THE MECHANISMS CONTROLLING NEUTROPHIL MIGRATION

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by

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Abstract

Neutrophil accumulation in the lung plays a significant role in the pathogenesis of inflammatory respiratory diseases including cystic fibrosis and COPD. The entry of neutrophils into the lung has been well characterised. However, the fate of these cells once inside the tissue microenvironment of the lung is not fully understood. An understanding of the interaction and signal transduction pathways controlling the migration of neutrophils within the lung could prove beneficial in the treatment of respiratory diseases. A variety of signal transduction pathways have been suggested to be involved in neutrophil migration. We aimed to determine the mechanisms controlling chemokinetic and chemotactic neutrophil migration in response to stimulation with CXCL8 and GM-CSF.

Stimulation with CXCL8 in the 3D collagen migration assay caused a concentration dependent migration which, depending on the assay used can be chemokinetic (non-gradient) or chemotactic (gradient). Stimulation with GM-CSF caused a concentration dependent chemokinetic migration. Both chemokinetic and chemotactic migration induced by CXCL8 signal via a GPCR. Chemokinetic migration in response to stimulation with both CXCL8 and GM-CSF is dependent on the mitogen activated protein kinase, ERK and the PI3-Kinases and is partially dependent on ROCK. Chemotactic migration in response to stimulation with CXCL8 is dependent on the PI3-Kinases and partially dependent on ERK. Further exploration of the specific class I PI3-Kinases highlighted that although both chemokinesis and chemotaxis are dependent on PI3-Kinase delta, there is a differential requirement for the PI3-Kinase alpha and gamma isoforms. Chemokinesis is dependent on the PI3-Kinase gamma isoform whereas chemotaxis is dependent on the PI3-Kinase alpha isoform.

In conclusion, this study highlights the similarities and differences between the signal transduction pathways needed for neutrophil chemokinesis and chemotaxis, in response to stimulation with CXCL8 and GM-CSF. Further examination of the mechanisms controlling neutrophil migration could be valuable in the treatment of conditions characterised by neutrophil influx.

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BAL	Bronchoalveolar lavage
BAX	BCL-2 associated X protein
BEBM	Bronchial epithelial cell basal medium
CF	Cystic fibrosis
CFTR	Cystic fibrosis conductance receptor
COPD	Chronic obstructive pulmonary disease
DH	Dbl homology
DOCK	Dedicator of cytokinesis
DPX	di-n-butylphthalate in xylene
ECM	Extracellular matrix
ELMO	Engulfment and cell motility
ERK	Extracellular signal-regulated kinase
ERM	Ezrin/radixin/moesin
FADD	Fas-associated death domain
FH	Formin-homology domain
fMLP	N-Formyl- Methionyl-Leucyl-Phenylalanine
FPR	Formyl peptide receptor
GAPs	GTPase activating proteins
GBD	GTPase binding domain
G-CSF	Granulocyte colony- stimulating factor
GDIs	Guanine nucleotide dissociation inhibitors
GEFs	Guanine nucleotide exchange factors
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
GRB2	Growth factor receptor bound protein 2
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid

HGFR	Hematopoietic growth factor receptor
ICAM	Intracellular adhesion molecule
JNK	c-Jun amino-terminal kinase
LAD	Leucocyte adhesion receptor
LARG	Leukaemia-associated Rho guanine nucleotide exchange factor
LIMK	LIM-Kinase
LTB_4	Lipid leucotriene B
MAPK	Mitogen activated protein kinases
MEM	Minimum essential media
MEK	Mitogen activated protein kinase kinase
mSOS	Mammalian son of sevenless protein
NOX	NADPH oxidase
PAK	p21-activated kinase
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
PI3-Kinases	Phosphoinositide 3-Kinases
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PIX	PAK-associated guanine nucleotide exchange factor
PLA	Phospholipase A
PMNs	Polymorphonuclear leucocytes
P-Rex	PtdIns(3,4,5)P_3 dependent Rac exchanger
PSGL-1	P-selectin glycoprotein ligand-1
PTEN	Phosphatase and tensin homologue
ROCK	Rho dependent kinase
RTK	Receptor tyrosine kinase
SDF-1	Stromal cell-derived factor 1

SH2	Src homology
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TORC	Target of Rapamycin complex
TRADD	TNFR associated death domain
TSC	Tuberous sclerosis
WASP	Wiskott Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein

List of abstracts

Posters

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Presentations

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Chapter 1: Main Introduction

1.1 Neutrophils

Neutrophils also known as polymorphonuclear neutrophilic leucocytes are the most abundant class of white blood cells in human peripheral blood, with approximately 3-5 $x10^9$ neutrophils per litre of blood (Amulic et al. 2012). They are characterized by their multi-lobed nucleus and neutrophilic granules, and have a vital role in immune defence (Amulic et al. 2012). Neutrophils are short-lived cells with an average circulatory lifespan of 5.4 days. However, this is reduced when activated, as the cells will die soon after completing phagocytosis (Pillay et al. 2010).

1.1.1 Neutrophil production

Neutrophil production occurs in the adult bone marrow under the influence of growth factors and cytokines particularly granulocyte colony-stimulating factor (G-CSF; Borregard 2010). Haematopoietic cells differentiate into myeloblasts, which are developmental cells committed to becoming granulocytes (Borregard 2010). As myeloblasts under go maturation into neutrophils, they synthesise proteins. In healthy individuals the release of mature neutrophils from the bone marrow into the blood stream is tightly regulated by chemokines and a subset of cells are stored in the bone marrow in case of infection (Amulic et al. 2012).

1.1.2 Neutrophil activation and recruitment to sites of infection

Bacterial-derived and host-produced inflammatory signals are released in abundance at sites of infections. These signals stimulate the endothelial cells near the site of inflammation to produce the adhesion molecules P-selectins, E-selectins and some members of the intracellular adhesion molecule (ICAM) integrin superfamily (Borregaard 2010).

Mature neutrophils constitutively express two proteins on their surface, the P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin (Kansas 1996, McEver and Cummings 1997). While circulating in the blood the two surface proteins of the neutrophils can engage with the P- and E-selectins, causing the neutrophils to tether to the blood vessel wall near to the site of infection (Kansas 1996, McEver and Cummings 1997). The neutrophils will then "roll' along the endothelium, until firm adhesion of the neutrophil to the endothelial cell wall is brought about by β2 integrins (LFA-1 and MAC-1) on the neutrophils engaging with their endothelial ligands, members of the ICAM-1 immunoglobulin superfamily (Campbell et al. 1998, Ley et al. 2007). Once firmly adhered to the endothelial cell wall, the neutrophil, under the control of the β2 integrins and ICAMs, crawls along the wall until it arrives at a preferred site for transendothelial migration (Phillipson et al. 2006). Following arrival at the desired site, the neutrophil will transverse the endothelial barrier, and upon entry into the interstitial space will be presented with a variety of chemoattractants and inflammatory stimulants, that will control neutrophil survival and migration to the site of infection (Amulic et al. 2010). Neutrophil migration will be discussed in more detail in section 1.5.

1.1.3 Phagocytosis

Once at the site of infection, neutrophils along with macrophages and dendritic cells are able to engulf the invading microorganisms (pathogens) in a process termed phagocytosis. This internalisation and resultant destruction of the microorganism is key to not only the innate immune response but also the development of the adaptive immune response (Flannagan et al. 2009). Phagocytosis begins by either direct recognition of the pathogen by pattern recognition of pathogen-associated molecules including carbohydrates, peptidoglycans or lipoproteins on the surface of the pathogen, or indirect recognition via opsonins (Flannagan et al. 2009). Opsonins are derived from the host and include components of the complement cascade that attach to the pathogen and are recognised by receptors including complement receptor 3 on the surface of the neutrophil (Flannagan et al. 2009). Following recognition via ligation of neutrophil surface receptors, the bound pathogen is surrounded by the membrane of the neutrophil and is then internalised in a membrane bound vesicle creating a phagosome (Janeway et al. 2005). Maturation of the phagosome occurs by sequentially binding to early and late endosomes, and a lysosome, forming a phagolysosome, which is fully equipped to destroy the internalised pathogen (Flannagan et al. 2009). During the maturation process the cellular pH is reduced, this acts to create a hostile environment preventing microbial growth, which is also a favourable environment for the hydrolytic enzymes contained within the lysosome (Huynh and Grinstein 2007).

1.1.3.1 Neutrophil granules

Neutrophils contain three main types of granules: azurophilic, specific and gelatinase. Azurophilic granules are the largest and they contain myeloperoxidase (which is critical

for oxidative burst), defensins, lysozyme, and serine proteases (Lacy 2005). Specific granules, which are characterised by the presence of lactoferrin, are smaller and contain a variety of antimicrobial compounds including lysozyme (Lacy 2005, Faurschou and Borregaard 2003). Smaller still are the gelatinase granules, which store the metalloproteases, gelatinase and leukolysin (Borregaard 2010). At sites of inflammation, these granules aid in the destruction of the invading pathogen in two main ways. Firstly, they can fuse with the phagosome increasing the antimicrobial activities of the compartment (Amulic et al. 2012). Alternatively, they can fuse with the plasma membrane causing the release of the antimicrobials into the tissue (Amulic et al. 2012). The extensive assortment of antimicrobial proteins contained within the three granule types, exert differential effects on the invading microorganisms, causing either direct or indirect death. For example, in defence against bacteria, lysozyme will degrade the cell wall of the bacteria and lactoferrin will alter bacterial growth by binding to iron, which is essential for growth (Markart et al. 2004, Corbin et al. 2008)

1.1.3.2 Oxidative burst

As the phagocyte ingests bacteria, its oxygen consumption increases, the phagocytic NADPH oxidase (NOX2), catalyses the transfer of electrons from NADPH to molecular oxygen, releasing the superoxide O_2^- (Quinn and Gauss 2004). The O_2^- can dismutate to H_2O_2, which can in turn react with O_2^- generating hydroxyl radicals and singlet oxygen (Quinn and Gauss 2004, Minakami and Sumimotoa 2006). Myeloperoxidase, contained within neutrophil granules can also convert H_2O_2 into hydrochlorous acid and chloramines (Minakami and Sumimotoa 2006). The resultant reactive oxygen species (hydroxyl radicals, hydrochlorous acid and chloramines) are highly toxic and can

effectively kill the invading microorganism, this will however cause the death of the neutrophil.

1.1.4 Apoptosis

Apoptosis is a programmed cell death responsible for controlling cell turnover to maintain normal immune system homeostasis (Savill et al. 1989). Neutrophil apoptosis is a non-inflammatory process. It is characterized by the loss of cytoplasmic granules, rounding of the nucleus and condensation of nuclear heterochromatin and can be instigated by extrinsic or intrinsic pathway stimuli (Savill et al. 1989, Kennedy and DeLeo 2009).

Intracellular signals from reactive oxygen species and/or from the neutrophil mitochondria bring about intrinsic constitutive apoptosis. One such example is the BCL-2 associated X protein (BAX) which following interaction with mitochondria leads to activation of the caspase cascade resulting in the apoptotic death of the cell (Sawatzky et al. 2006, Murphy et al. 2003).

Extracellular signals in the form of soluble protein factors including tumour necrosis factor alpha (TNFα) and FAS ligand bind to membrane-bound receptors on the cell surface to initiate extrinsic apoptosis (Kennedy and DeLeo 2009).

1.1.4.1 FAS/FAS-ligand

Soluble FAS-ligand and the transmembrane protein FAS are expressed by a variety of cells including neutrophils, and macrophages. FAS contain a binding domain for FAS-

ligand and a FAS-associated death domain (FADD; Kennedy and DeLeo 2009). Binding of FAS ligand to FAS induces clustering of FADDs to the cytoplasmic side of the plasma membrane, which activates a caspase cascade leading to apoptosis of the neutrophil (Kennedy and DeLeo 2009).

1.1.4.2 TNFα/ TNFα receptor

Like FAS, the transmembrane protein TNFα receptor (TNFR) contains a TNFα binding domain and a TNFR-associated death domain (TRADD; Murphy et al. 2003, Harper et al. 2003). Binding of the TNFR to TNFα causes clustering of TRADDs at the cytoplasmic side of the plasma membrane. However, unlike the FAS/FAS-ligand pathway the TNFα/TNFR pathway can activate apoptosis downstream of TRADD by either a caspase dependent or independent pathway (Murphy et al. 2003, Maianski et al. 2003).

1.1.4.3 Resolution of inflammation

In addition to controlling cell turnover for normal immune system homeostasis, apoptosis is also vital for resolution of inflammation following microorganism invasion.

Following phagocytosis, neutrophils undergo apoptosis and must be quickly cleared. This prevents the release of the cytotoxic molecules, which would cause damage to the host tissues. These apoptotic neutrophils are ingested by macrophages (Savill et al. 1989). Macrophages have been shown to secrete FAS ligand in response to phagocytosis; this induces further leucocyte apoptosis assisting in the resolution of inflammation (Brown and Savill 1999). Macrophage engulfment of the apoptotic

neutrophils not only reduces the neutrophils present, but also produces signals to prevent further neutrophil recruitment, and reprograms the macrophages to an anti-inflammatory phenotype (Brown and Savill 1999, Amulic et al. 2012).

1.2 The involvement of neutrophils in airway disease

Neutrophil accumulation has been associated with the severity and progression of a number of airway diseases including cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease (Cowburn et al. 2008). In addition, a subset of asthma termed 'noneosinophilic' is characterized by an elevated sputum neutrophil count. These airway diseases will be discussed in further detail below.

1.2.1 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder characterized by airway obstruction and chronic bacterial infection, which results in bronchiectasis, respiratory failure and premature death (Stockley et al. 2007). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance receptor (CFTR), which is expressed in epithelial cells including those of the lung, sweat ducts, pancreas, liver, vas deferens and the gut (Stockley et al. 2007). The defects in the CFTR cause a continual build up of thickened airway mucus and ineffective mucocillary and cough clearance resulting in the mucus becoming an ideal breeding ground for bacteria (Knowles et al. 2002).

Neutrophils are the primary effector cells responsible for the inflammation and lung damage seen in patients with CF (Chmiel et al. 2002). Although bacterial products initiate the inflammatory response, host mediators are responsible for the persistence of neutrophil influx. These mediators including the chemokine CXCL8, the chemoattractant LTB4 and the complement factor C5a have all been shown to be present at extremely high concentrations in the bronchoalveolar lavage (BAL) and sputum of patients with CF (Lawrence and Sorrell, 1993; Muhlebach et al. 1999; Chmiel et al. 2002). CXCL8 released primarily from epithelial cells is thought to be the predominant chemoattractant, however the substantial influx of neutrophils is controlled by a whole host of chemoattractants. Therefore, inhibition of any one chemoattractant would not be sufficient to reduce the influx of neutrophils (Khan et al. 1995; Kube et al. 2001; Chmiel et al. 2002). The activated neutrophils cause a substantial release of proteases, other mediators and oxygen free radicals. These cause significant damage to the airway wall and increase the obstruction of the airway lumen, as the local inhibitors and disposal mechanisms are overwhelmed, including the ability of macrophages to scavenge apoptotic neutrophils (Chmiel et al. 2002). Inflammation is a key contributor to the pathogenesis of CF. It is the major cause of the chronic destructive changes responsible for the morbidity and mortality in CF, forming a vicious cycle between airway obstruction, chronic bacterial infection and inflammation. This cycle eventually spins out of control resulting in the death of the CF patient (Chmiel et al. 2002).

1.2.2 Bronchiectasis

Bronchiectasis is characterized by the abnormal widening of the bronchial wall, as a result of the loss of structural proteins including elastin from the bronchial wall, muscle

and cartilage (Stockley et al. 2007). The main symptoms of bronchiectasis include a chronic cough, excess sputum and recurrent lower respiratory tract infections with breathlessness and wheeze. The 'coughing up' of blood from the respiratory tract termed, haemoptysis is also present in some cases (Stockley et al. 2007).

In bronchiectasis, ciliated epithelial cells in the bronchial wall become damaged and are then replaced by goblet cells. This results in the production of large volumes of mucus, which are difficult to clear as the mucus is less elastic and more vicious compared to mucus in healthy lungs and there are less ciliated cells to perform the cilary beat needed to remove the mucus (Stockley et al. 2007). The build up of mucus provides a breeding ground for bacteria that would ordinarily be cleared by the cilary beat. The bacteria multiple, and bacteria products and host cell mediators such as CXCL8 attract large numbers of neutrophils to the site of infection (Stockley et al. 2007). This results in chronic neutrophilic inflammation, which can cause a significant amount of damage to the tissue, as the large numbers of neutrophils release significant quantities of enzymes including elastase and oxygen free radicals contained within their nuclei, which the lung cannot neutralise (Stockley et al. 2007). This damage to the tissue further increases the widening of the bronchial wall, which in turn further weakens the lungs ability to defend against bacterial pathogens, which in turn increases inflammation. This then results in an increasingly severe and vicious cycle of damage, infection and inflammation (Stockley et al. 2007).

1.2.3 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) causes a progressive narrowing of the airways, which is not fully reversible and is a mixture of emphysema, chronic

obstructive airway disease and chronic bronchitis with the exact contributions of each varying from person to person (Rabe et al. 2007). The primary cause of COPD is smoking, usually for a substantial period. Therefore, COPD usually occurs in middle-aged patients that have been smoking since adolescence (Rabe et al. 2007). The characteristic symptoms of COPD include a chronic and progressive shortness of breath, a chronic cough and sputum production. Diagnosis occurs when patients present with a combination of these symptoms in conjunction with airflow limitation, which is measured using spirometry (Rabe et al. 2007). Based on the spirometry results patients can be divided into four stages ranging stage I, mild to stage IV, very severe (Rabe et al. 2007).

In patients with COPD, there is an increase in neutrophils and neutrophil activation markers (Riise et al. 1995; Balbi et al. 1997; Pesci et al. 1998). Cigarette smoke exposure has been shown to stimulate the differentiation and maturation of neutrophils within the bone marrow and this leads to peripheral blood leucocytosis and neutrophilia (van Eeden and Hogg 2000). The blood leucocyte count correlates inversely to $FEV_{1,}$ so the greater the numbers of neutrophils the lower the FEV_1 (Yeung and Buncio 1984). The increased blood neutrophils are recruited to the airway, where they can cause tissue destruction, as they release high concentrations of elastase. The elastase breaks down the connective tissue, which leads to airflow obstruction (Ko et al. 1997). The neutrophil respiratory burst also proves harmful in COPD. Oxidative molecules are released by the neutrophils, these are normally controlled by antioxidants, however, in COPD there is a reduced plasma antioxidant capacity and a high number of neutrophils releasing oxidative molecules, so these molecules can not be neutralised and will damage the lung cells causing them die. This leads to obstruction of the airway (Weiss et al. 1981; Rahman et al. 1996). Neutrophils are central to the pathogenesis of COPD,

as they are the only cells that contain all the products shown to directly cause the pathological features of COPD (Stockley et al. 2007).

1.2.4 Asthma

Asthma is an airway disease characterized by chronic airway inflammation, airway hyper-responsiveness and variable airflow obstruction (Haldar and Pavord 2007). The main symptoms of asthma include breathlessness, a tight chest, wheeze and coughing.

Analysis of the sputum from asthmatics has typically associated infiltration of eosinophils with onset of the disease (Pavord et al. 1999; Gibson et al. 2001; Pignatti et al. 2005). The chronic airway inflammation is usually caused by an increase in eosinophils. It was initially thought that eosinophilic airway inflammation was closely related to airway dysfunction, which encompasses airway hyper-responsiveness and variable airflow obstruction, with the inflammation suggested to directly cause the airway dysfunction (Haldar and Pavord 2007). However, the sputum eosinophil count is thought to be normal in as many as 25% of patients with steroid naïve symptomatic asthma and 50% of patients treated with high doses of corticosteroids. Therefore, this absence of eosinophilic airway inflammation in a large proportion of patients suggests that airway dysfunction is controlled by factors independent of eosinophilic airway inflammation (Gibson et al. 2001; Green et al. 2002; Haldar and Pavord 2007). A new model of asthma where airway dysfunction and eosinophilic airway inflammation are separate independent domains has been suggested, with asthma potentially being grouped into eosinophilic and noneosinophilic (Haldar and Pavord 2007).

Noneosinophilic asthma is common in patients with occupational asthma, refractory asthma and patients with mild or more severe exacerbations (Turner et al. 1995; Wenzel at al. 1999; Anees et al. 2002). Noneosinophilic asthma in itself can be divided into neutrophilic and paucigranulocytic based on the presence or absence of a sputum neutrophil count >61% (Simpson et al. 2006). In neutrophilic asthma fixed airflow obstruction and a decline in lung function in smokers has been associated with elevated neutrophil counts. Although, it is thought that this subtype of asthma represents a more stable phenotype with a reduction in exacerbation frequency and severity, as compared to eosinophilic asthma as eosinophils are thought to be the driving force behind severe exacerbations (Stanescu et al. 1996; Woodruff et al. 2001). There is much debate about the effectiveness of corticosteroids in noneosinophilic asthma with some reporting a significantly smaller improvement in airway hyper-responsiveness and associated symptoms in noneosinophilic asthma in comparison to eosinophilic asthma. However, more work needs to be carried out to determine the responsive of noneosinophilic asthma to corticosteroids (Pavord et al. 1999).

1.3 Cytokines and neutrophils

Cytokines are small proteins secreted by cells, which act on specific membrane-expressed, signal transducing receptors. Cytokines can be divided into groups based on their supposed function and include chemokines, growth factors, lymphokines and interleukins.

1.3.1 Chemokines

Chemokines are a group of 48 8-10kDa chemotactic cytokines that are capable of stimulating and activating cells enabling them to migrate (Teran 2000; Palmqvist et al. 2007). They are expressed by a variety of cells, primarily immune cells (Palmqvist et al. 2007). Chemokines cause directed migration of phagocytic and lymphocytic cells to precise locations, particularly trafficking to lymphoid and inflammation sites (Pease and Williams 2006). They have been divided into four subgroups based on the position of the cysteine residues (Pease and Williams 2006). The two largest groups are CC and CXC, with the smaller C and CX3C containing only a few chemokines.

Chemokines signal via receptors belonging to the G-protein coupled receptor super family. There are 29 chemokine receptors divided into the groups CCR, CXCR, CR and CX3CR. Chemokine receptor binding is usually class restricted, i.e. CXC receptors are only activated by CXC chemokines. However, there are some exceptions (Pease and Williams 2006).

