neutrophils reverse migrated during the resolution of the inflammation. Although zebrafish ALOX12 has been suggested as zebrafish ortholog of human ALOX15, their amino acid sequence is only 44% similar. Moreover, zebrafish ALOX12 has a similar degree of amino acid conservation with all other human ALOX isoforms (39 to 47%) (Peters-Golden and Brock, 2003). It could therefore be interesting to look at the effect of PGE2 on the upregulation of the 6 other zebrafish ALOX genes, by performing RT PCR on zebrafish larvae incubated in PGE2, as performed in the study I described above (Loynes et al., 2018). Once 15-LO ortholog of human in zebrafish is clearly identified, this gene could be knocked out with CRISPR Cas9 and the sterile laser injury assay I developed in chapter 3 could be used to verify the potential anti-inflammatory effects of this gene in neutrophil inflammation.

As mentioned in human and mice, 15-LO can convert LTA4, the product of 5-LO in LXA4. Ultimately, the optogenetic tool I developed, and described in chapter 7, is controlling the production of LTA4 by triggering the translocation of 5-LO to the membrane of the nucleus. LTA4H is the enzyme that produce LTB4 from LTA4. It has been shown in zebrafish larvae knocked out for LTA4H, that LTB4 production was abrogated and LXA4 production was taking place instead (Tobin et al., 2010). It could be interesting to use LTA3 (Leukotriene A3 methyl ester), a suicide inhibitor of LTA4H and if needed a fish overexpressing the ortholog of 15-LO to turn the LTB4 producing optogenetic tool into a LXA4 optogenetic producing tool and further analyse the resolution of neutrophil inflammation by modifying the production pattern of LXA4.

It has been suggested that macrophage efferocytosis of neutrophils (the phagocytosis of apoptotic neutrophils by macrophages) might be a mechanism leading to the resolution of the inflammation. This phenomenon was first observed in new-born babies with airway inflammation (Grigg et al., 1991). In their study, the authors used light microscopy and electronic microscopy to show evidence of the ingestion of apoptotic neutrophils by macrophages. The authors suggested that this mechanism “might represent a mechanism by which tissue injury is reduced during the resolution of […] inflammation”. Their hypothesis was

correct, and we now know that efferocytosis is an immunosuppressive process. A study showed that efferocytosis of UV-irradiated, apoptotic neutrophils by macrophages actively inhibits the pro-inflammatory production of IL-1β, GM-CSF, TNF-α and stimulates the production of anti-inflammatory TGF-β and PGE_2 (Fadok et al., 1998). This was shown *in vitro*, by stimulating the cells with LPS and zymosan and then quantifying the pro and anti-inflammatory mediators by ELISA. Efferocytosis was observed in zebrafish larvae in the context of *Mycobacterium marinum* infection. In this study a transgenic line displaying eGFP positive macrophages Tg(*mpeg1:*eGFP) was crossed with a transgenic line displaying DsRed2 positive zebrafish Tg(lyz:DsRed2). The authors were able to identify efferocytosis by observing the engulfment of red fluorescent cells (neutrophils) by the green fluorescent cells (macrophages). Our laboratory possesses a zebrafish line expressing mCherry under Mpeg1 (Macrophage Expressed Gene 1), a macrophage specific promoter. It would be interesting to use this Tg(*mpeg1*:mCherry) zebrafish line to study the impact of efferocytosis on the resolution of neutrophil swarms. For this, efferocytosis of neutrophils could be visualised and quantified during swarms.

During my research, I rationalised elements of previous experiments to elucidate the findings and discovered a new molecular pathway underlying the generation of neutrophil swarms. I have shown that Cx43 hemichannels drive purinergic activation of calcium influx in neutrophils clustering during swarms *in vivo*. This, in turn, triggers the production of LTB4 creating a gradient source necessary for the generation of neutrophil swarms (Figure 8.1). I then demonstrated that Cx43 driven neutrophil accumulation at infected wounds was important to defend the organism against pathogens. The experiments which I have suggested above will help further understanding the role of neutrophils during inflammation. Neutrophil swarming is likely to be important for wound healing, and wound defence against pathogens. The beneficial or detrimental role of neutrophils during these physiological processes is likely to depend on their migratory pattern and the pro-inflammatory and anti-inflammatory functions they exert.

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