CXCL8 is an important chemokine in neutrophil activation and migration. CXCL8 belongs to the CXC subfamily of chemokines and is approximately 8kDa in size. In response to a number of pro-inflammatory stimuli such as IL-1, TNF, LPS and viruses, CXCL8 is released primarily by macrophages and epithelial cells, but can also be produced by a number of other cell types including T cells, neutrophils and keratinocytes. The receptors CXCR1 and CXCR2 are both receptors for CXCL8.

1.3.2 Growth factors

Growth factors are a group of cytokines that stimulate cellular growth, proliferation and differentiation.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 14kDa protein, which causes the activation and aids the survival of neutrophils, eosinophils, basophils and macrophages. GM-CSF is produced and secreted by a variety of cells including macrophages, mast cells, fibroblasts and T cells.

GM-CSF exerts its effects via the GM-CSF receptor also known as Cluster of Differentiation 116. This receptor is a heterodimer consisting of an α chain, which is the binding site for GM-CSF, and a β chain, which is shared with the IL-3 and IL-5 receptors and this chain functions to activate signal transduction (Geijsen et al. 2001). The GM-CSF, IL-3 and IL-5 receptors all belong to the hematopoietic growth factor receptor super-family (Cosman et al. 1990).

1.3.3 N-Formyl-Methionyl-Leucyl-Phenylalanine

N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) is chemoattractant, produced by bacteria that can also be released by the mitochondria of dead cells (Marasco et al. 1984). It is a potent inducer of leucocyte chemotaxis and cell activation (Marasco et al. 1984). The receptor for fMLP is the formyl peptide receptor (FPR), which is a G-protein coupled receptor and is expressed by a variety of cells including neutrophils, monocytes/macrophages, endothelial cells and fibroblasts (Migeotte et al. 2006).

1.4 Hierarchy of intracellular signalling

As leucocytes are recruited from the blood to sites of infection, they require mechanisms to enable them to respond to the directional signals they receive from a wide variety of chemoattractants, to ensure they arrive at the appropriate destination (Foxman et al. 1997).

In the control and coordination of the neutrophil migration from the blood to the site of infection, chemoattractants can be divided into two groups, referred to as the intermediary chemoattractants and the end target chemoattractants. The intermediary/regulatory chemoattractants are mediators secreted by endothelial tissue and immune cells and include CXCL8 and lipid leucotriene B4 (LTB4). They control the adherence of neutrophils to the endothelium and migration out of the microvasculature into the infected tissue (Campbell et al. 1997; Foxman et al. 1997; Heit et al. 2002). The end target chemoattractants are produced by, or in response to bacterial infection, and include formyl peptides such as fMLP and the complement factor C5a. They are responsible for attracting the neutrophils to the final site of infection (Campbell et al. 1997; Foxman et al. 1997; Heit et al. 2002).

Although neutrophils will initially encounter the intermediary chemoattractants, once through the vasculature and in the tissue they will be exposed to both intermediary and end target chemoattractants. Therefore, for efficient migration to occur a hierarchy of chemoattractants must exist (Campbell et al. 1997; Foxman et al. 1997; Heit et al. 2002).

Using the transwell assay the ability of neutrophils to migrate towards a second agonist was examined. An equal concentration of chemoattractant was added to both the top and bottom wells, which made a uniform concentration of the first agonist and the

second agonist was then added to the bottom well only (Campbell et al. 1997). Increasing the uniform concentration of fMLP inhibited chemotaxis towards the intermediary chemoattractant CXCL8. However, increasing the uniform concentration of CXCL8 had no effect on chemotaxis towards fMLP, indicating that fMLP-induced signalling had dominance over CXCL8-induced signalling (Campbell et al. 1997).

The under agarose assay has also been used to understand the dominance of end target attractants over intermediary attractants, as the positioning of the chemoattractants and cells within the wells can be easily manipulated. In the under agarose assay it has been shown that neutrophils migrated towards fMLP and C5a even if CXCL8 or LTB4 were present in the neutrophil- containing well; however, neutrophils did not migrate towards CXCL8 or LTB4 if either fMLP or C5a were present in the cell- containing well (Foxman et al. 1997). In addition, cells migrated towards distant CXCL8 to the same extent in the presence or absence of local LTB4 and vice versa (Foxman et al. 1999). When migrating down a gradient of local agonist in the under agarose assay neutrophils have been shown to prioritise signals from a distant gradient, which changes their chemotaxis towards the distant source. This is thought to be due to cellular memory, which alters the perceived strength of the local agonist, and enables the cells to preferentially respond to gradients of novel (distant) attractants (Foxman et al. 1999).

When neutrophils were added to the middle well in the under agarose assay and different chemoattractants CXCL8, LTB4, fMLP and C5a were added to the wells either side, the neutrophils preferentially migrated to the end target chemoattractants fMLP and C5a. However, when neutrophils were pre-treated with a p38MAPK inhibitor neutrophils preferentially migrated towards CXCL8 and LTB4, and this migration was inhibited with wortmannin (Heit et al. 2002). Therefore, it was concluded that end target attractants signal via p38MAPK and intermediary attractants

signal via PI3-Kinases (Heit et al. 2002). PTEN has been suggested as a potential 'molecular switch' between p38MAPK and PI3-Kinase signalling, as when neutrophils were exposed to opposing gradients of CXCL8 and formyl peptides, PTEN was shown to be distributed throughout the cell circumference. This inhibited PI3-Kinase activity and caused the preferential migration of neutrophils towards the formyl peptides (Heit et al. 2008). In contrast, when the neutrophils were treated with a p38MAPK inhibitor PTEN localised normally in the rear of the cell (also known as the uropod) and the neutrophils migrated towards CXCL8 (Heit et al. 2008).

Therefore, as concluded by Foxman et al. (2007), the cell's ability to ignore a local agonist source to respond to a distant one is controlled by the dominance of end target-derived attractants over intermediary attractants.

1.5 Neutrophil migration

Immune surveillance and migration to sites of infection are essential for neutrophil function. The failure of neutrophils to adhere to blood vessels needed for transmigration to the site of infection results in a severe immunodeficiency, with a propensity for life threatening bacterial infections, as seen in leucocyte adhesion deficiency (LAD; Etzioni et al. 1999). LAD is a rare autosomal recessive disorder resulting from heterogeneous mutations in the common chain β2 subunit CD18. It is characterized by recurrent bacterial and fungal infections, marked neutrophilia and delayed separation of the umbilical cord (Etzioni et al. 1999).

Leucocyte migration is initiated by the recognition of an extracellular signal released from another cell or group of cells. Extracellular signals include chemokines, growth

factors, chemoattractant lipids and peptides and the extracellular matrix. This causes the intracellular machinery to be stimulated enabling the control of cell migration towards the chemoattractant source (Procko and McColl 2005). Cell migration is dependent on the assembly, disassembly and reorganization of the actin cytoskeleton (Raftopoulou and Hall 2004). Actin filaments and specialized actin-binding proteins make up the actin cytoskeleton; the filamentous actin is organized into three main structures (Van Aelst and D'Souza-Schorey 1997). These are:

(1) Filopodia- finger like protrusions consisting of a tight bundle of long actin filaments that form in the direction of the protrusion

(2) Lamellipodia- thin protrusive actin sheets that form at the edge of the cytoskeleton

(3) Actin stress fibres- bundles of actin filaments that transverse the cell and are linked through focal adhesions to the extracellular matrix (Van Aelst and D'Souza-Schorey 1997).

Cell migration is brought about by the extension of the lamellipodia, the formation of a new adhesion site at the front of the cell, contraction of the cell body and the detachment of adhesions from the back of the cell (Raftopoulou and Hall 2004).

There are three main forms of leucocyte migration random, chemokinetic and chemotactic. Both random and chemokinetic migration are random motions the difference being chemokinesis occurs in the presence of usually chemical stimuli, whereas random motion occurs in the absence of such stimuli. As with chemokinesis, chemotaxis is in response to a chemical stimulus, primarily a chemoattractant, but unlike chemokinesis, the migration is directed towards the source of the stimulus (Knall et al. 1997; Manes et al. 2005).

1.5.1 Overview of chemokine- and growth factor-induced migration

This section provides an overview of the signal transduction pathways and there connection together in chemokine- and growth factor- induced migration with diagrams showing pathways. The components of the pathways will be discussed in further detail following this section. All abbreviations are listed on page xii and will be also be defined in the detailed sections.

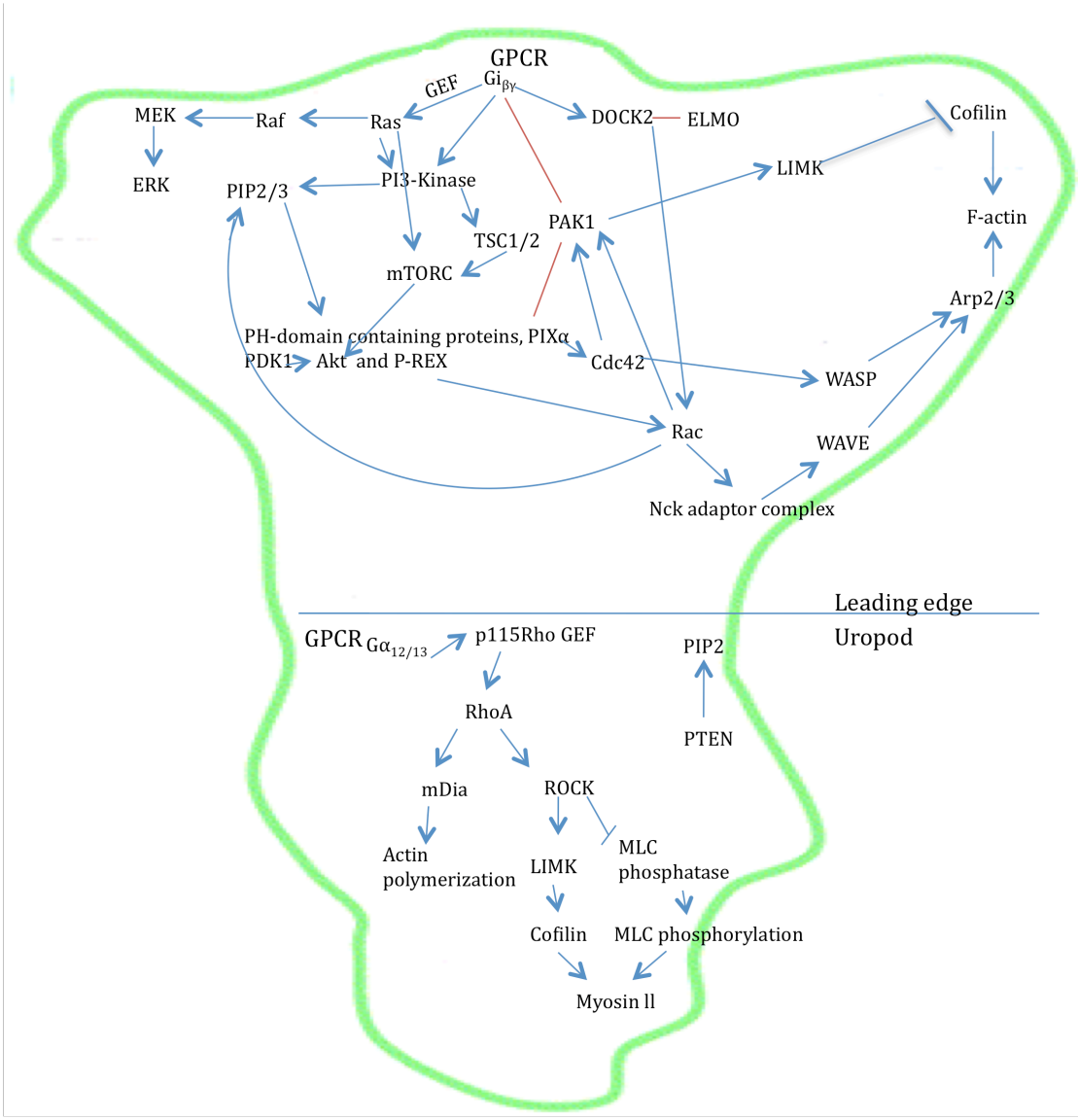


Figure 1.1. Overview of signal transduction pathways induced in chemokine stimulated migration. Key: blue arrow represents activated by, blue line represents blocked by and red line represents binding. A chemokine binds on the cell surface to its GPCR, causing the activation of the GPCR, which causes the exchange of GDP for

GTP on the Gα subunit of the G-protein, resulting in the dissociation of the Gα and Gβγ subunits. The Gβγ subunit can bind directly to the class IB PI3-Kinase causing its activation. This leads to the accumulation of PIP3 at the leading edge and the exclusion of PIP_2 and PTEN to the rear of the cell. PI3-Kinase can also activate mTORC via TSC1/2. The accumulation of PIP3 recruits and activates the PH-domain containing proteins including P-Rex, PDK1 and PIXα. PIP3 also recruits the PH-domain containing protein Akt, which is activated via phosphorylation of two sites, threonine 308 by PDK1 and serine 473 by mTORC. P-Rex and DOCK2/ELMO binding can both cause the activation of Rac at the front of the cell. A positive feedback loop exists between Rac and PIP3 resulting in elevated PIP_3 accumulation and Rac activation. Rac activates PAK and WAVE (via an NCK-adaptor complex). Free Gβγ subunits can also bind to PAK enabling it to interact with PIXα, which stimulates Cdc42 activity. Activated Cdc42 in turn activates PAK, as well as WASP. PAK phosphorylates LIMK, which stabilizes actin polymerisation by preventing cofilin from severing ADP-bound actin. WASP and WAVE activate Arp2/3 which initiates the formation of new actin filaments by controlling the nucleation of ATP bound G-actin to F-actin, which pushes the membrane forward. Ras causes the activation of Raf and mTORCs. Raf activates MEK; this in turn activates the MAPK, ERK, which plays an unknown role in migration. At the rear of the cell, the $G\alpha_{12}$ and $G\alpha_{13}$ G proteins of the GPCR activate the guanine nucleotide exchange factor, p115 Rho GEF, which in turn exchanges GDP for GTP on Rho causing its activation. Rho activates ROCK and LIMK; these activate downstream effectors that regulate the assembly of actin-myosin filaments to bring about the detachment and movement of the uropod. ROCK activation via Rho also causes the activation of ERM proteins that link the actin cytoskeleton and plasma membrane proteins.

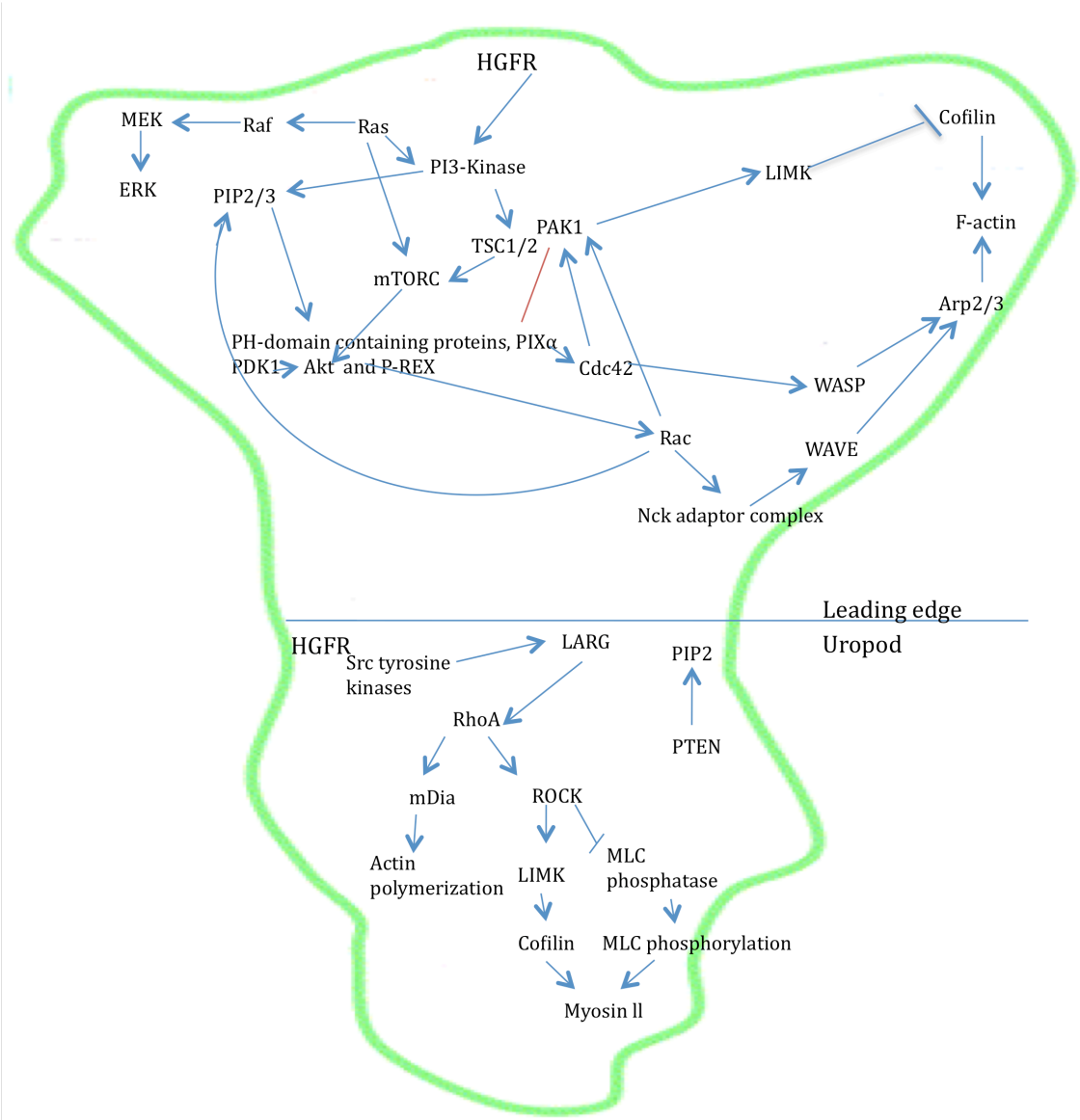


Figure 1.2 Overview of signal transduction pathways induced in growth factor stimulated migration. Key: blue arrow represents activated by, blue line represents blocked by and red line represents binding. A hematopoietic growth factor binds to its HGFR; this causes conformational changes to the receptor, which enables cross phosphorylation of the two JAK2 molecules bound to the receptor. The phosphorylated JAK2 molecules in turn phosphorylate tyrosine residues, which act as a docking site for the class IA P13-Kinase, this interaction causes the PI3-Kinase to localise to the plasma membrane in close proximity with Ras, which is capable of directly activating the p110 subunit. Class IA PI3-Kinase activation leads to the accumulation of PIP_3 at the leading edge and the exclusion of PIP_2 and PTEN to the rear of the cell. The accumulation of PIP_3 recruits and activates the PH-domain containing proteins including P-Rex, PDK1 and PIXα, as well as recruiting Akt. Akt is activated via phosphorylation at two sites, firstly, threonine 308 by PDK1 and secondly, serine 473 by mTORC (activated via TSC1/2, which is activated by PI3-Kinase). Cdc42 is activated by PIXα, interacting with PAK, which in turn activates PAK. Cdc42 also activates WASP. The PH-domain containing protein, P-Rex activates Rac, which in turn activates PAK and WAVE via an Nck adaptor complex. A feedback loop between PIP3 and Rac results in an increased

accumulation of PIP3 and activation of Rac. PAK activated by both Rac and Cdc42, phosphorylates LIMK, which inhibits cofilin function, stabilizing actin polymerisation. Arp2/3 is activated by WASP and WAVE, which controls the nucleation of ATP bound G-actin to F-actin to form new actin filaments. Ras can cause the activation of Raf and mTORC. Raf activation in turn activates, MEK, which activates ERK, however the role of ERK in migration is unknown. At the rear of the cell src tyrosine kinases phosphorylated on the HGFR, bind to LARG, which then activates Rho. Rho regulates the assembly of actin-myosin filaments leading to detachment and movement of the uropod, by activating ROCK and LIMK, which in turn activate downstream effectors.

1.5.2 The G-protein coupled receptor family

Chemokine binding to its receptor is the initial step in the control of chemokine-induced migration. The G-protein coupled receptor (GPCR) family encompasses chemokine receptors. This family is characterized by a seven-transmembrane receptor coupled to pertussis toxin sensitive Gi proteins (Niggli 2003). Although responsible for the activation of the downstream effectors controlling migration chemokine receptors are uniformly distributed along the cell membrane and are therefore not responsible for the distribution of the pathways that occur at the front and back of the cells to enable coordinated cell migration (Servant et al. 1999; Merlot and Firtel 2003). The G proteins are composed of the subunits Gα, Gβ and Gγ. Upon activation, the receptor catalyzes the exchange of GDP for GTP on the Gα subunit, which causes its dissociation from the Gβγ subunits of the G protein complex (Neptune and Bourne 1997; Lehmann et al. 2007). This dissociation enables Gα and Gβγ subunits to bind to downstream effector proteins. One of the Gβγ subunits main targets in neutrophils is the class I phosphoinositide 3-kinases (Stephens et al. 1997; Parent 2004).

1.5.3 The hematopoietic growth factor receptor family

The hematopoietic growth factor receptor (HGFR) family contains the cell surface receptors for erythropoietin, thrombopoietin, some colony stimulating factors and most interleukins. They are characterized by a conserved 200 amino acid extracellular cytokine-binding domain (Cosman et al. 1990). Activation of the receptor occurs via specific ligand binding to the α chain of the receptor (Muto et al. 1996; Guthridge et al. 1998; Martinez-Moczygemba and Huston 2003). This binding recruits the βc chain of the receptor to the bound ligand resulting in a conformational change (Muto et al. 1996; Guthridge et al. 1998; Martinez-Moczygemba and Huston 2003). Two Janus kinase 2

(JAK2) molecules are bound to the HGFR and the conformational change enables the JAK2 molecules to cross phosphorylate each other (Kaushansky 2006). The phosphorylated JAK2 molecules in turn phosphorylate receptor tyrosine residues, which serve as docking sites for adaptors of a variety of signalling pathways including p85 and p110 subunits of the class IA PI3-Kinases, and the Ras activating proteins src homology protein (SHC), growth factor receptor bound protein 2 (GRB2) and mammalian son of sevenless protein (mSOS; Kaushansky 2006).

1.5.4 The receptor tyrosine kinase family

Growth factors including platelet derived growth factor, vascular derived growth factor, fibroblast growth factor and insulin signal via receptor tyrosine kinases (RTK) to control important cellular functions including migration, metabolism, survival, differentiation and proliferation (Lemmon and Schlessinger 2010). The binding of growth factors to their corresponding RTK induces activation by causing the dimerisation and autophosphorylation of the cytoplasmic domains of the receptor (Kazlauskas and Cooper 1990; Brachmann et al. 2005). The autophosphorylation of the receptor enables the recruitment and activation of a variety of signalling molecules downstream of the receptor. These signalling molecules contain src 2 homology domains (SH2) or phosphotyrosine domains, which can be directly or indirectly recruited to the receptor (Schlessinger and Lemmon 2003). Direct recruitment to the receptor occurs via binding to phosphotyrosines, whereas indirect recruitment occurs via docking proteins, which have been phosphorylated by RTKs (Schlessinger and Lemmon 2003). As receptors contain numerous phosphotyrosines and there is a large variety of docking proteins, RTKs are capable of recruiting and activating a wide range

of downstream signalling molecules responsible for the many cell functions performed in response to growth factor signalling (Lemmon and Schlessinger 2010).

1.5.5 Phosphoinositide 3-Kinases

Signalling via the phosphoinositide 3-Kinases (PI3-Kinases) has been shown to be important for a variety of cellular functions. PI3-Kinases are homologous in the catalytic subunit, which consists of a catalytic core domain, linked to a PI kinase homology domain and a C2 domain. They can be divided into three classes (namely I, II and III) based on substrate specificity, structure and mode of regulation (table 1.1; Vanhaesebroeck et al. 2001; Curnock et al. 2002).

	Catalytic	Regulatory	Regulated by	Substrates
Class I				
IA	p110α,β,δ	p85α,β; p55γ	Tyrosine Kinases, Ras, Gβγ?	PtdIns, PtdIns(4)P, PtdIns(4,5)P_2
IB	p110γ	p101	Gβγ, Ras	PtdIns, PtdIns(4)P, PtdIns(4,5)P_2
Class II	PI3K-C2α,β,γ		Tyrosine Kinases, Chemokines? Integrins?	PtdIns, PtdIns(4)P
Class III	VpS34	p150	Constitutive?	PtdIns

Table 1.1. Table summarising the PI3-Kinases. Adapted from Vanhaesebroeck et al. 2001.

Class I PI3-Kinases have been suggested to be the main class involved in cell migration. They are heterodimers made up of a catalytic subunit and a regulatory subunit. Based on sequence similarity class I PI3-Kinases can be further subdivided into two groups termed Class IA and Class IB.

1.5.5.1 Class IA PI3-Kinases

In Class IA PI3-Kinases the p110 subunit is in a complex with a regulatory subunit that has two-src homology 2 domains (SH2; Vanhaesebroeck et al. 2001). Activation occurs via growth factor binding to an RTK. This causes the dimerisation and autophophorylation of the receptor, enabling the binding of the SH2 domains of the regulatory subunit. This in turn, causes the recruitment of the class IA P13-Kinase (Kazlauskas and Cooper 1990; Brachmann et al. 2005; Procko and McColl 2005). This interaction causes the PI3-Kinase to localise to the plasma membrane in close proximity with Ras, which is capable of directly activating the p110 subunit (Kazlauskas and Cooper 1990; Brachmann et al. 2005; Procko and McColl 2005). The activation and downstream effectors of the Class IA PI3-Kinases are shown in figure 1.3. Class IA contains three p110 catalytic isoforms p110α, p110β and p110δ, which are encoded by three separate genes. There are over seven regulatory subunits generated by the expression and alternative splicing of the three genes p85α, p85β and p55γ. All the splice variants form fully functional complexes with the p110 subunits (Vanhaesebroeck et al. 2001; Moraes and Downey 2003). p110α and p110β are ubiquitously expressed in all cells however p110δ is expressed primarily in leucocytes.

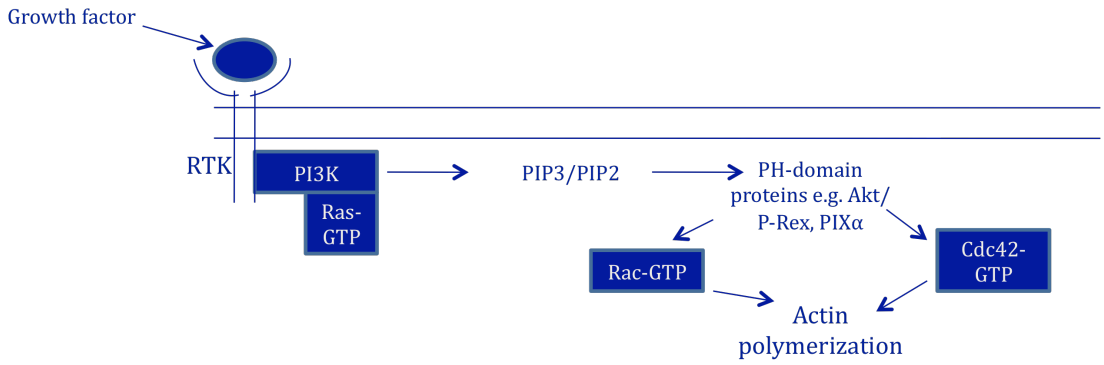


Figure 1.3. A schematic diagram summarising the activation of Class IA PI3-Kinases and its downstream effectors.

1.5.5.2 Class IB PI3-Kinases

The heterodimer between the p101 regulatory subunit and p110γ catalytic subunit is the only member of this class. Binding of the Gβγ subunit of the GPCR directly to the heterodimer causes its activation (Stephens et al. 1997). It is unknown whether the Gβγ subunit binds to the regulatory subunit, which in turn causes the activation of the catalytic subunit, or whether direct binding occurs between the Gβγ and catalytic subunits (Vanhaesebroeck et al. 2001). The catalytic subunit is expressed only in leucocytes. The activation and downstream effectors of the Class IB PI3-Kinases are shown in figure 1.4.

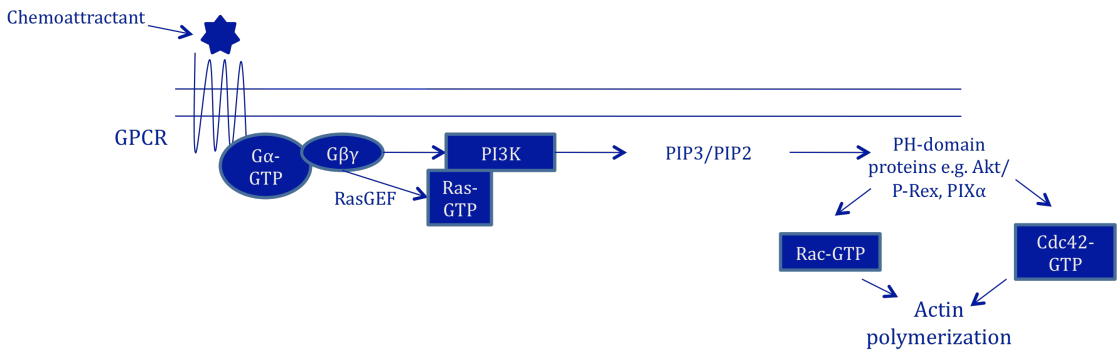


Figure 1.4. A schematic diagram summarising the activation of Class IB PI3-Kinases and its downstream effectors.

Although the activation of class IA and IB is thought to be via these two distinct receptor types, this may not be the case. As PI3-Kinase γ has been shown to be activated indirectly via tyrosine kinase receptors in macrophages and in neutrophils and PI3-Kinase δ contributes to phosphatidylinositol (3, 4, 5)- trisphosphate (PIP3) accumulation in response to GPCR activation (Randis et al. 2008). GPCRs are also thought to be capable of stimulating class IA PI3-Kinase activity as stimulation of the heterotrimeric G-proteins can induce tyrosine kinases (Vanhaesebroeck et al. 2001). Therefore, the activation of the two class I subtypes may not be as clear cut as first thought and there could indeed be an overlap between the two subtypes with signalling pathways not restricted to a particular type of receptor (Randis et al. 2008).

1.5.5.3 The involvement of Class I PI3-Kinases in neutrophil migration

The broad spectrum PI3-Kinase inhibitor wortmannin has been shown to inhibit the migration of neutrophils in 3D migration assays in response to stimulation with the N-formyl chemotactic peptide, fNLPNTL and the cytokine stromal cell-derived factor 1 (SDF-1; Niggli and Keller 1997; Bastian et al. 2006). However pre-treatment with wortmannin prior to the addition of the chemoattractant fMLP has generated conflicting results, with some studies indicating that migration is unaffected, and others reporting that migration is significantly reduced. In three-dimensional collagen lattices, pre-treatment with wortmannin prior to the addition of fMLP had no effect on migration in comparison to stimulation with fMLP alone. However, in Dunn chambers pre-treatment with wortmannin prior to the addition of fMLP inhibited the proportion of cells moving in response to fMLP, but had no effect on speed or direction (Bastian et al. 2006; Ferguson et al. 2007).

In addition to observing the generalised involvement of PI3-Kinases using wortmannin, many authors have studied the specific class I catalytic isoforms in order to determine whether some functions such as chemotaxis versus chemokinesis are isoform specific. The catalytic isoform p110γ has been shown to play a role in neutrophil migration in both inhibitor and mouse model experiments. Treatment with the p110γ selective inhibitor, AS-252424 significantly reduced the proportion of human neutrophils migrating in the Dunn chamber in response to stimulation with fMLP. But had no effect on the migratory index, speed or direction suggesting that inhibition of PI3-Kinase γ causes a failure of chemokinesis but not the ability of cells to navigate once they are moving (Ferguson et al. 2007). The p110γ selective inhibitor, AS-041164 dose dependently decreased the *in vivo* recruitment of mouse neutrophils into the peritoneum, in response to stimulation with the chemokine CCL5, in comparison to stimulation alone, highlighting the importance of PI3-Kinase γ in neutrophil recruitment (Ferrandi et al. 2007). Purified neutrophils from PI3-Kinase $\gamma^{-/-}$ mice, in comparison to their heterozygous littermates, had a substantially decreased *in vitro* migration in Zigmond chambers and a decreased ability to translocate up an *in vitro* chemotactic gradient in response to fMLP and C5a stimulation (Sasaki et al. 2000; Hannigan et al. 2002). Polymorphonuclear leucocytes (PMNs) were examined from wild type and PI3-Kinase $\gamma^{-/-}$ mice with or without the addition of the PI3-Kinase γ selective inhibitor AS-605240 using a transwell filter. The migratory activity when stimulated with CXCL2 and CXCL3 was significantly reduced in the PI3-Kinase $\gamma^{-/-}$ cells compared with the wild type cells. This reduction in migratory index was also seen when the wild type cells were treated with AS-605240 confirming that the reduction in migration was due to specific inhibition the isoform (Reutershan et al. 2010).

The catalytic isoform p110δ has also been studied in relation to neutrophil migration. Treatment with the PI3-Kinase δ selective inhibitor IC-87114 in the under agarose assay inhibited fMLP-induced PIP3 production and chemotaxis but had no effect on chemokinesis, indicating a role for PI3-Kinase δ in the directional component of chemotaxis but not random movement (Sadhu et al. 2003).

The *in vivo* recruitment of mouse neutrophils into the cremaster muscle in response to the chemokines MIP-2 (CXCL2) and KC (CXCL1) was entirely dependent on the class I PI3-kinases, however it appears that the different isoforms caused non-overlapping distinct events (Liu et al. 2007). The early response (first 2 hours) of neutrophil recruitment to MIP-2 and KC was dependent on PI3-Kinase γ, whereas, the more prolonged response (over 4 hours) was dependent on PI3-Kinase δ (Liu et al. 2007).

1.5.6 Target of Rapamycin complex

The target of Rapamycin complex (TORC) belongs to a PI3-Kinase-related family of serine/threonine kinases, which are thought to be critical regulators of cell polarity and chemotaxis (Lee et al. 2005; Kamimura et al. 2008; Liu et al. 2010). There are two distinct mTORCs in mammals, mTORC1 and mTORC2, with mTORC2 being implicated in chemotaxis (Liu et al. 2010). The involvement of TORC2 in migration was initially observed in the amoebae *Dictyostelium discoideum* whereby chemoattractant stimulation lead to the activation of a RasC-TORC2- Akt pathways at the front of the cell, which controls cAMP production, causing modulation of the F-actin cytoskeleton (Kamimura et al. 2008; Charest et al. 2010). When any of the TORC2 proteins are downregulated in *Dictyostelium* cells, it causes defects in chemotaxis, including the loss of speed, cell polarity and directionality (Lee et al.

2005). More recently, a role for TORC2 has been highlighted in neutrophil migration in response to stimulation with fMLP. Neutrophils that are deficient in mTORC2 display substantially reduced chemotaxis, in addition to a reduction in F-actin polarity (Liu et al. 2010). It is thought TORC2 could exert its effects at both the front and rear of the cell through two independent pathways. The first acts at the front of the cell regulating F-actin through an unknown pathway. The second acts at the rear of the cell to phosphorylate myosin via a cAMP and RhoA-dependent pathway (Liu et al. 2010). The mechanisms controlling mTORC2 regulation have only begun to be revealed; it is however known that mTORC2 activation requires PI3-Kinase and the tuberous sclerosis (TSC)1/2 complex and is thought to play a role in the cytoskeletal organisation by controlling actin polymerisation (Martelli et al. 2010, Jacinto et al. 2004). mTORC2 phosphorylates Akt on serine 473 enhancing phosphorylation on threonine 308 by phosphoinositide-dependent kinase 1 (PDK-1; Sarbassov et al. 2005).

1.5.7 Phosphoinositides

Following activation by either tyrosine kinases receptors (RTK) or GPCRs, class I PI3-Kinases are responsible for controlling the phosphorylation of phosphatidylinositol (4,5)-bisphophate (PIP2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3; Puri et al. 2005). In response to stimulation with fMLP and CXCL8, neutrophils polarize rapidly with simultaneous accumulation of PIP3 along the leading edge of the cell and PTEN in the rear of the cell (uropod; Heit et al. 2008). PIP3 accumulates strongly at the leading edge of the cell, which in part is due to the preferential activation of the PI3-Kinases at the leading edge (Merlot and Firtel 2003; Sasaki and Firtel 2006). In addition, exclusion of phosphatase and tensin homologue from the leading edge results

in the localisation of PTEN in the uropod and cell sides. This contributes to the high levels of PIP3 seen at the leading edge as PTEN dephosphorylates PIP3 into PIP2 resulting in a PIP3 gradient at the cell front (Sasaki and Firtel 2006). PIP3 plays an important role in the development of cell polarity, which is essential for cell motility and directional sensing (Lehmann et al 2007).

A positive feedback loop where the Rho GTPase, Rac can function both upstream and downstream of PIP3 is thought to enhance the accumulation of PIP3 and activation of downstream signals. PIP3 binds to and activates the PH-domain containing protein P-REX, which in turn activates Rac (Weiner et al. 2002, Srinivasan et al. 2003). Activated Rac can control the phosphorylation of PIP2 to PIP3 increasing accumulation, which via P-REX increases the activation of Rac (Weiner et al. 2002, Srinivasan et al. 2003).

1.5.8 Phosphatase and Tensin homologue

Phosphatase and tensin homologue (PTEN), has been shown co-localise with active Rho in the uropod of neutrophils undergoing chemotaxis. It functions to dephosphorylate PIP_3 into PIP_2, restricting PIP_3 to the front of the cell (Li et al. 2005; Charest and Firtel 2006). The localisation of PI3-Kinase at the front and PTEN in the uropod of the cell generates a PIP3 gradient at the front of the cell, which plays an important role in regulating the site-directed actin dynamics (Disanza et al. 2005). Polarized PTEN distribution in the uropod is reduced in cells with inhibited Cdc42 or ROCK function, leading to the suggestion that Cdc42 and Rho-ROCK may have important roles in the regulation of PTEN localization (Li et al. 2005). Coexpression of PTEN with Rho in Jurkat cells enhanced the dephosphorylation of PIP_3 induced by PTEN in comparison to those without active Rho (Li et al. 2005). This suggests that

Rho regulates the phosphatase activity of PTEN. It is thought that PTEN does not contribute to the directionality of the cell due to its location at the rear but it does have an effect on the cell's velocity (Lacalle et al. 2004).

1.5.9 Pleckstrin Homology-domain containing proteins

The Pleckstrin Homology (PH)- domain is a protein domain containing 100 amino acids (Mayer et al. 1993). This domain is conserved between a large group of proteins and they are therefore termed PH-domain containing proteins. PtdIns (3,4,5) P_3 dependent Rac exchanger (P-REX), PAK-associated guanine nucleotide exchange factor (PIXα) and phosphoinositide-dependent protein kinase 1 (PDK1) are all members of the PH-domain family of proteins (Merlot and Firtel 2003, Martelli et al. 2010). The PH-domain proteins can bind with phosphoinositides, including for example PIP3 to PDK1 (Martelli et al. 2010). The PH-domain containing proteins can act as GTPase activating proteins and guanine nucleotide exchange factors for the RhoGTPases. The PH-domain containing protein recruitment and polarization occurs at the same time as actin rearrangement, suggesting a link between the two (Weiner et al. 2002).

1.5.10 Akt

Akt also known as protein kinase B, belongs to a family of serine/threonine kinases characterised by a PH-domain in the N-terminus (Chen et al. 2010). In neutrophils under basal conditions, Akt is cytosolic. However, when cells are exposed to a chemoattractant gradient, Akt is recruited to the plasma membrane of the leading edge, where it interacts with PIP3 (Parent 2004). Once recruited to the plasma membrane the

Akt is phosphorylated at two sites and phosphorylation of both is required for activation. The first is in the activation loop at threonine 308 (Thr 308) by PDK1, attracted to the plasma membrane by PIP3, and the second is in the COOH-terminus at serine 473 (Ser 473) by mTOR2 (Alessi et al. 1997, Rane et al. 2001).

Akt has three isoforms Akt1, Akt2 and Akt3. A study by Chen et al. (2010) highlighted an important role for Akt2 in neutrophil migration, as depletion of Akt2 resulted in a significant impairment in the ability of neutrophils to migrate. In contrast, depletion of Akt1 had no effect on the migration of neutrophils (Chen et al. 2010). In fMLP-stimulated neutrophils, Akt co-localizes with F-actin at the leading edge of the cell. In contrast, in cells from mice lacking PI3-Kinase γ there is poor co-localisation between Akt and F-actin at the front of the cell suggesting an important role for PI3-Kinase γ in the intracellular co-localisation of Akt and F-actin at the leading edge (Hannigan et al. 2002).

1.5.11 Dedicator of cytokinesis 2

A PI3-Kinase dependent pathway is utilised mainly by neutrophils and macrophages. Lymphocytes however, show a very low dependence on PI3-Kinase for migration. Therefore, in order for these cells to migrate other pathways, independent of PI3-Kinase must exist (Nombela-Arrieta et al. 2004). Dedicator of cytokinesis (DOCK) 2 is a member of the CDM protein family (Ced-5 *C.elegans*, DOCK180 in humans and myoblast city in *D.melanogaster*), displaying 60% homology to DOCK180 and is highly expressed in lymphocytes (Fukui et al. 2001; Reif and Cyster 2002). As with PI3-Kinases, activation of DOCK2 occurs downstream of the G protein coupled receptor (Nombela-Arrieta et al. 2004). Overexpression of DOCK2 increases the

activation of Rac, which is the most predominant downstream effector, highlighting an important role for DOCK2 in both Rac activation and cell migration (Reif and Cyster 2002). Rac activation is dependent on the binding of DOCK2 to the scaffolding engulfment and cell motility protein (ELMO1), via the interaction of the C-terminus and SH3 domain of DOCK2 and the proline rich sequence in the C-terminus of ELMO1 (Gumienny et al. 2001; Sanui et al. 2003). Once bound to ELMO1, DOCK2 acts as guanine nucleotide exchange factor for Rac causing the exchange of GDP for GTP enabling the activation of Rac (Nishikimi et al. 2009). The activation of DOCK2 is shown in figure 1.5.

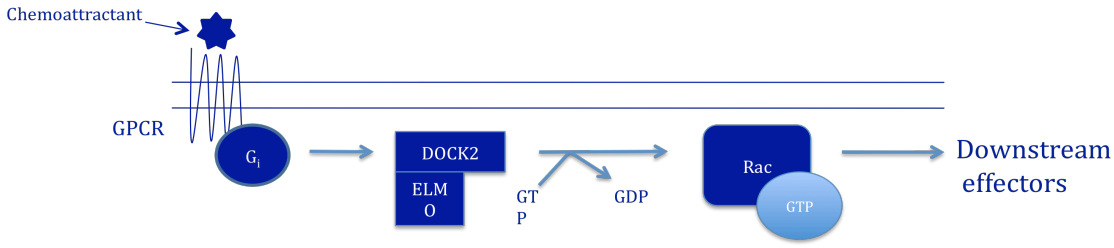


Figure 1.5. A schematic diagram summarising the activation of DOCK2.

Inhibition of DOCK2 severely impairs the migration of B and T lymphocytes, however inhibition of PI3-Kinase only causes a minor reduction in the migration of the T cells. This indicates that the main pathway used for cell migration is the DOCK2 pathway, which is sufficient to cause a large amount of migration independent of PI3-Kinase activity (Fukui et al. 2001; Nombela-Arrieta et al. 2004; Nombela-Arrieta et al. 2007). However, inhibition of both DOCK2 and PI3-Kinase activity in combination caused a greater reduction in T cell migration than when cells were treated with DOCK2 or PI3-Kinase inhibitors alone. This highlights an important, but largely independent role for

each during migration (Nombela-Arrieta et al. 2004; Nombela-Arrieta et al. 2007). In addition, inhibition of PI3-Kinase in T cells only slightly reduces chemokine induced F-actin formation. Whereas DOCK2 deficient cells have a greatly reduced chemokine induced F-actin formation, which again is enhanced when DOCK2 and PI3-Kinase are both inhibited, resulting in an absence of F-actin formation (Nombela-Arrieta et al. 2004).

Although thought to be primarily involved in lymphocyte migration fMLP-induced activation of Rac is substantially reduced in $DOCK^{-/-}$ neutrophils. This indicates that DOCK2 plays a major role in chemoattractant induced Rac activation in neutrophils (Kunisaki et al. 2006). When neutrophils are stimulated with fMLP, DOCK2 translocates rapidly to the plasma membrane at the leading edge of the cell in a PI3-Kinase dependent manner (Nishikimi et al. 2009). DOCK2 remains at the leading edge of the cell during migration. However, only the initial translocation is induced by PI3-Kinase activity. DOCK2 accumulation at the leading edge of the cell is maintained by the negatively charged phospholipid phosphatidic acid that acts as a lipid anchor binding positively charged effector proteins such as DOCK2 (Nishikimi et al. 2009). Taken together these observations demonstrate that chemokine-induced neutrophil migration is dependent on both PI3-Kinases and DOCK2 mediated signalling.

1.5.12 Rho GTPases

Rho GTPases have been implicated as important signal transduction factors involved in leukocyte migration. Their main role is to control the actin cytoskeleton assembly by regulating the signal transduction pathways responsible for the assembly of actin-myosin filaments, lamellipodia and filopodia (Hall 1998). They cycle between a GDP-

bound inactive form and a GTP-bound active form. The cycle is shown in figure 1.6. Three groups of proteins are responsible for regulating the GTPase cycle. The first group of proteins are the guanine nucleotide exchange factors (GEFs), which cause the exchange of GDP to GTP to enable the activation of the GTPase. These include Smg, Dbl, Ost and Tiam-1 all of which belong to a family of proteins which share the Dbl homology (DH) domain (Matsui et al. 1996; Van Aelst and D'Souza-Schorey 1997; Moon and Zheng 2003). The second group of proteins are the GTPase activating proteins (GAPs), which cause the exchange of GTP for GDP. Examples of GAPs include Ras GAP-associated p190, Rho GAP and Rho GAP p122 (Matsui et al. 1996; Moon and Zheng 2003). The third group of proteins are the guanine nucleotide dissociation inhibitors (GDIs), which block the GTPase cycle as they stabilize the GDP-bound form, an example of which is Rho GDI (Matsui et al. 1996; Moon and Zheng 2003). Activation of the Rho GTPases enables them to interact with downstream targets responsible for leukocyte migration (Raftopoulou and Hall 2004).

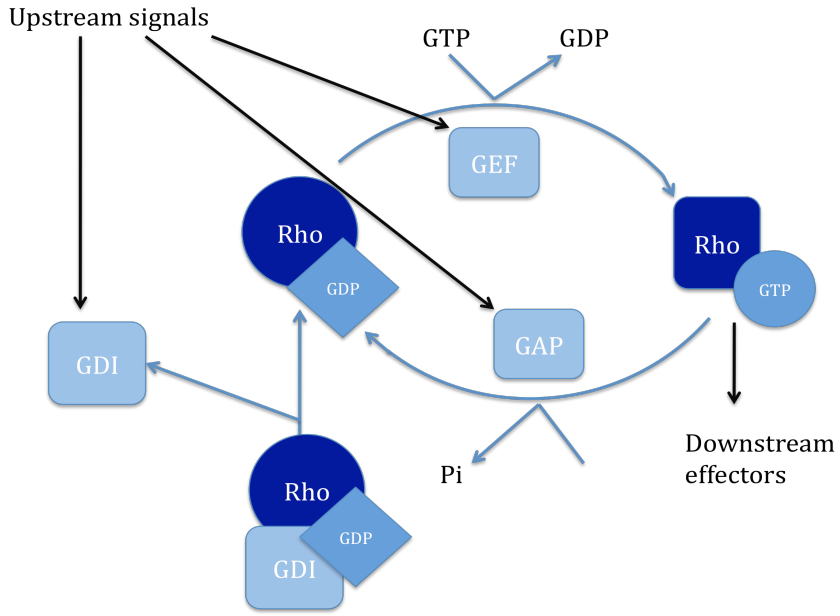


Figure 1.6. A diagram summarising the GTPase cycle between the GDP bound and GTP bound forms.

1.5.12.1 Ras

Ras has been proposed to be an activator of class I PI3-Kinases. It has been shown to co-immunoprecipitate with the class I PI3-kinases via GTP dependent binding of the p110 catalytic subunit of the class I PI3-kinases with the Ras effector site (Rodriguez-Viciana et al. 1994; Sasaki et al. 2004). Ras/ PI3-Kinase activation is dependent on a Ras, PI3-Kinase, F-actin feedback loop although Ras can be initially activated without PI3-Kinase and indeed Ras activation and PI3-Kinase translocation both occur upstream of PI3-Kinase signalling (Sasaki et al. 2004). The activation and regulation of PI3-Kinases by Ras enables the accumulation of large quantities of PIP_3 at the front of the cell, causing a concentration gradient within the cell, which enables the translocation of PH containing domains to the front of the migrating cell (Rodriguez-Viciana et al. 1997). This means Ras is capable of controlling the actin cytoskeleton as it causes the activation of PI3-Kinases, which function upstream of Rac (Rodriguez-Viciana et al. 1997). Inhibition of Ras causes defects in the directional movement of cells, indicating that Ras in an important component in the ability of cells to recognise and migrate towards a chemotactic stimuli (Sasaki et al. 2004).

1.5.12.2 Rho

Rho acts at the rear of the cell to regulate the assembly of actin-myosin filaments. This brings about the detachment and movement of the uropod (Nobes and Hall 1995). Rho causes the activation of the two downstream effectors mDia and Rho dependent kinase (ROCK), which is summarised in figure 1.7 (Vicente-Manzanares et al. 2003). The Gα subunit of the GPCR causes the activation of Rho. Gα can be further divided into 20 different subunits, which include the G_{12} and G_{13} subunits. These subunits activate the

guanine nucleotide exchange factor, p115 Rho GEF, which then in turn exchanges GDP for GTP on Rho causing its activation (Kozasa et al. 1998). It is also thought in the absence of a GPCR that Rho can be activated via src tyrosine kinases activating the leukaemia-associated Rho guanine nucleotide exchange factor (LARG), which in turn activates Rho (Guilluy et al. 2011).

mDia a member of the DRF family of proteins is the mammalian homologue of the *Drosophila melanogaster* diaphanous protein (Palazzo et al. 2001). There are two isoforms of the mammalian mDia: mDia1 and mDia2, both of which contain a small GTPase binding domain and two formin-homology domains (FH1 and FH2; Palazzo et al. 2001). In the presence of GTP bound Rho the interaction between the N and C terminus of mDia becomes disrupted enabling the binding of Rho to the N-terminus resulting in the activation of mDia (Ishizaki et al. 2001). The activation of mDia in turn enables profilin to bind to the FH1 region of mDia, which significantly increases the polymerisation of actin (Ishizaki et al. 2001). The activation of mDia1 is vital for T cell chemotaxis, as cells from mDia1$^{-/-}$ mice show decreased chemotaxis towards the T cell chemokines CXCL12 and CCL21. However, B cell chemotaxis towards CXCL12 is not impaired when B cells were obtained from mDia1$^{-/-}$ mice indicating that although mDia1 is important in T cell migration it is not however important in B cell migration (Sakata et al. 2007). Therefore, mDia may or may not play a role in the migration of neutrophils.

The effector ROCK can cause the activation of two different pathways. In the first pathway, ROCK binds to and phosphorylates the myosin binding subunit of the myosin light chain phosphatase, which causes its activity to be inhibited. This indirectly increases the amount of myosin phosphorylation, which causes actomyosin assembly, stress fibre formation and contraction (Kawano et al. 1999; Bishop and Hall. 2000). The

second pathway involves ROCK and cofilin. ROCK is not able to directly phosphorylate cofilin. Therefore, phosphorylation must be achieved through another kinase. ROCK causes the phosphorylation of LIMK, which in turn phosphorylates cofilin causing its inactivation leading to actin-filament stabilization (Bishop and Hall 2000).

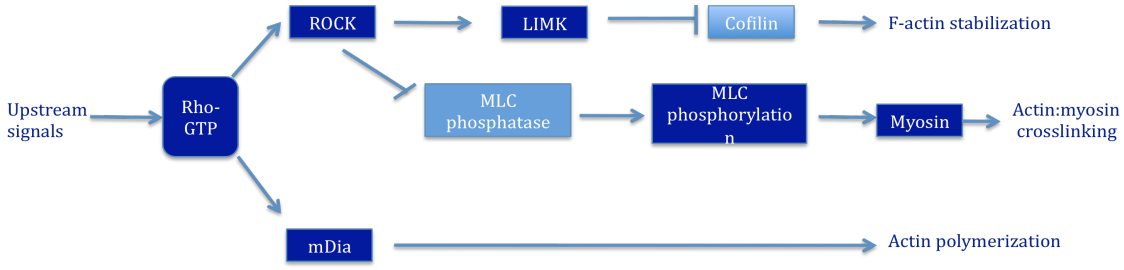


Figure 1.7. The downstream effectors of active Rho.

The Rho-ROCK pathway plays a vital role in cell detachment. However, detachment can only occur when other pathways are involved, as ROCK only induces disorganised actin bundles. mDia activation corrects the orientation of the actin bundles induced by ROCK enabling cell migration to occur (Watanabe et al. 1999).

The ROCK inhibitor Y27632, reduces myosin light chain phosphorylation and fNLPNTL- and fMLP-induced neutrophil polarity, chemokinesis and chemotaxis (Niggli 1999; Alblas et al. 2001). In addition, the Rho inhibitor c3-exoenzyme inhibited fMLP-induced random migration of neutrophils, although the cell body is still highly motile the inhibitor prevents uropod detachment preventing the cell from moving (Alblas et al. 2001).

The involvement of ROCK and Rho in neutrophil migration was determined in Transwell and Boyden chambers; in contrast, the effect of ROCK and Rho inhibitors

was examined in eosinophils in the 3D collagen assay (the three migration assays will be discussed in section 1.1.5 of the introduction). In eosinophils GM-CSF-induced migration could occur independently of Rho and ROCK activity, whereas, in contrast CCL11-induced migration was dependent on both ROCK and Rho (Muessel et al. 2008). The levels of migration were also very different: when stimulated with GM-CSF the majority of the cells migrate, whereas when treated with CCL11 only a very small number of cells were able to migrate (Muessel et al. 2008). Therefore, as eosinophils and neutrophils are similarly related cells ROCK and Rho may not be required for growth factor and chemokine-induced neutrophil migration in 3D collagen assays.

1.5.12.3 Cdc42

Cdc42 acts at the front of the cell to regulate the polymerization of actin to form filopodial protrusions (Nobes and Hall 1995). Upon activation of G-protein coupled receptors, free Gβγ subunits can bind to p21-activated kinase (PAK). This interaction enables PAK to interact with the PH-domain containing protein PIXα. Binding of PAK and PIXα activates Cdc42, which in turn activates PAK. (Szczur et al. 2006).

Cdc42 activates the two main effectors Wiskott-Aldrich syndrome protein (WASP) and PAK both of which activate signal cascades that induce the extension of the filopodia at the leading edge of the cell; this is shown in figure 1.8 (Bishop and Hall 2000; Raftopoulou and Hall 2004; Mitsushima et al. 2006).

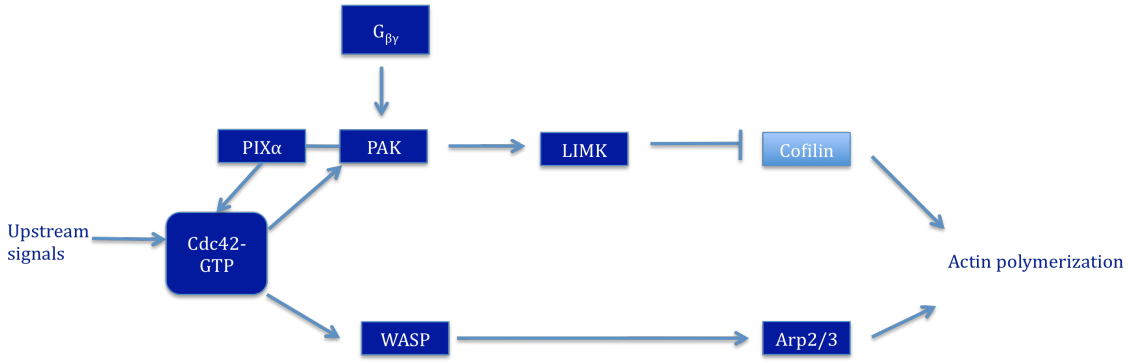


Figure 1.8. The activation and downstream effectors of Cdc42.

Cdc42 binds directly to the GTPase binding domain (GBD) of the WASP protein causing its activation (Higgs and Pollard 2000; Takenawa and Miki. 2001). WASP is a major activator for the Arp2/3 complex. However, it can only cause activation of the complex in the presence of PIP2 and GTP-Cdc42 (Higgs and Pollard 2000; Raftopoulou and Hall 2004). Arp2/3 is a major modulator of actin polymerisation resulting in the formation of filopodia (DesMarais et al. 2004). WASP can also bind to profiling, which acts in conjunction with Arp2/3 resulting in faster actin polymerisation (Raftopoulou and Hall 2004).

PAK phosphorylates LIM-Kinase (LIMK), which in turn phosphorylates cofilin resulting in its inactivation (Raftopoulou and Hall 2004). Cofilin plays a key role in maintaining and extending the lamellipodial and filopodial protrusions at the leading edge of cells undergoing migration as it enhances the treadmilling rate of actin filaments (Disanza et al. 2005). Therefore, the inactivation of cofilin causes actin polymerization and prevents filament treadmilling at the front of the migrating cell. This enables the cell to contract and move. In fMLP stimulated neutrophils the activity of PAK increases over a rapid period of time resulting in a highly polarised cell front containing F-actin and PAK (Dharmawardhane et al. 1999).

Loss of function mutations of Cdc42 have shown it is not vital for the cell to move but is vital for maintaining directional migration towards a chemoattractant source when the cell is moving. In Cdc42 $^{-/-}$ neutrophils increased motility was observed whereas directed migration was defective suggesting that Cdc42 is needed for direction not motility (Sepp and Auld 2003; Szczur et al. 2006).

1.5.12.4 Rac

Rac acts at the front of the cell to regulate the polymerization of actin to form lamellipodial protrusions (Nobes and Hall 1995). Rac can be activated as described earlier by interaction with DOCK2 and ELMO; in addition the PH-domain containing protein PtdIns(3,4,5)P_3-dependent Rac exchanger (P-REX) can also activate Rac. P-Rex activity is regulated by the Gβγ subunit of the GPCR and PIP_3, thus linking the GPCR, PI3-Kinase and Rac pathways (Parent 2004).

There are two main forms of Rac involved in cell migration these are Rac1 and Rac2; both of these have different functions that enable the control of migration. Rac1 and Rac2 are over 90% homologous, Rac2 is the most predominant isoform and is expressed only in cells of hematopoietic lineage. In contrast, Rac1 is ubiquitously expressed (Sun et al. 2004; Zhang et al. 2009). Rac activation causes the activation of the two effectors WASP family verprolin-homologous protein (WAVE) and p65PAK both of which activate a series of downstream effectors that mediate the polymerization of actin to produce lamellipodial protrusions; this is shown in figure 1.9 (Nobes and Hall 1995; Raftopoulou and Hall 2004).

WAVE does not contain a GBD domain like its family member WASP. Therefore, unlike WASP that directly binds to Cdc42, WAVE instead binds to Rac indirectly through an Nck-adaptor complex (Weiner et al. 2007). Once activated WAVE stimulates the Arp2/3 complex, which induces actin polymerization (Weaver et al. 2003).

As with Cdc42, Rac activates PAK, which phosphorylates and activates LIMK. Activated LIMK phosphorylates and inactivates cofilin, which in turn causes actin polymerization (Raftopoulou and Hall 2004).

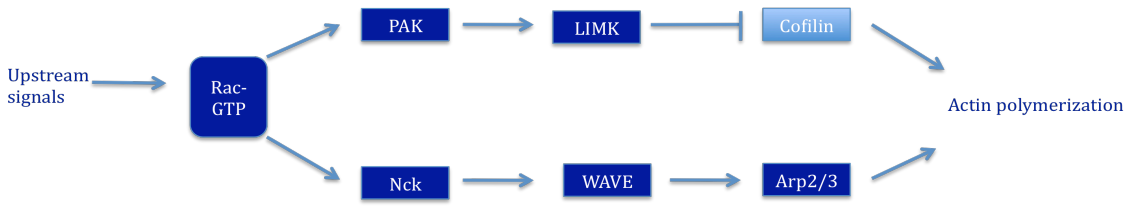


Figure 1.9. The downstream effectors of active Rac.

Analysis of directed neutrophil migration in Rac1$^{-/-}$ and Rac2$^{-/-}$ mice has highlighted isoform dependent differences in migration. Rac1$^{-/-}$ neutrophils migrate at a normal speed in comparison to wild-type neutrophils but are unable to migrate chemotactically towards the source of the chemoattractant. They instead migrate randomly, coinciding with the formation of many randomly orientated lamellipodia (Sun et al. 2004; Zhang et al. 2009). In contract to Rac1$^{-/-}$ neutrophils, Rac2$^{-/-}$ neutrophils have a significantly reduced speed in comparison to wild type and Rac1$^{-/-}$ neutrophils. However, the neutrophils that are still able to migrate can orient towards the chemoattractant source. In addition, these neutrophils display major defects in F-actin assembly (Sun et al.

2004; Zhang et al. 2009). Therefore, Rac1 is essential for gradient detection and orientation towards the chemoattractant source whereas Rac2 is responsible for the actin assembly needed to power the migration (Sun et al. 2004).

As well as the differences in migration, there was also a significant decrease in phosphorylated Akt in the leading edge of $Rac1^{-/-}$ neutrophils compared to neutrophils from $Rac2^{-/-}$ and wild type mice (Sun et al. 2004; Zhang et al. 2009). The immunolocalization of P-Akt to the leading edge was also dependent on Rac1 (Zhang et al. 2009). The reduction in P-Akt at the leading edge in $Rac1^{-/-}$ neutrophils could be due to the PIP3-Rac positive feedback loop. In the feedback loop Rac can function both upstream and downstream of PIP3, which leads to increased PIP3 phosphorylation and accumulation. This therefore increases the activation and activity of downstream effectors (Weiner et al. 2002, Srinivasan et al. 2003). In the $Rac1^{-/-}$ neutrophils, no positive feedback can be generated, which reduces PIP3 accumulation. PIP3 attracts both Akt and PDK1 to the plasma membrane and is responsible for Akt phosphorylation via PDK1 (Alessi et al. 1997, Rane et al. 2001). Therefore, inhibition of Rac would cause the non-direct reduction of phosphorylated Akt compared to wild type cells.

Rac1 function has also been implicated as being essential for regulating uropod function at the rear of the cell by activating Rho and myosin (Pestonjamasp et al. 2006). The expression of activated Rac can inhibit Rho function, which is likely to be responsible for maintaining Rac activity at the front of the cell and Rho activity at the rear of the cell whilst it is undergoing migration (Sander et al. 1999).

1.5.13 Mitogen-activated protein kinases

The Mitogen-activated protein kinases (MAPKs) are known regulators of a variety of physiological process, including chemotaxis. The three main groups of serine/threonine kinases, which make up the MAPKs, are extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun amino-terminal kinase (JNK; Suzuki et al. 1999; Kampen et al. 2000).

In growth factor induced leukocyte migration, binding of growth factors to RTKs causes Ras localisation and activation. Ras is able to recruit the Raf family kinases resulting in their activation (Pullikuth and Catling 2007). Raf is then able to activate the mitogen activated protein kinase kinases, MEK1 and 2 which are located upstream of ERK 1 and 2. ERK then localises in the cytoplasm enabling the regulation of the cytoskeleton and cell motility (Pullikuth and Catling 2007). In chemokine induced migration, PI3-Kinase activity is thought to be necessary for activation of the MAPK pathway, as Raf1 and B-Raf are activated in a Ras dependent manner and are upstream regulators of MEK, which in turn activates MAPK (Pullikuth and Catling 2007; Knall et al. 1996). Raf1 and B-Raf are activated by CXCL8 in a dose dependent manner and this activation can be inhibited in the presence of wortmannin. Therefore, indicating that PI3-Kinases regulate Raf1 and B-Raf activation in human neutrophils, which in turn regulate MAPKs (Knall et al. 1996). The activation of ERK is shown in figure 1.10.

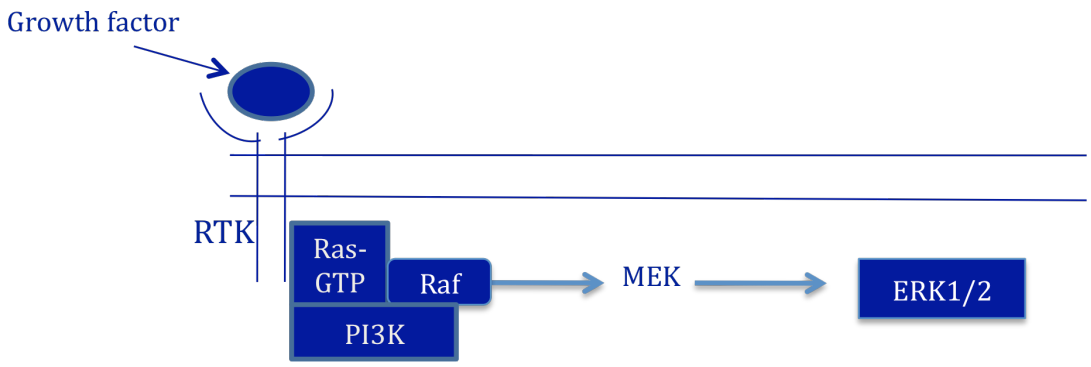


Figure 1.10. A schematic diagram summarising the activation of ERK.

In growth factor- induced migration, ERK but not p38MAPK, has been shown to be important in neutrophil signalling and migration. The growth factor, granulocyte colony stimulating factor (G-CSF)-induced random neutrophil migration within a Boyden chamber, which was dependent on MEK/ERK signalling and independent of p38MAPK signalling (Nakamae-Akahori et al. 2006). In addition, ERK1 and ERK2 were shown to be strongly phosphorylated by GM-CSF in human neutrophils, whereas p38MAPK was only weakly phosphorylated (Suzuki et al. 1999). In contrast, fMLP- and CXCL8-induced chemotactic migration has been shown to be dependent on p38MAPK but not ERK. In transwell chambers, neutrophil migration was suppressed by pre-treatment with the p38MAPK inhibitor SB203580, but not by the MEK/ERK inhibitor PD98059. In addition, fMLP stimulated phosphorylation of Akt has been shown to be inhibited by SB203580 but not PD98059 (Rane et al. 2001; Fujita et al. 2005).

Myosin light chain kinase, which acts at the rear of the cell to phosphorylate myosin to bring about rear detachment and contraction, has been suggested as a potential downstream effector of MEK/ERK. This is because treatment of human leucocytes with the MEK inhibitor PD98059 significantly inhibits myosin light chain kinase activity (Mansfield et al. 2000).

1.5.14 Ezrin/radixin/moesin proteins

The ezrin/radixin/moesin (ERM) family of proteins act as a link between the actin cytoskeleton and plasma membrane proteins (Oshiro et al. 1998; Ivetic and Ridley 2004). In leukocytes the main ERM protein expressed is moesin. Activation of the ERM proteins by a variety a kinases including protein kinase C and ROCK occurs via the phosphorylation of the C-terminal (Manes et al. 2005). The ERM proteins can act both upstream and downstream of Rho GTPases that has lead to the theory that between the two types of proteins a positive feedback loop exists (Ivetic and Ridley 2004).

1.5.15 Actin polymerization

Actin polymerization is vital for the movement of cells as it is the assembly and disassembly of the actin cytoskeleton that enables the cell to move. Actin filaments are orientated so the growing barbed ends face the plasma membrane and net chemoattractant concentration, while the pointed ends are located at the rear of the cell (Wang 1985). Profilin catalyzes the exchange of ADP for ATP by binding to the ATP-G-actin in a complex that associates at the barbed ends shifting G-actin distribution (Pantoloni and Carlier 1993). The Arp2/3 complex initiates the formation of new actin filaments by controlling the nucleation of ATP-bound G-actin to F-actin. (Ryan et al. 2012). This F-actin growth at the barbed end pushes the membrane forward, while capping proteins antagonize this process by binding the barbed ends, terminating elongation and promoting branching (Park et al. 2010). As filaments age they undergo severing, which catalyses actin filament disassembly into monomers that recycle back to filaments by depolymerization creating new barbed ends that serve as new sites for elongation (Park et al. 2010, Ryan et al. 2012). Cofilin acts to sever and depolymerise

actin from filaments by increasing the rate of ADP-G-actin depolymerisation at the pointed ends (Disanza et al. 2005). LIMK stabilizes actin polymerization by preventing cofilin from severing ADP-bound actin and promoting actin depolymerisation (Park et al. 2010).

The integration and coordination of the activities of all proteins interacting with and affecting the different steps of actin polymerization is needed to promote cell motility (Disanza et al. 2005).

1.5.16 Adhesion receptors

Integrins are connected to bundles of actin filaments and act as transmembrane linkers between the cytoskeleton and extracellular matrix (ECM) by mediating leucocyte adhesion to extracellular matrix proteins including collagens, laminins and fibronectin (Alberts et al. 2002). Integrins are heterodimers composed of two noncovalently associated transmembrane glycoprotein subunits, α and β (Alberts et al. 2002).

In mammals at present there are thought to be 24α and 9β subunits, which can generate 24 distinct integrins (Alberts et al. 2002). In neutrophil transendothelial migration from the vasculature into tissue the involvement of integrins as well as other adhesion receptors has been well characterised. Integrins are inactive in circulating leucocytes. However, the β_2 subunit, which forms subunits with four different α subunits (αD, αL, αM and αX) unlike the other β subunits is constitutively expressed at high levels exclusively by leucocytes and is the primary integrin for adhesion to the endothelial lining (Lindbom and Werr 2002; Kay et al. 2008).

The role of intergrins in leucocyte migration within the tissue has not been well defined. Although, following leucocyte emigration into the tissue from the vasculature the β_1 subunit is known to be rapidly up regulated and is therefore thought to be the major integrin for migration within the ECM (Werr et al. 1998). While their exact role is not well defined, integrins have been suggested as important components of cell migration. As efficient migration requires repeated attachment and detachment of the leucocyte to the ECM components, which is achieved via integrin binding (Lindbom and Werr 2002). Although the β_2 subunit has been suggested as the main integrin, both the β_2 and β_1 subunits have been highlighted in leucocyte chemotaxis in the ECM and 3D matrices of proteins of the ECM including fibronectin, collagen and laminin. In addition, the integrins $\alpha_L\beta_2$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ have all been shown to be polarized at the front of migrating cells (Werr et al. 1998; Harler et al. 1999; Werr et al. 2000; Sixt et al. 2001; Lindbom and Werr 2002; Manes et al. 2005). Furthermore, the integrin $\alpha_2\beta_1$ is thought to have a dominant role in neutrophil migration in the ECM as an interaction between the integrin and collagen within the membrane promotes forward movement (Werr et al. 2000).

Migration independent of integrins has been demonstrated in some conditions, although this migration was not as efficient as integrin dependent migration and therefore integrins remain an integral component of neutrophil migration (Malawista and de Boisfleury Chevance 1997; Lindbom and Werr 2002).

1.6 Migration assays

Characterisation of the signal transduction pathways controlling cell migration could potentially lead to effective therapeutic treatments in a variety of conditions. Therefore, it is important to be able to study cell migration. There are a variety of in vitro and in vivo techniques currently utilised to examine cell migration, of which the most frequently used are described below.

1.6.1 In vitro methods

1.6.1.1 The Boyden chamber assay

The Boyden chamber assay, also known as the transwell, is a system whereby two compartments are separated by a porous membrane (figure 1.11; Boyden 1962; Chen 2005). Polycarbonate or cellulose nitrate membranes can be used between the two compartments and these come in a range of pore sizes from 2-12μm. The appropriate pore size to use depends on the cell type being examined, as it needs to be larger than the cell nuclei but not so large that the cells will easily drop through. In the case of lymphocytes, macrophages and leucocytes the ideal membrane pore size is between 3-5μm. Whereas for endothelial cells, epithelial cell, fibroblasts and tumour cells the ideal pore size is 8μm.

Once the compartments have been filled usually with cells in the top and chemoattractant in the bottom the chamber is incubated. The incubation time depends on the cell being examined, as some cells can migrate more quickly than others can. For example, stimulated neutrophils can migrate more quickly than eosinophils and T cells (Boyden 1962; Chen 2005).

Following incubation for the appropriate length of time, the porous membrane between the two compartments is fixed and stained and the number of cells that have migrated to the bottom side of the membrane are counted (Boyden 1962; Chen 2005).

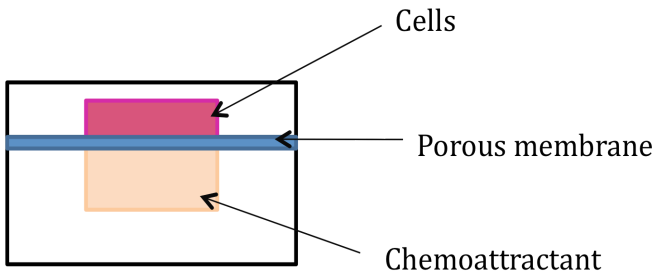


Figure 1.11 Diagram showing the set up of a Boyden chamber well

Most modern chemotaxis assays are variants of the Boyden chamber and these include the Zigmond and Dunn chambers. Zigmond chambers contain compartments side by side whereas the Dunn chambers contain concentric rings (Blow 2007). The Boyden chamber has many advantages including ease of use and the ability to quickly screen many compounds at many concentrations (Heit and Kubes 2003). However, the Boyden chamber also has a number of disadvantages, which include only one sharp chemotactic gradient can be formed and the migration cannot be observed in real time which means the speed of migration and path the cells take cannot be determined (Heit and Kubes 2003).

1.6.1.2 The under agarose assay

In the under agarose assay a series of wells set distances apart are cut into hardened agarose in a culture dish (Nelson et al. 1975). In the majority of experiments, chemoattractant is added into a central well and leucocytes are added into the outer two wells, either side of the central well (figure 1.12; Heit and Kubes 2003). Placing the neutrophils in the middle well and the chemoattractant in the outer wells can be used to examine the response of leucocytes to two opposing gradients (Heit and Kubes 2003). The completed dishes are then incubated for 2-18 hours depending on cell type (Nelson et al. 1975). For analysis, cells can be fixed and stained, recorded and analysed by phase contrast video microscopy or live cell imaged. The advantages of the under agarose assay are that it gives the researcher total control over positioning, timing and intensity of chemoattractant signals. It is also easy to set up two or more competing chemoattractant signals or to adapt the system for live imaging, which records the movement of 'chemotaxing cells' in real time (Heit and Kubes 2003).

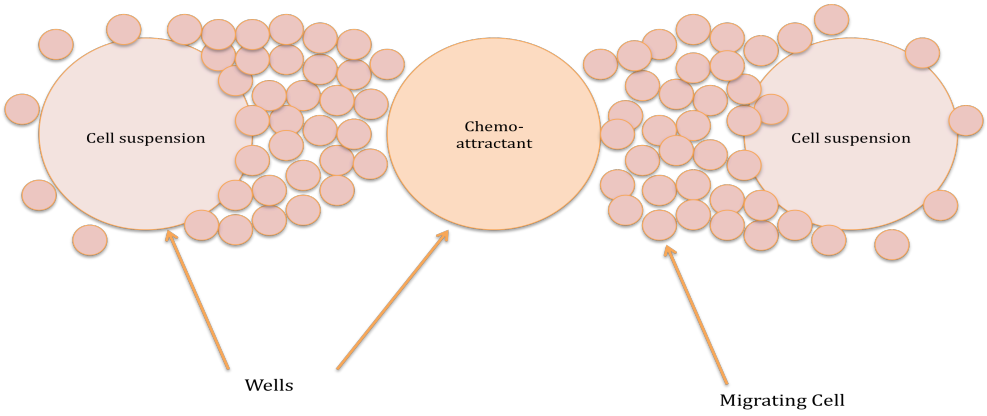


Figure 1.12 Diagram showing the set up and migration in the under agarose assay

1.6.1.3 The 3D migration assay

Friedl and Brocker (2004) developed the 3D migration assay. Chambers are constructed on a glass microscope slide by painting a wax/Vaseline mix in an open rectangle and sealing a glass coverslip over the top. 10x MEM, sodium bicarbonate, cell-free collagen and cell suspension in media are mixed and added into the migration chamber. Chemokinesis and chemotaxis can be studied by either adding the chemoattractant into the gel for chemokinesis or on the edge of the polymerized gel for chemotaxis (figure 1.13). The chamber is then placed vertically into an incubator to allow the collagen to polymerize and the gas conditions to reach equilibrium. The chamber is sealed and placed onto a microscope stage. The z-position of the stage is focused until it is in the middle of the lattice. The migration is then recorded by time-lapse, which allows the migration of the cells to be continuously visualized. The time of recording is dependent upon the migration speed of the cell type being studied. The individual migration paths of the cells can be analyzed using computer assisted cell tracking.

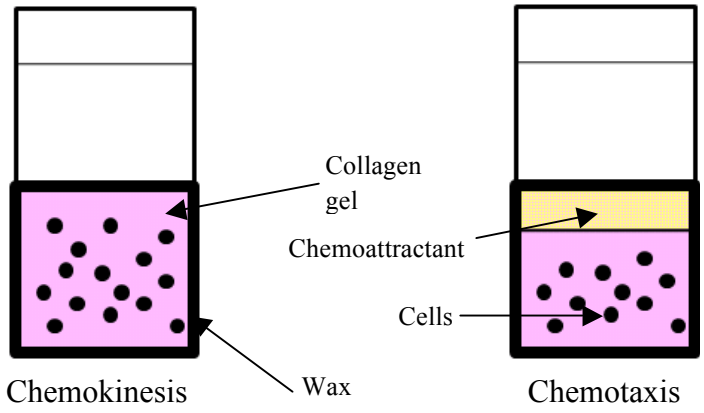


Figure 1.13 Diagrams demonstrating how the 3D migration gel is set up for both chemokinesis and chemotaxis.

The advantages of this assay are that it mimics aspects of in vivo-like scaffolding and reconstruction of cell adhesion and motility, cell patterning and cell communication. Cells are incorporated within a transparent 3D extracellular matrix to be monitored by time-lapse video microscopy and quantitative real-time data assessment.

1.6.2 In vivo methods

1.6.2.1 Intravital microscopy

The part of the animal being observed is dissected surgically, spread over a glass plate under a microscope, superfused with an isotonic salt solution and maintained at body temperature (Entschladen et al. 2005). The area can then be filmed using the microscope to determine cell influx and migration. The advantages of this method are that the cells can be visualised in an in vivo setting meaning the observations are the most physiologically relevant to true migration.

1.7 Hypotheses and aims

Hypotheses

1. Growth factor and chemokine mediated migration of neutrophils occurs via different signal transduction pathways.
2. Different signal transduction pathways control chemokinetic and chemotactic neutrophil migration.
3. The signal transduction events controlling neutrophil migration are different in a 3D environment compared to a 2D environment.

Aims

- Use the 3D migration assay for both non-gradient and gradient migration to determine the differences between growth factor and chemokine mediated signalling events.
- Use the 3D migration assay and Boyden chamber assay to dissect out the signal transduction events that control neutrophil migration in a three dimensional environment compared to a two dimensional environment.

Chapter 2- Materials and Methods

2.1 Reagents

All chemicals and reagents were purchased from Fisher Scientific (Loughborough, UK) unless otherwise stated.

Hanks' balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), EDTA, minimum essential media (MEM), sodium bicarbonate, RPMI, trizol, see blue plus 2 pre-stained marker and primers were from Invitrogen (Paisley, UK). Bronchial epithelial cell basal medium (BEBM) was obtained from TCS cellworks (Buckingham, UK). Collagen I was purchased from Nutacon (Leimuiden, The Netherlands).

Histopaque, Laemmli buffer, Gills' haematoxylin, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and immobilion-P transfer membrane were from Sigma-Aldrich (Poole, UK). CXCL8 and granulocyte macrophage-colony stimulating factor (GM-CSF) were from R&D systems (Abingdon, UK). Dextran was purchased from Pharmacosmos (Denmark).

Boyden migration assay equipment was from Neuroprobe (Leamington Spa, UK) and cellulose nitrate membrane was from Sartorius (Epsom, UK).

Western blot running equipment was from Biorad (Hertfordshire, UK) and semi-dry blotter was obtained from Geneflow (Fradley, UK). Enhanced chemiluminescence (ECL) reagent and hyperfilm were purchased from GE, Healthcare (Little Chalfont, UK).

Omniscript RT kit, Taq PCR mastermix, TAE buffer and QIAquick gel extraction kit were obtained from Qiagen (Crawley, UK).

Inhibitors	What it inhibits	Company	Concentration used	Specificity (from datasheet)	Reference
Pertussis toxin	$G\alpha_{i/o}$ subunit of the GPCR		100ng/ml		Spangrude et al. 1985
SB203580	p38MAPK	Calbiochem (Nottingham, UK)	10μM		Heit et al. 2002
SB239063	p38MAPK	Calbiochem (Nottingham, UK)	10μM		Underwood et al. 2000
PD98059	MEK, directly upstream ERK	Calbiochem (Nottingham, UK)	50μM		Hii et al. 1999
Y27623	ROCK	Alexis, (Exeter, UK)	10μM		Muessel et al. 2008
Wortmannin	PI3-Kinase	Calbiochem (Nottingham, UK)	50nM		Heit et al. 2002
PIK-75	PI3-Kinase α	Calbiochem (Nottingham, UK)	1μM	IC50: 0.3nM α, 40nM γ, 100nM C2β, 850nM β	Knight et al. 2006
PIK-294	PI3-Kinase δ	Symansis (Oxfordshire, UK)	1μM, 10μM	IC50: 10nM δ, 160nM γ, 490nM β, 10μM α	Knight et al. 2006
AS-605240	PI3-Kinase γ	Calbiochem (Nottingham, UK)	10μM	IC50: 8nM γ, 60nM α, 270nM β, 300nM δ	Reutershan et al. 2010

Table 2.1 List of inhibitors

Antibody	Molecular weight	Company	Monoclonal/ Polyclonal	Dilution	Secondary antibody
P-ERK	42-44kDa	Santa Cruz Biotechnology (Wembley, UK)	Monoclonal	1:1000	Anti-Mouse HRP
ERK	42-44kDa	Santa Cruz Biotechnology (Wembley, UK)	Polyclonal	1:1000	Anti-Rabbit HRP
P-Akt Ser^{473}	60kDa	Cell Signalling Technology (Hertfordshire, UK)	Polyclonal	1:1000	Anti-Rabbit HRP
Akt Ser^{473}	60kDa	Cell Signalling Technology (Hertfordshire, UK)	Polyclonal	1:1000	Anti-Rabbit HRP

Table 2.2 List of antibodies

2.2 Neutrophil separation

Whole human peripheral blood was taken from healthy non-asthmatic individuals with the approval of Leicestershire, Northamptonshire and Rutland Research Ethics Committee into a 50ml syringe containing heparin (1000U/ml). Dextran (composition: HBSS, 5mM EDTA, 30mM HEPES, 6% (w/v) dextran) was added to the 50ml syringe via a filling tube, and this was mixed by tipping the syringe gently back and forth. The syringe was clamped using a clamp stand at a 45-degree angle for 45 minutes to allow the majority of the red blood cells to sediment to the bottom of the syringe. The plasma layer was removed into a fresh 50ml tube; following this, the tube was centrifuged at 201 x g for 10 minutes at room temperature with the brake off. The supernatant was removed and the cell pellet was resuspended in a small volume of wash buffer

(composition: HBSS, 5mM EDTA, 30mM HEPES, 2% FBS), which had been warmed to room temperature. The resuspended pellet was layered over an equal volume of histopaque in a universal tube. This was centrifuged at 394 x g for 25 minutes at room temperature without the brake. The buffy coat containing mononuclear cells was removed and the sides wiped with a cotton bud to remove any residue. The cell pellet was added to a 50ml tube using ice-cold wash buffer. This was spun at 232 x g for 8 minutes at 4^{o}C. The supernatant was discarded and the cell pellet was resuspended in ice-cold distilled water for 30 seconds to lyse any remaining red blood cells, and was then toped up with cold wash buffer (composition: 2X HBSS, 10mM EDTA, 30mM HEPES). This was centrifuged at 232 x g for 8 minutes at 4^{o}C. The cell pellet was resuspended in a small volume of RPMI containing 2% heat-inactivated FBS. The cell count and percentage purity were determined using a haemocytometer and Kimura stain (composition: 5% (wt/wt) toulidine blue, 0.03% light green, saturated saponin and 0.7M phosphate buffer, pH 6.4). Kimura stain was used to count the cells as it allows white blood cells to be easily distinguished. For example, in neutrophils the stain clearly shows the multi-lobes, whereas, in contrast eosinophils will appear green. As eosinophils were the main contaminant affecting purity, the Kimura stain enabled easy and clear distinction between the two cell types, which meant the purity of the neutrophils within the solution could be calculated. Following the separation protocol the resultant neutrophil solution was usually between 96-98% pure.

2.3 Cell culture

BEAS-2B human bronchial epithelial cells were maintained in 25-cm^2 tissue culture flasks with bronchial epithelial cell basal medium (BEBM). Cells were used between

passages 15-25 and were split when the cells were 90% confluent. For use in experiments the cells were resuspended in RPMI with 2% FBS and were used at a concentration of $12x10^6$cells/ml.

2.4 Migration assays

2.4.1 3D collagen gel assay

3D collagen gels were constructed as described by Freidl and Brocker (2004) and Muessel et al. (2008). Purified neutrophils (6×10^6 cell/ml) were resuspended in RPMI containing 2% FBS and used directly in the migration assays. Chambers were constructed from a microscope slide with a coverslip sealed on with a paraffin wax and Vaseline mix. In all experiments control cells were unstimulated neutrophils. Cells were mixed with collagen I (3mg/ml), 10x MEM and sodium bicarbonate (7.5% w/v).

For non-gradient migration assays (see figure 2.1a) the chemoattractants were added to the gel, the gel was then loaded into a chamber and incubated at 37^{o}C for 1 hour prior to sealing the chamber and videotaping.

For gradient migration assays (figure 2.1b) the gel was loaded into approximately 3/4 of the chamber and incubated at 37^{o}C for 1 hour prior to videotaping. The chemoattractant of interest was then added to the final ¼ and the chamber was sealed.

For the inhibitor experiments, the neutrophils were pre-treated with the appropriate inhibitor for 30-120 minutes 37^{o}C in a CO_2 incubator. Following this incubation period, the cells were then added to gels in either the non-gradient or gradient assays as described above.

Neutrophil migration was recorded by time-lapse videomicroscopy using a Zeiss Axiovert 25 microscope (Germany) with a heated stage at magnification 20x, a QImaging Retiga 1300 CCD camera (BC, Canada) and the Improvision software Openlab (figure 2.2). To find an area to film the slide was placed under the microscope and the microscope was focused. When the collagen gel sets the cells are suspended in a series of layers and a middle layer where cells were able to move above and below was chosen for filming. In the non-gradient assay this area was chosen randomly. In the gradient assay, the migration was filmed with the edge of the area being the interface between the cell layer and chemoattractant solution. The microscope was then switched to the camera and using the Openlab software took a picture every 2 seconds for 20 minutes producing a migration movie.

The movie was then transferred into the Improvision software Volocity for analysis. A series of parameters were created within the Volocity software, objects were excluded if they were less than 10μm, more than 100μm, were touching the edge of the field of view or had failed to move for at least 3 minutes throughout the 20 minute filming. Following the exclusions, between 50-100 cells remained in the field of view. The software would show tracks for each of these cells movements from the point of origin to its location at the end of the 20 minutes of filming (figure 2.3). These tracks were checked manually as the software can mistakenly track the same cell twice. In addition, to the tracks the software calculates a variety of results including the distance moved, speed and migration time for each of the cells. The percentage of migration was calculated as the number of cells that had migrated more than 10 μm from the point of origin divided by the total number of cells.

Figure 2.3 is an example of the movement of cells, represented by the tracks in the 20 minute filming, although the tracks represent 2 dimensional migration, as the collagen forms layers, the cells can move freely into and out of the layer being filmed. This means that the migration does represent a 3 dimensional migration, as the cells moving in and out would be included within the results.

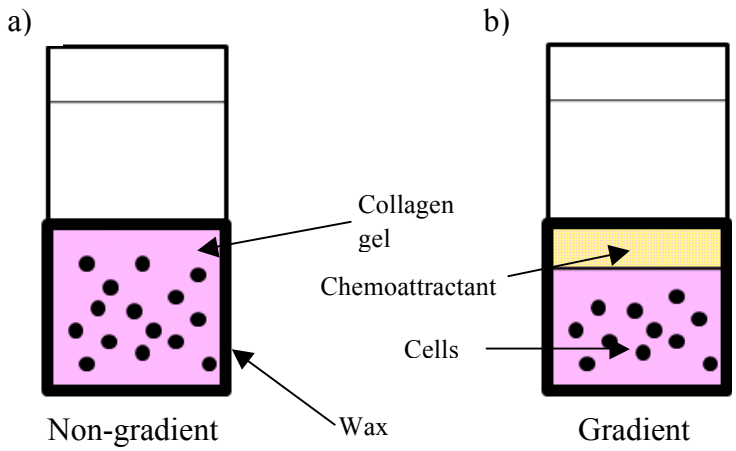


Figure 2.1 Diagrams demonstrating the non-gradient (a) and gradient (b) migration gels, as they would appear on the microscope slides.

Figure 2.2 Photograph showing the computer and microscope set up.

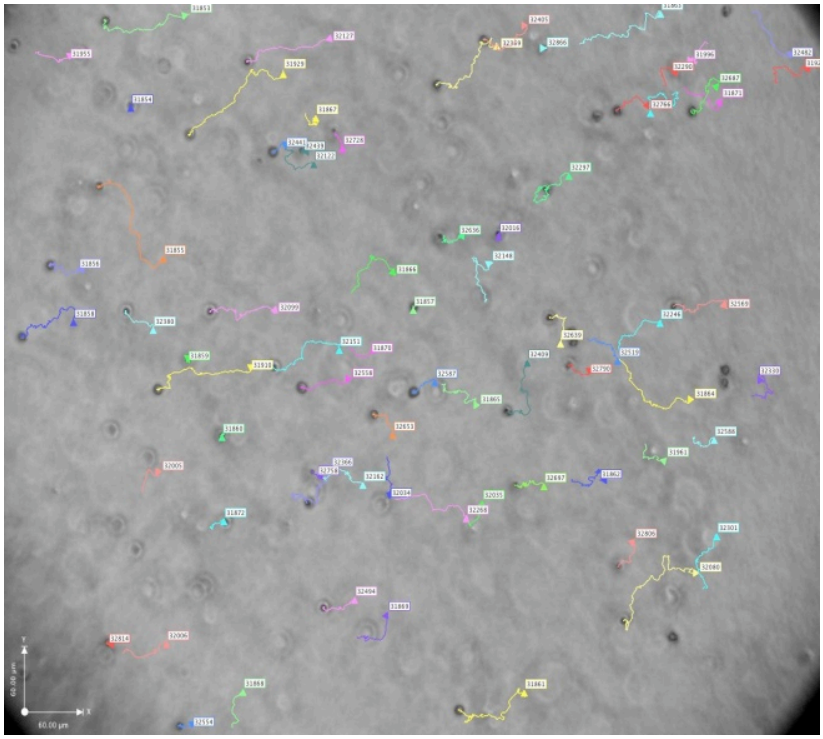


Figure 2.3 Snapshot of analysis showing movement tracks of cells from point of origin.

2.4.2 Boyden chamber assay

The bottom wells of a 48 well Boyden chamber plate were filled with warmed chemoattractant or control reagent (figure 2.4). A 5µm pore cellulose nitrate filter membrane was lowered onto the top of the filled wells with the middle section making contact first. The silicone gasket was then placed on top of the membrane. This was followed by the top plate, which was held down firmly until all the screws had been attached and tightened to prevent air bubbles from entering the wells. The neutrophils suspended in RPMI with 2% FBS at $4x10^6$cells/ml, were then added to the appropriate wells (figure 2.5). The cells were ejected in a rapid motion to dislodge any air bubbles in the well. The wells were then checked for trapped air bubbles, which are visible as an abnormally large meniscus. If air bubbles were detected, the cell solution was removed using a suction line and the well was refilled. It was important to ensure that there were no air bubbles in the wells, as this would affect the migration of the cells through the

membrane. The chamber was incubated for 90 minutes at 37^{o}C with 5% CO_2. For the inhibitor experiments, the neutrophils were pre-stimulated with the appropriate inhibitor for 90 minutes at 37^{o}C in a CO_2 incubator, following this incubation period the cells were then added to the top of the Boyden chamber as described above. After the appropriate incubation time the membrane was removed from the chamber and placed in IMS for 15 minutes to fix the cells. The membrane was then stained using Gills' haematoxylin for 3 minutes making sure that the membrane was fully submerged to allow equal staining. The membrane was washed in water then placed in solution containing Hydrochloric acid (HCl) (composition: 280mls IMS, 100mls H_2O and 3.8mls HCl) to remove excess stain. The membrane was washed in a running water bath in order for the blue colour of the stain to develop. After blueing the membrane was cleared in graded alcohols in the order 30% IMS for 2 minutes, 70% IMS for 2 minutes, 100% IMS for 5 minutes, Propran-1-ol 5 minutes and finally the membrane was left in xylene overnight to allow the membrane to clear. The membrane was finally laid onto a microscope slide with the migrated cells oriented upwards and a cover slip was attached using di-n-butylphthalate in xylene (DPX) mounting medium. The cells were counted using a Zeiss Axioskop 40 microscope at 40X resolution. 10 high power fields were counted for each well and the mean number of cells per well was calculated. Three wells were counted for each condition. If any of the total cell counts from each of the three wells was more or less than 10 cells away from the other two results it was not included in the average of the three wells. The total cell counts were averaged to express migration as a count/high powered field.

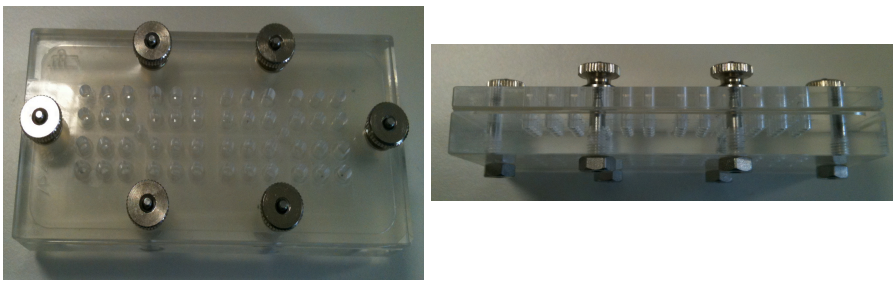


Figure 2.4. Photograph of the 48 well Boyden chamber from above (left) and the side (right).

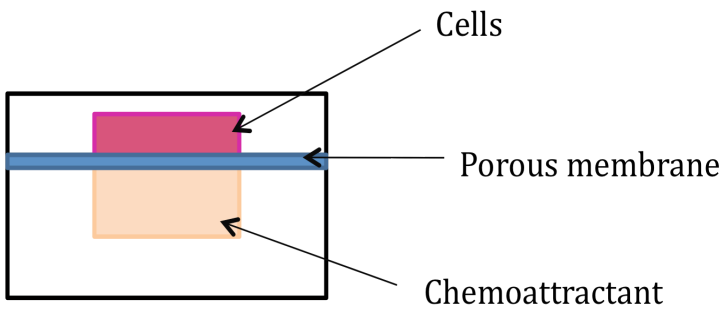


Figure 2.5. Diagram demonstrating the set up of one well of the Boyden chamber

2.5 Western blotting

2.5.1 Protein isolation

Neutrophils were left untreated or stimulated with CXCL8 or GM-CSF with/without inhibitors for 2 minutes. The cells were pelleted in Eppendorfs, the supernatant was discarded and the pellet was resuspended in RIPA buffer (composition: 10mM Tris,

100mM NaCl, 1% Triton-X, 0.1% (w/v) Deoxycholate, 5mM EDTA, 10mM NaF, 2mM Na_3VO_4, 10µg/ml Leupeptin, 10µg/ml Aprotinin, 100µM Phenylmethylsulfonyl fluoride) or Laemmli buffer. After the addition of RIPA buffer, the Eppendorfs were incubated on ice for 20 minutes, after which they spun at 13,000 x g for 10 minutes in the cold room to pellet the cell debris. The supernatants were removed, retained in fresh Eppendorfs and frozen at -20^{o}C until needed. After the addition of Laemmli buffer, the Eppendorfs were heated at 99^{o}C for 5 minutes before storing them at -20^{o}C until needed.

2.5.2 Electrophoresis

Resolving and stacking gels (see table 2.3 and 2.4) were freshly made. The resolving gel was loaded into Bio-Rad apparatus glass plates and 500µl of isobutanol was added to ensure the gel was level and enable it to set. The isobutanol was poured off and the gel was washed once with distilled water. The stacking gel was poured on top of the solidified resolving gel and the comb was put in place. Once the stacking gel had set, the comb was removed and the plates were put in the gel running tank containing running buffer (see table 2.5). 10µl of SeeBlue Plus2 pre-stained marker was loaded along with 20-40µl of sample protein. Prior to addition to wells sample proteins in RIPA buffer were mixed 1:1 (v/v) with Laemmli buffer and were incubated at 99^{o}C for 5 minutes. The gel was run at 100V, 400mA for approximately 2 hours.

Resolving Gel	8%	10%	12%
Water	2.98ml	2.4ml	1.82ml
1.5M Tris, *pH 8.8*	3.36ml	3.36ml	3.36ml
10% SDS	90µl	90µl	90µl
10% APS	200µl	200µl	200µl
30% Acrylamide	2.33ml	2.92ml	3.5ml
TEMED	10µl	10µl	10µl

Table 2.3 Resolving gel composition

Stacking Gel	
Water	3.6ml
1M Tris, *pH 6.8*	625µl
10% SDS	50µl
10% APS	100µl
30% Acrylamide	665µl
TEMED	8µl

Table 2.4 Stacking gel composition

10x Running Buffer	Final Concentration	Add
Tris	25mM	30.3g
Glycine	192mM	144g
SDS	0.1%	10g
Water		1000ml
pH 8.3, store at 4^{o}C		

Table 2.5 Running buffer composition

2.5.3 Membrane blotting

The gel was removed from the gel-running unit and was soaked in transfer buffer (see table 2.6) for 15 minutes. During this time Immobilion-P transfer membrane was cut to size and prepared for transfer by soaking in methanol for 15 seconds, followed by distilled water for 2 minutes and finally transfer buffer for 10 minutes. Filter paper was also soaked during this time. The transfer sandwich was assembled on a semi-dry blotter in the order filter paper, membrane, gel, filter paper. The blotter was then run at 15V for 30 minutes. Following transfer the membrane was submerged in methanol for 15 seconds, and then was allowed to dry fully on blue roll for 15 minutes before adding back to methanol for a further 15 seconds followed by 2 minutes in distilled water.

The membrane was then blocked in 1x TBS+0.1% Tween (see table 2.7) + 5% BSA or milk depending on the primary antibody being used for 1 hour at room temperature. It was then incubated with the corresponding primary antibody in 5% BSA overnight at 4^{o}C if milk was used for blocking the membrane was washed 3 times for 10 minutes each time using TBS+0.1% Tween20.

Following incubation with the primary antibody overnight the membrane was washed with TBS+0.1% Tween20 three times for 10 minutes each time. The membrane was subsequently incubated with secondary antibody in blocking solution for 1 hour. The membrane was washed a further three times for 10 minutes each time with TBS+0.1% Tween20.

Transfer Buffer	Final Concentration	Add
Tris	12.5mM	1.54g
Glycine	200mM	14.44g
Methanol		100ml
Water		900ml

Table 2.6 Transfer buffer composition

10x TBS buffer	Final Concentration	Add
Tris	250mM	30.3g
NaCl	150mM	90.9g
Water		1000ml
pH 7.5, store at room temp		

Table 2.7 Tris-buffered saline (10xTBS) composition

2.5.4 Protein detection

The membranes were developed using ECL detection reagents. Reagents 1 and 2 were added at a 1:1 ratio and added to the membrane for 1 minute before being removed. The membrane was wrapped in Saran wrap ready for detection onto ECL Hyperfilm.

2.5.5 Probing of membranes with additional antibodies

Membranes were added to stripping buffer (Table 2.8) and incubated in a water bath at 50^{o}C for 30 minutes with gentle shaking. They were then washed three times for 10 minutes each time in TBS + 0.1% Tween 20 before blocking and incubating as described above with additional antibodies.

Stripping buffer	Final Concentration	Add
2-β-mercaptoethanol	100mM	350µl
SDS	2%	10ml
Tris 1M pH 6.8	62.5mM	3.12ml
Water		36.53ml

Table 2.9 Stripping buffer composition

2.5.6 Densitometry

Densitometry was carried out on the western blots to quantify the fold change compared to the control. A Syngene genius bio imaging system was used for the densitometry. The Syngene programme Gene snap was used to take a picture of the western blot film and the Syngene programme Gene tools was used to measure the bands. Manual measurements were taken by drawing rectangles of equal size around each band of the western blot and one of the background. The background measurement was then subtracted from the band measurements. The fold change was then calculated by dividing each measurement by the control (unstimulated cells), so in the case of the control the fold change was 1.

2.6: PCR

2.6.1 RNA isolation

Neutrophil samples were spun down, and the pellet was lysed in Trizol. This was kept at -20^{o}C until required. The lysate was thawed on ice and then a mix of chloroform and isoamyl alcohol was added. The tube was vortexed until the two phases were

completely mixed. The tubes were centrifuged at 13,000 x g for 15 minutes. The clear phase was transferred to a new tube, isopropanol was added, and the solution was mixed well by hand. The tubes were left at room temperature for 5 minutes. The tubes were then spun at 13,000 x g for 15 minutes. The RNA pellet was visible as a grey/white shadow at one side of the tube. The isopropanol was removed and ice cold 75% ethanol was added to wash the pellet. This was centrifuged at 13,000 x g for 15 minutes. Following which, as much ethanol as possible was removed and the pellet was left to air dry. RNAse free water was then added to the dried pellet to resuspend the pellet and this was heat inactivated for 5 minutes at 57^{o}C. The RNA sample was then transferred to ice.

2.6.2 Primer design

The following primers were designed and used in the PCR

PI3Kα

LEFT PRIMER AGGGACCTCAATTCACCTCA
RIGHT PRIMER ACATCAAATTGGGCATCCTC

PI3Kβ

LEFT PRIMER AGCGTGGGTAAATACGATGG
RIGHT PRIMER CAGTCTTGTCGCAAAGTCCA

PI3Kδ

LEFT PRIMER CTGGTGCAGGTGCTCAAGTA
RIGHT PRIMER AAGTGCATCAGCTCCTTGGT

PI3Kγ

LEFT PRIMER TTGTGGCCAAAACATACCAA
RIGHT PRIMER AATCACAGCGAACCTCTGCT

2.6.3 RT-PCR

The concentration of RNA was measured using a spectrophotometer. The reverse transcription reaction was set up as shown in table 2.9. This was incubated at 37ºC for one hour.

	Add
RNAse free water + RNA sample (0.1µg)	12µl
10x buffer	2µl
DNTP mix	2µl
RNAse inhibitor	1µl
Random primers	2µl
Reverse transcriptase	1µl

Table 2.9 Reverse transcription reaction

Following this, the PCR reaction was prepared as shown in table 2.10. This was then run using the PCR programme shown in table 2.11.

	Add
RNAse free water	15µl
Forward primer	2.5µl
Reverse primer	2.5µl
cDNA	5µl
Taq PCR mastermix	25µl

Table 2.10 PCR reaction

Stage	Step	Temperature (°C)	Time	Stage cycles
1	1	94	3 minutes	1
2	1	94	30 seconds	35
2	2	58	30 seconds	
2	3	72	1.5 minutes	
3	1	72	3 minutes	1

Table 2.11 PCR stages

2.6.4 Gel electrophoresis

A 2 % gel was made by adding 2g of agarose to 100ml of 1x TAE buffer, this was heated in a microwave oven until a transparent liquid was obtained. The solution was left to cool to approximately 50° C before adding ethidium bromide. The cooled solution containing ethidium bromide was then poured into a mould, a comb was put in place and the gel was left to set. While the gel was setting, the samples to be run were prepared. 2µl of gel loading buffer was added to 10 µl of each PCR reaction and 2µl of gel loading buffer was added to 1µl of 100bp size marker and 9 µl TAE buffer. When the gel had set, the comb was removed and the gel was put into the gel running container. The container was filled with 1xTAE buffer, making sure that the gel was covered. The size marker and samples were loaded in the gel. The gel was then run at 90 V for 1 hour. The Gel was removed from the tank and the bands were visualised under UV light.

2.6.5 Gel extraction for DNA sequencing

The DNA was extracted from the gel using the QIAquick gel extraction kit. Briefly as per the manufacturer's handbook the DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colourless tube. 3 volumes of Buffer QG were added to 1 volume of gel (100mg ~100µl) and this was incubated at 50^{o}C for 10 minutes. To help dissolve the gel, vortexing was used to mix the gel every 2-3 minutes during the incubation. After the gel slice had dissolved completely, the colour of the mixture was checked, as it should still be yellow. If the colour of the mixture was orange or violet 10µl of 3M sodium acetate, pH 5.0, was added. 1 gel volume of isopropanol was added to the sample and mixed. A QIAquick spin column

was placed in a 2ml collection tube. To bind the DNA, the sample was applied to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the QIAquick column was placed back into the same tube. 0.5ml of Buffer QG was added to the QIAquick column and this was centrifuged for 1 minute. The flow-through was discarded and the QIAquick column was placed back onto the same tube. Adding 0.75ml of buffer PE and centrifuging for 1 minute washed the column. The flow-through was discarded and the column was placed back into the same tube. The column was centrifuged for 1 minute. The QIAquick column was place into a clean 1.5ml microcentrifuge tube and the DNA was eluted by adding 30µl of water to the centre of the QIAquick membrane. The column was left to stand for 1 minute then centrifuged for 1 min after which the samples were stored at -20^{o}C. The samples were then sent to the Protein Nucleic Acid Chemistry Laboratory at the University of Leicester for DNA sequencing.

2.7: Statistics

Results are expressed as means ±SEM. The n number represents the number of times the experiments were repeated. Each of the repeated experiments were carried out on different days with different donors. Significance ($p<0.05$) was determined using ANOVAs with Bonferroni correction on the commercially available software program, GraphPad Prism. The use of a parametric test such as an ANOVA assumes that the data are normally distributed. This is the case for the majority of biological data and in the experiments carried out there was no reason to believe that the data was not normally distributed, as the statistics indicated the predicted outcome in the majority of experiments. Normality cannot be tested for in these experiments as normality tests

such as chi-squared have no power when the n number is low as it is in the experiments presented in the thesis. However, parametric tests are relatively insensitive to deviations from normality. Parametric tests are also used for similar experiments within the literature. This indicates that the data generated was thought to be normally distributed, these include in human migration experiments towards CXCL8 and fMLP, with and without a series of inhibitors, and eosinophil migration dose response experiments when treated with GM-CSF (Knall et al. 1997, Heit et al. 2008, Muessel et al. 2008, Sadhu et al. 2003).

Chapter 3: Establishing neutrophil migration

3.1 Introduction

Neutrophils are the primary target for the chemokine CXCL8/IL-8 and activation of neutrophils in response to CXCL8 has been previously demonstrated to induce both chemokinesis and chemotaxis (Knall et al. 1997; Heit et al. 2002). In addition, the growth factor GM-CSF has also been shown to induce neutrophil chemokinesis and chemotaxis (Gomez-Cambronero et al. 2003). Therefore, to address the first hypothesis that growth factor- and chemokine-mediated migrations occur via different signal transduction pathways the chemokine, CXCL8 and growth factor, GM-CSF were examined.

In previous studies with CXCL8 and GM-CSF migration has typically been measured using either the Boyden chamber or under agarose method. These assays have provided a valuable insight into the signal transduction pathways involved in controlling neutrophil migration. The main method used in this thesis is the 3D collagen migration assay, which unlike the Boyden chamber and under agarose assay, more closely mimics the environment that the cells would experience within tissues structures such as the lung, while enabling the migration to be filmed.

This chapter details migration in response to CXCL8 and GM-CSF. As neutrophil migration in 3D migration assays has not previously been examined, the initial experiments were carried out to establish the normal migration pattern of migration when stimulated with CXCL8 and GM-CSF in the non-gradient and gradient assays. In addition to CXCL8- and GM-CSF-induced migration, this chapter also examines the

effects on migration when both CXCL8 and GM-CSF are added together and the migration of neutrophils in response to epithelial cells.

3.2 CXCL8-induced neutrophil migration

In the non-gradient and gradient migration assays human neutrophils were stimulated with increasing concentrations of CXCL8; these were 10ng/ml (1.25nM), 50ng/ml (6.25nM), 100ng/ml (12.5nM) and 300ng/ml (37.5nM).

Non-gradient assay

The neutrophils migrated in a dose dependent manner, with the CXCL8 concentrations of 50ng/ml, 100ng/ml and 500ng/ml all causing significant migration in comparison to unstimulated neutrophils (figure 3.1a.). 100ng/ml of CXCL8 evoked the highest percentage of neutrophil migration with an average of 62±11% of the cells moving. The neutrophils migrated in a chemokinetic manner in the non-gradient assay in response to CXCL8. This is illustrated by the vector diagram, which shows the direction of migration of each neutrophil, and was split approximately equally in all directions (figure 3.1b). In addition, to the dose dependent increase in the percentage of neutrophil migration in the non-gradient assay, the average velocity of the neutrophils also increased (figure 3.2.) Neutrophils stimulated with CXCL8 at the concentrations 100ng/ml and 300ng/ml move significantly faster than the control unstimulated cells (0.10±0.015μm/s). 300ng/ml of CXCL8 caused the highest response with an average velocity of 0.17±0.032 μm/s.

Gradient assay

Treatment of neutrophils with increasing concentrations of CXCL8 induced a dose dependent migration with both 100ng/ml and 300ng/ml causing a significant migration compared to the unstimulated neutrophils (10±9%, figure 3.1a). In contrast to the non-gradient assay, 300ng/ml of CXCL8 caused the highest percentage of neutrophil migration (73±5%). The neutrophil migration in the gradient assay was chemotactic, as highlighted by the vector diagram, which showed that the majority of the neutrophils moved towards the source of the CXCL8 (at the right of the gel; figure 3.1b). The dose dependent increase in the percentage of neutrophil migration was accompanied by a dose dependent increase in the average velocity of the neutrophils (figure 3.2). Upon stimulation with CXCL8 at the concentrations 100ng/ml and 300ng/ml neutrophils moved significantly faster than the unstimulated neutrophils (0.08±0.015μm/s). As with the percentage of neutrophil migration 300ng/ml induced the highest velocity (0.18±0.009 μm/s) in comparison to the control neutrophils.

Reproducibility

The reproducibility of the assay was established using the intra and inter coefficient of variation. The intra coefficient of variation was calculated by producing eight gels over the course of one day using neutrophils obtained from one donor. These eight gels contained 100ng/ml of CXCL8 and the coefficient of variation was 6%. (figure 3.3a). The inter coefficient of variation was calculated by using the same donor for the same gels but on different days. The gels used for comparison were the non-gradient 100ng/ml of CXCL8 and the coefficient of variation for the gels was 16% (figure 3.3b).

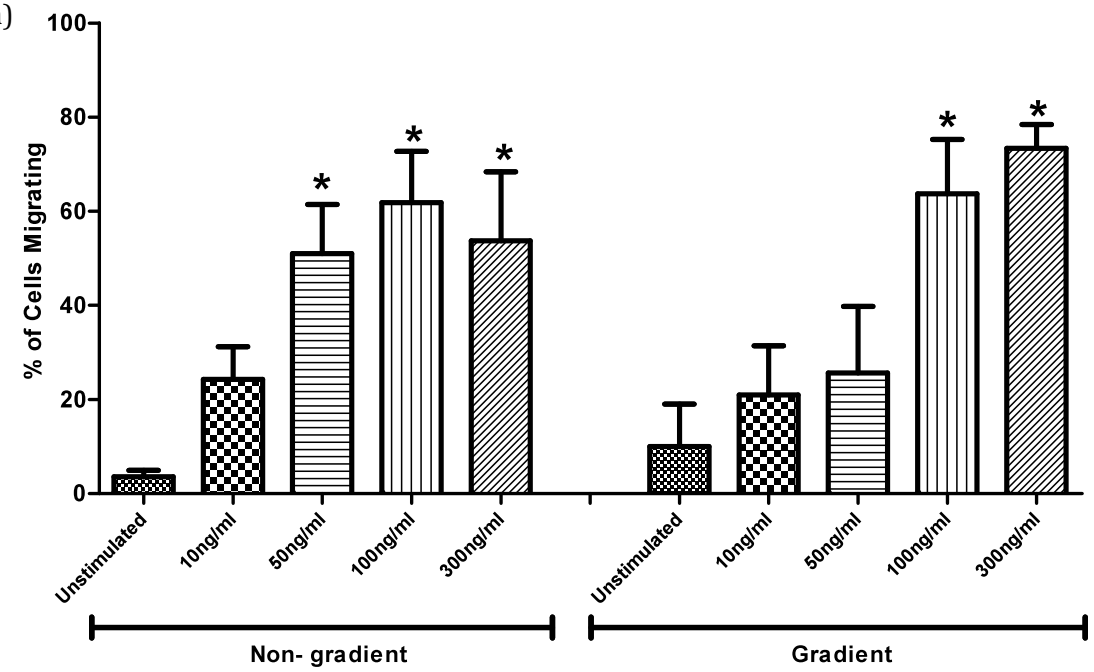
Migration in each section

In order to establish the effects of the chemokine gradient on the neutrophil migration, the movie of the gel as demonstrated in figure 3.4a was divided into four equal sections (section 1 nearest the source of the CXCL8) and the migration in each section was quantified. The percentage of migration decreased the further the neutrophils were from the gradient source with 84±4% of cells migrating in section 1 compared to 58±12% migrating in section 4 (figure 3.4). This trend was not significant; however, it does highlight the development of a gradient across the gel in the course of the 20-minute filming.

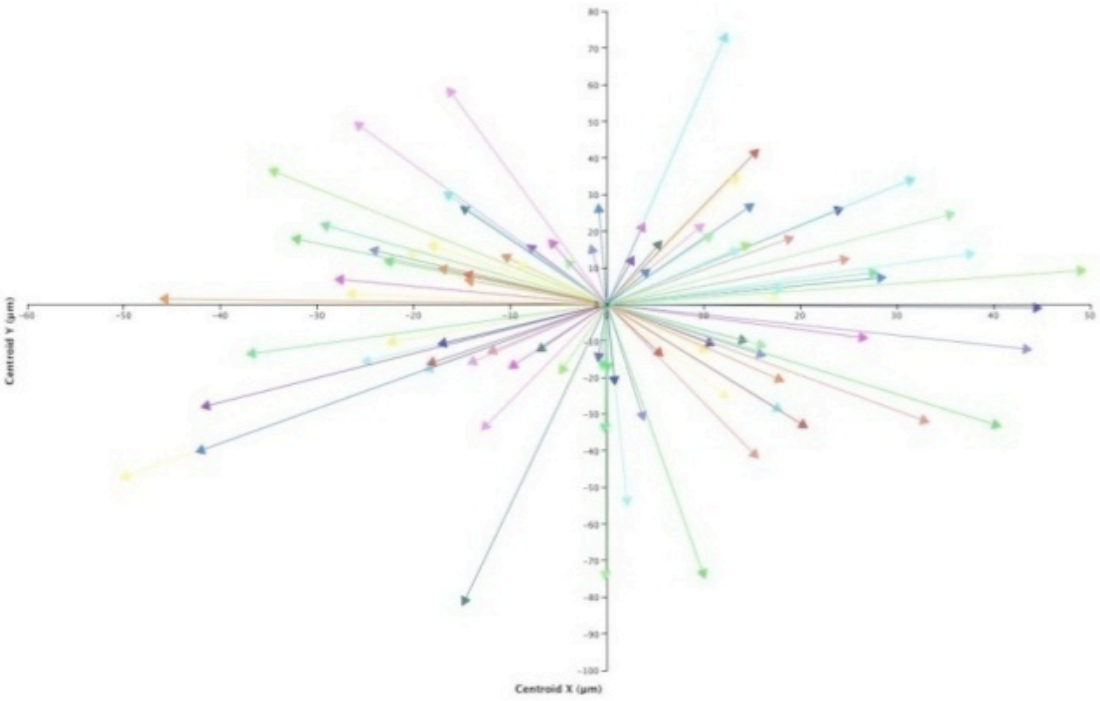
Boyden chamber

CXCL8 stimulates the migration of neutrophils through the membrane of the Boyden chamber in a dose dependent manner. Treatment with 10ng/ml of CXCL8 failed to increase the migration of neutrophils compared to cells that were unstimulated. Treatment with 50ng/ml and 100ng/ml significantly increased the proportion of cells migrating as measured by the cell number/ high-powered field (figure 3.5). 100ng/ml of CXCL8 induced the highest migration with 126±7 cells/high powered field compared to unstimulated neutrophils (32±3 cells/high powered field; figure 3.5).

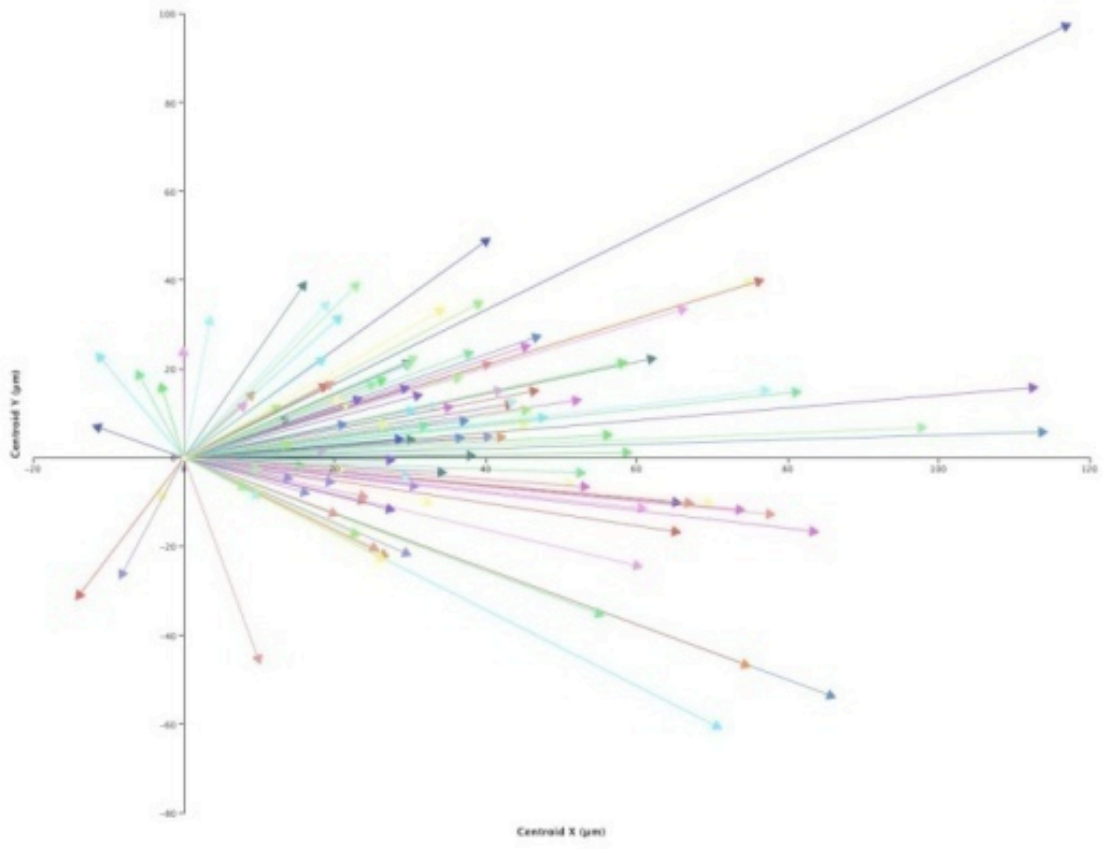
a)



b)



Non-gradient



Gradient

Figure 3.1. The percentage of neutrophil migration increases with higher concentrations of CXCL8 (a) in both the non-gradient and gradient assay. Results shown are mean ±SEM (n=3) except for non-gradient unstimulated cells and 100ng/ml where n=5. *Significantly greater as compared to unstimulated cells, $p<0.01$. The direction of movement for each neutrophil in the non-gradient and gradient assay in response to 100ng/ml of CXCL8 is illustrated in the vector diagrams (b)

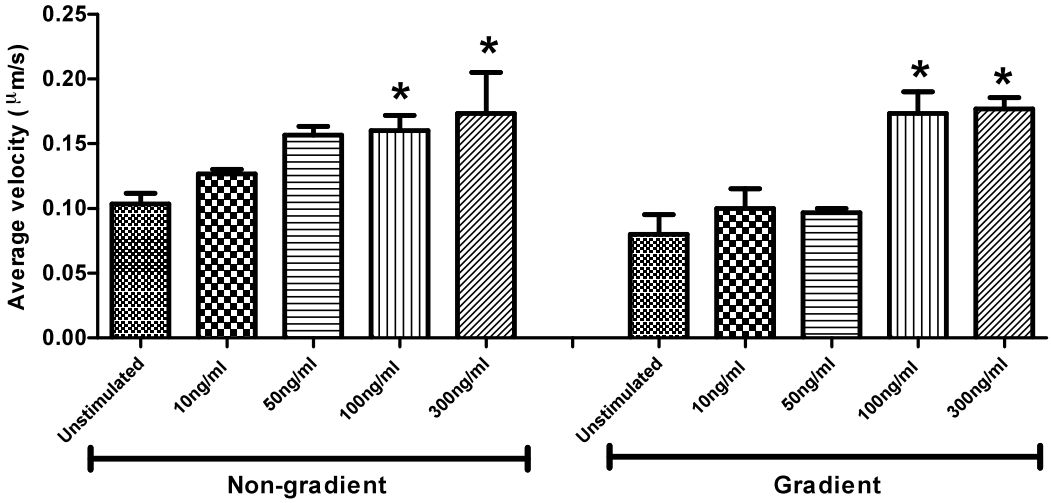
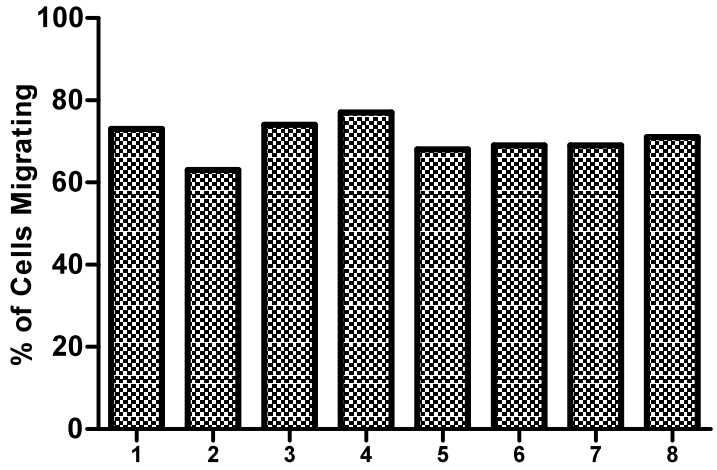


Figure 3.2. The average velocity of neutrophil migration increases with higher concentrations of CXCL8 in both the non-gradient and gradient assay. Results shown are mean ±SEM (n=3) except for non-gradient control and 100ng/ml where n=5. *Significantly greater as compared to unstimulated cells, $p<0.05$.

a)



b)

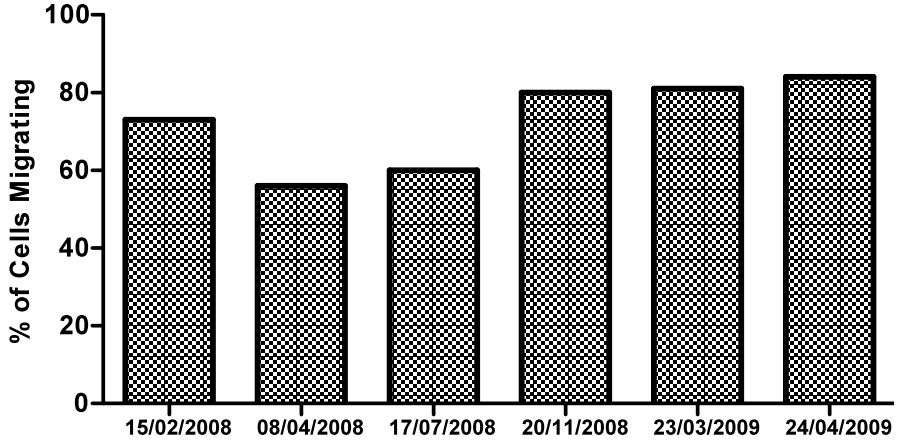


Figure 3.3. Coefficient of variation with CXCL8 stimulation. Intra: Over the course of a day cells obtained from one donor were used to produce eight non-gradient gels with 100ng/ml CXCL8 (a). The coefficient of variation was 6%. **Inter**: Over the course of a year blood was obtained from the same donor and was used to produce non-gradient 100ng/ml CXCL8 gels (b). The coefficient of variation was 16% (n=6).

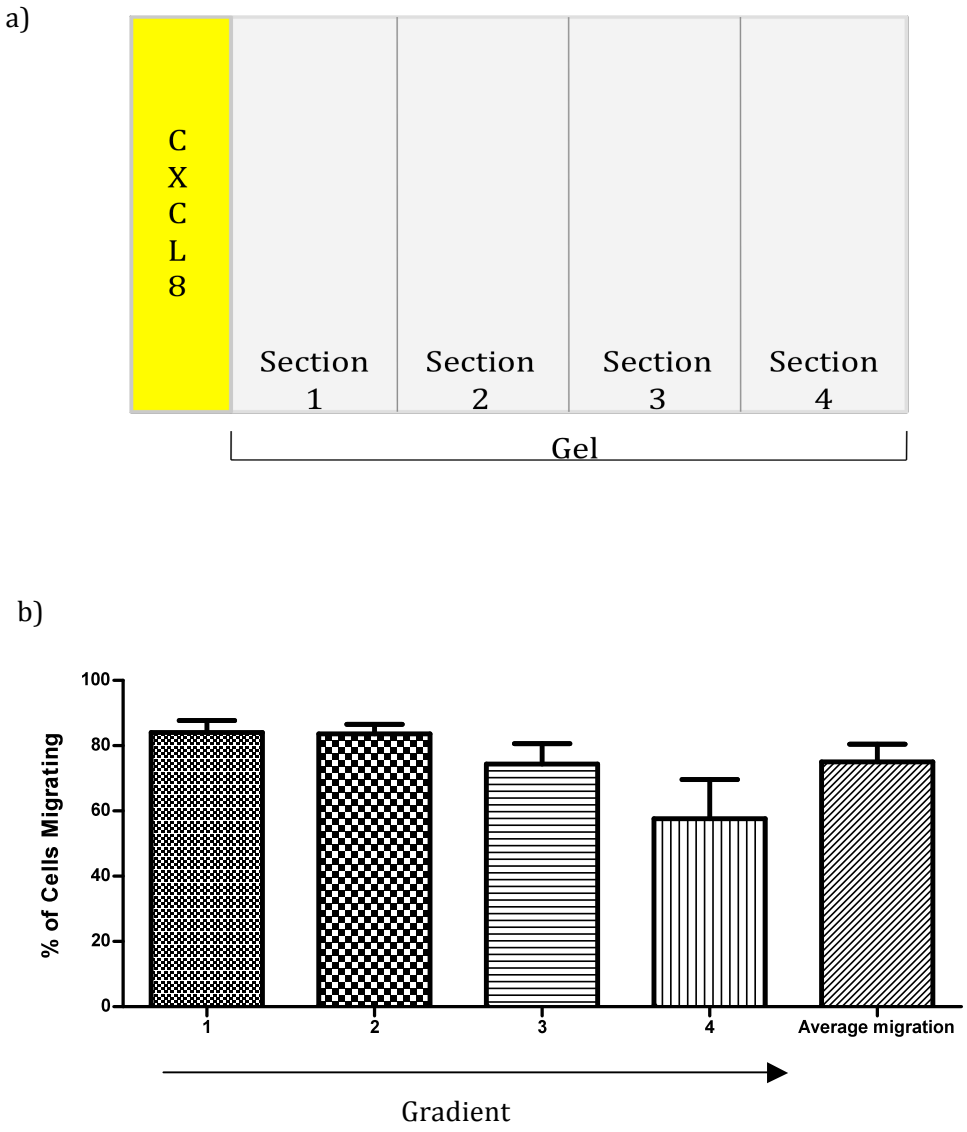


Figure 3.4. Neutrophil migration in each section of gradient CXCL8 gels. During analysis the screen was split into 4 equal sections as illustrated in a) and the percentage of cells migrating in each section was calculated b). 1 is the quarter nearest to the chemoattractant and 4 is the furthest away. The average migration over the whole gel is also shown. Results shown are mean ±SEM (n=5).

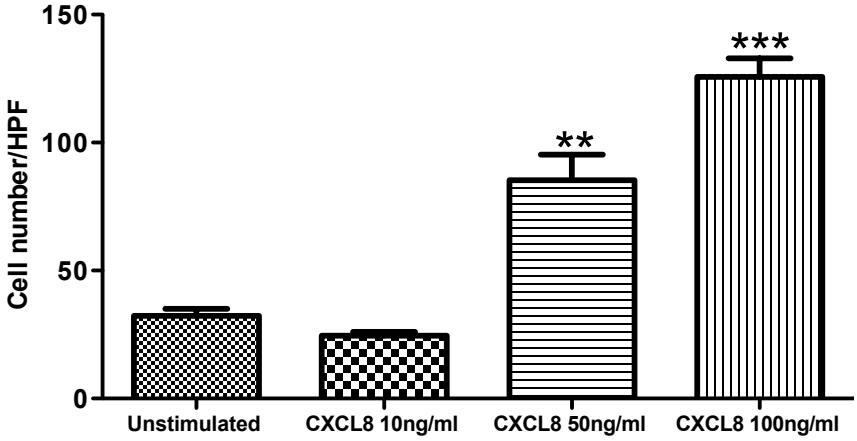


Figure 3.5. The number of cells moving through the membrane in the Boyden chamber increases with higher concentrations of CXCL8. Results shown are mean ±SEM (n=3). **/***Significantly greater as compared to unstimulated cells (**$p<0.01$, ***$p<0.001$).

3.3 GM-CSF-induced neutrophil migration

In the non-gradient and gradient migration assays human neutrophils were treated with increasing concentrations of GM-CSF; these were 0.05ng/ml (3.57pM), 0.5ng/ml (35.7pM), 5ng/ml (0.357nM) and 50ng/ml (3.57nM).

Non-gradient assay

Stimulation of neutrophils with increasing concentrations of GM-CSF from 0.05-50ng/ml induced a vigorous dose dependent migration, with all the concentrations evoking a significant response in comparison to the control neutrophils (7±4%, figure 3.6a). GM-CSF at a concentration of 5ng/ml elicited the highest response with 79±45% of neutrophils migrating. The migration of the neutrophils as expected in the non-gradient assay was chemokinetic. The vector diagram in figure 3.6b illustrates this. In conjunction with an increase in the percentage of migration, the velocity of migration also increased in a dose dependent fashion (figure 3.7). Although, only 5ng/ml and 50ng/ml of GM-CSF caused a significant increase in the velocity, compared to the control neutrophils (0.10±0.015μm/s). Stimulation with 5ng/ml induces the highest velocity with an average of 0.203±0.009μm/s. This was somewhat faster than the highest average velocity seen in response to stimulation with CXCL8, which was 0.17±0.032 μm/s, this difference however was not significant.

Gradient assay

Only a minor degree of migration was induced in response to the varying concentrations of GM-CSF in the gradient assay. The highest concentration of 50ng/ml caused the only significant response with 33±6% of neutrophils migrating. The migration in response to 50ng/ml GM-CSF was also chemokinetic in the gradient assay (figure 3.6). Therefore, although the signal is coming from just one direction the neutrophils respond to the stimulation and move freely in all directions. As with the percentage of migration, the average velocity of migrating neutrophils was only significantly increased in comparison to the control neutrophils, when stimulated with the highest GM-CSF concentration 50ng/ml (figure 3.7). Treatment with 50ng/ml of GM-CSF induced an average neutrophil velocity of 0.14±0.02μm/s, surprisingly, only slightly higher than the average velocity of the control neutrophils, which was 0.10±0.004μm/s. This was slower than the highest average velocity seen in response to stimulation with CXCL8, which was 0.18±0.009 μm/s; this difference however was not significant.

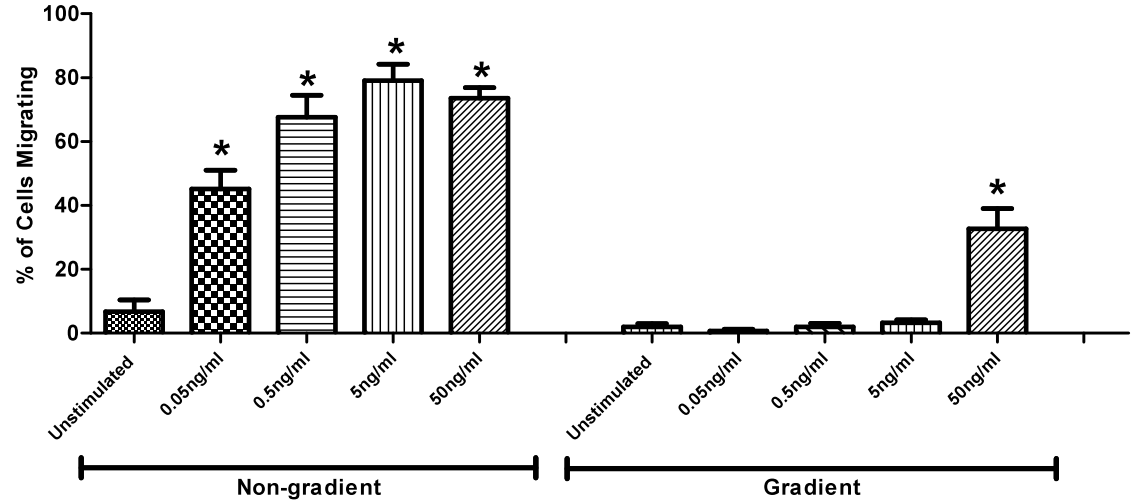
Reproducibility

The reproducibility of the assay was established using the intra and inter coefficient of variation. The intra coefficient of variation was calculated by producing eight gels over the course of one day using neutrophils obtained from one donor. These eight gels contained 50ng/ml of GM-CSF and the coefficient of variation was 7% (figure 3.8a). The inter coefficient of variation was calculated by using the same donor for the same gels but on different days. The gels used for comparison were the non-gradient 50ng/ml of GM-CSF and the coefficient of variation for the gels was 9% (figure 3.8b).

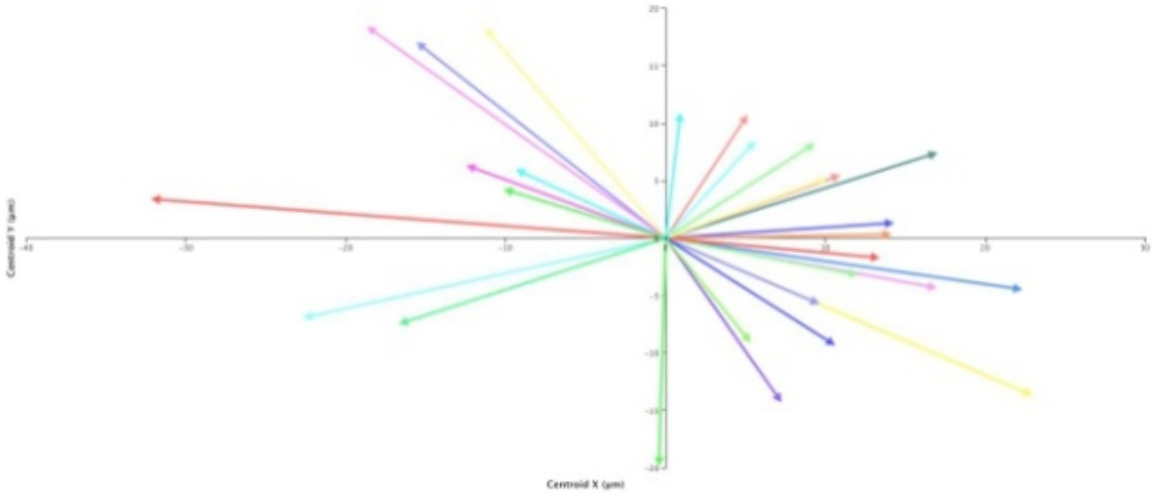
Migration in each section

In order to establish the effects of the cytokine gradient on the neutrophil migration, the movie of the gel was divided into four equal sections as demonstrated in figure 3.4a and the migration in each section was quantified. The percentage of migration decreased the further the neutrophils were from the gradient source, with 74±2% of cells migrating in section 1 compared to 48±7% of cells migrating in section 4 (figure 3.9). This trend was not significant; however, it does highlight the development of the gradient across the gel in the course of the 20-minute filming.

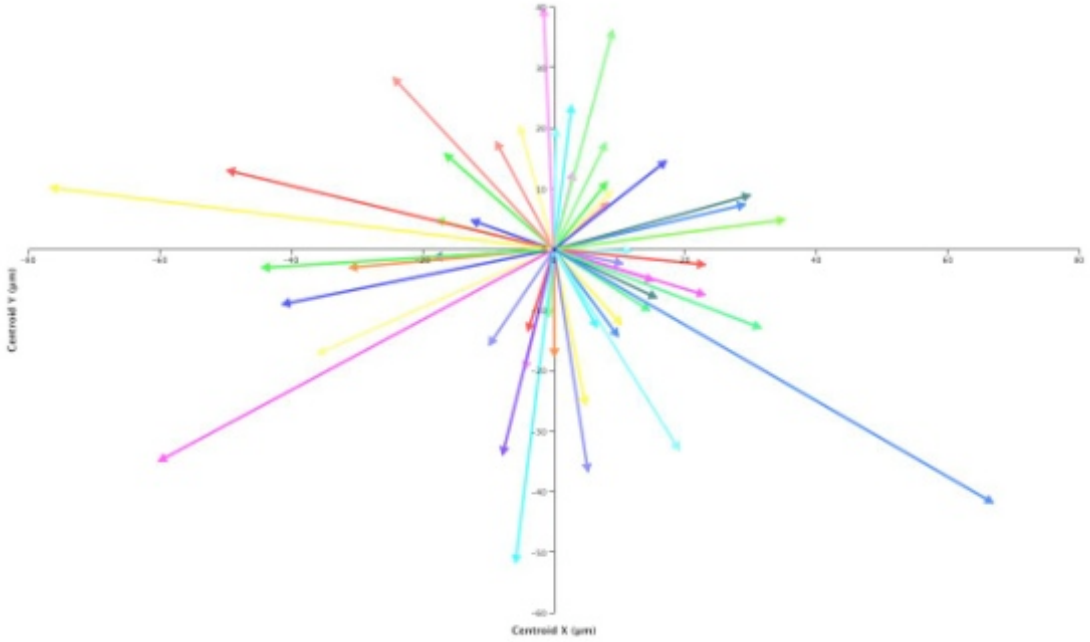
a)



b)



Non-gradient



Gradient

Figure 3.6. The percentage of neutrophil migration increases with higher concentrations of GM-CSF (a) in both the non-gradient and gradients assay, Results shown are mean ±SEM (n=3) except for gradient unstimulated cells and 50ng/ml where n= 7 and 6 respectively. *Significantly greater as compared to unstimulated cells, $p<0.01$. The direction of movement for each neutrophil in the non-gradient and gradient assay in response to 50ng/ml of GM-CSF is illustrated in the vector diagrams (b).

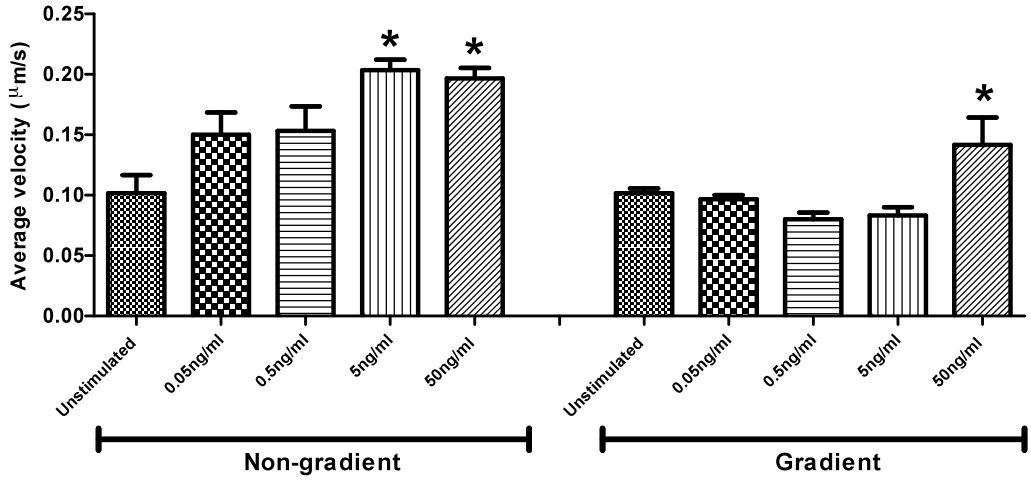
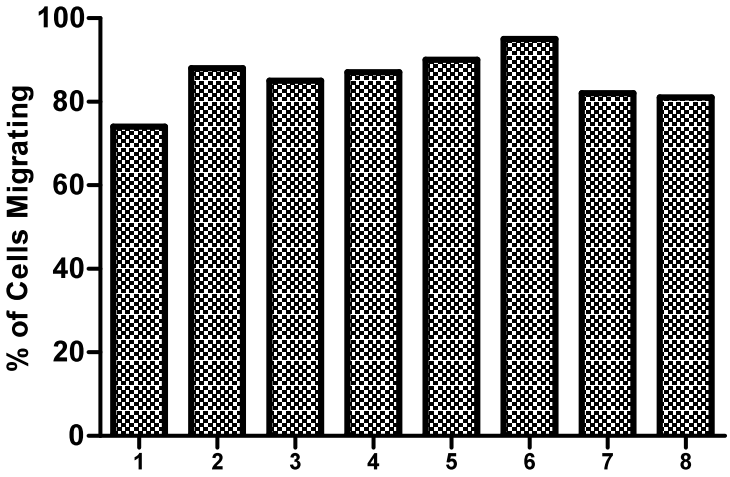


Figure 3.7. The average velocity of neutrophil migration increases with higher concentrations of GM-CSF (a) in both the non-gradient and gradients assay, Results shown are mean ±SEM (n=3) except for gradient unstimulated cells and 50ng/ml where n= 7 and 6 respectively. *Significantly greater as compared to unstimulated cells, $p<0.05$.

a)



b)

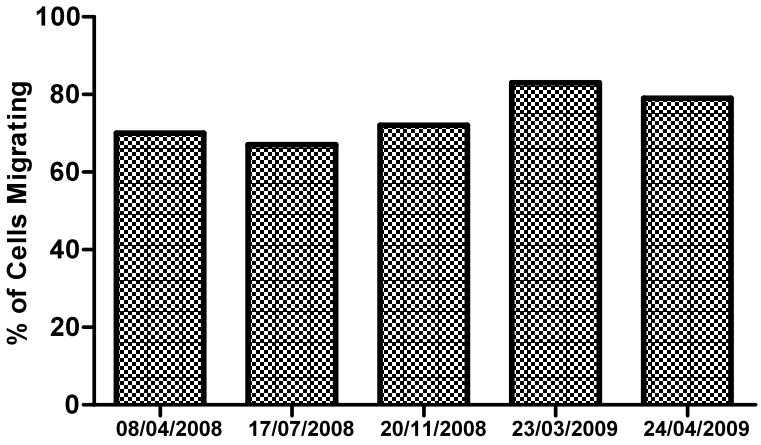


Figure 3.8. Coefficient of variation for GM-CSF. Intra: Over the course of a day cells obtained from one donor were used to produce eight non-gradient gels with 50ng/ml GM-CSF (a). The coefficient of variation was 7% **Inter**: Over the course of a year, blood was obtained from the same donor and was used to produce non-gradient 50ng/ml GM-CSF gels (b). The coefficient of variation for was 9% (n=5).

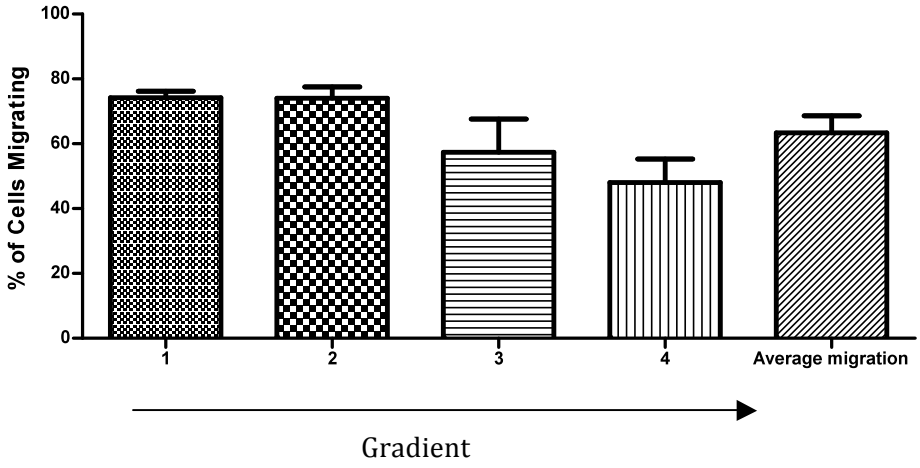
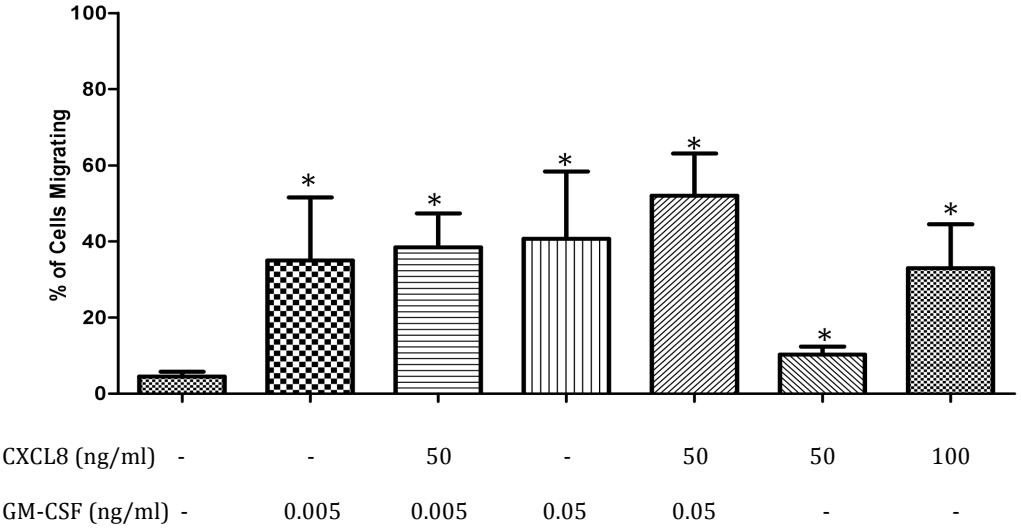


Figure 3.9. Neutrophil migration in each section of gradient GM-CSF (b) gels. During analysis, the screen was split into 4 equal sections and the percentage of cells migrating in each quarter was calculated. 1 is the section nearest to the chemoattractant and 4 is the furthest away. The average migration over the whole gel is also shown. Results shown are mean ±SEM (n=5).

3.4 CXCL8 and GM-CSF in combination

To determine if there was any synergy between CXCL8 and GM-CSF when added in combination, GM-CSF was added into the gel (as in the normal set up of a non-gradient assay), and CXCL8 was added at the edge of the gel (as in the normal set up of a gradient assay). Stimulation of neutrophils with this combination of CXCL8 and GM-CSF had no significant effect on the percentage (figure 3.10a) or directionality of neutrophil migration (figure 3.10b). The directionality of migration, determined as a percentage, was the number of cells that moved towards the CXCL8 over the total number of cells as shown in the vector diagram of migration. In the case of the unstimulated cells as less than 10 cells moved a percentage was not determined, therefore, it appears as if no cells moved towards the chemoattractant, however the same could also be said for the movement of cells away from the chemoattractant.

a)



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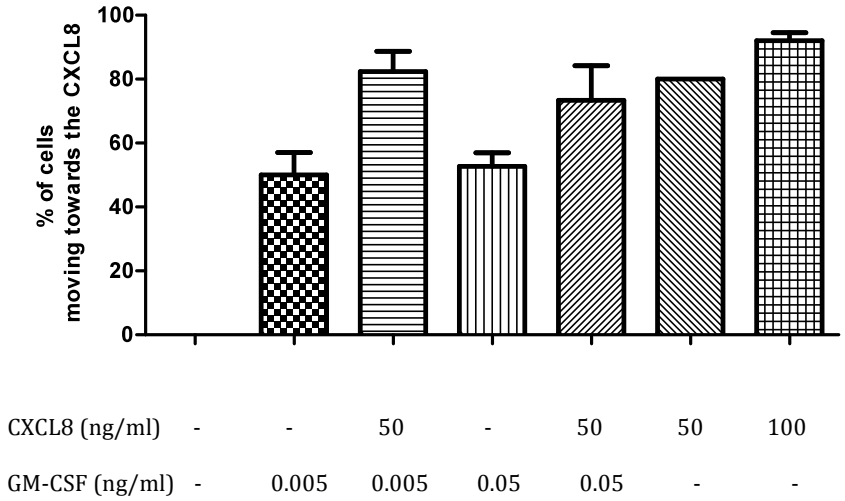


Figure 3.10. CXCL8 and GM-CSF in combination To determine if there is any synergy between CXCL8 and GM-CSF when added together GM-CSF was added into the gel as in the non-gradient assays and CXCL8 was added at the edge of the gel as in the gradient assays. a) the migration of cells in response to stimulation with CXCL8 and GM-CSF alone or in combination. b) the percentage of cells migrating directionally towards the chemoattractant. Results shown are mean ±SEM (n=3). *Significantly greater as compared to unstimulated cells, $p<0.05$.

3.5 Neutrophil migration in response to epithelial cells

Migration assays were carried out to determine whether neutrophils would migrate towards BEAS-2B epithelial cells (figure 3.11). The epithelial cells were treated with 50μg/ml of poly-IC overnight, which mimics viral disease and causes the release large amounts of cellular mediators including CXCL8, IL-4, IL-5 and IFNγ. The epithelial cells were then added to the gradient assay in the same way as the chemoattractant. Neutrophils significantly migrated towards the epithelial cells treated with poly-IC (50μg/ml; 67±7%, n=3; p=<0.001) in comparison to unstimulated epithelial cells (6±2%), RPMI alone (6±4%), and RPMI with poly-IC (10±5%). In addition, 92±2% of the neutrophils were migrating directionally towards the poly-IC treated epithelial cells. Treatment of neutrophils with anti-CXCR1 or anti-CXCR2 alone failed to inhibit neutrophil migration. However, treatment of neutrophils with anti-CXCR1 and anti-CXCR2 in combination caused a significant reduction in neutrophil migration (from 69±3% to 30±7%).

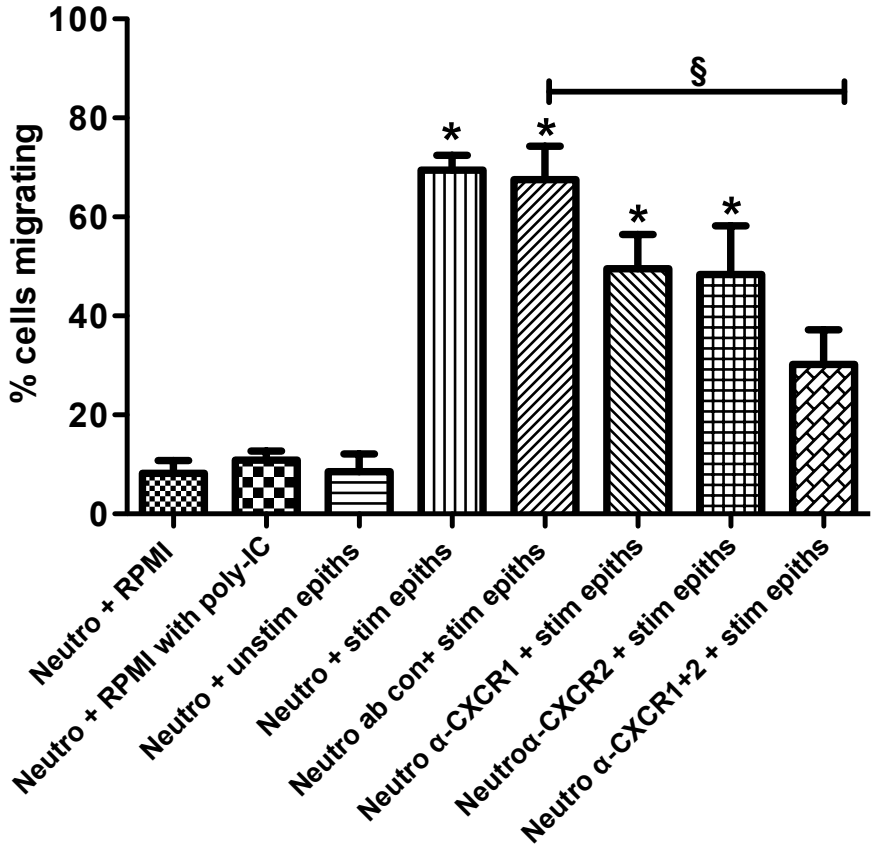


Figure 3.11. Neutrophils will migrate chemotactically in response to epithelial cells that have been treated with poly-IC overnight. Neutrophils were added to the gel as normal and epithelial cells were added to the edge immediately before filming. Unstimulated epithelial cells, RPMI containing poly-IC and RPMI alone were all used as negative controls. Results shown are mean ±SEM (n=7) except for α-CXCR1, where n=4, and α-CXCR2 alone and in combination where n=5. *Significantly greater as compared to control cells (neutro + RPMI, neutro + RPMI with poly-IC and neutro + unstim epiths) $p<0.01$. §Significantly reduced by antibodies $p<0.01$.

3.6 Discussion

Previous studies have demonstrated neutrophil migration in response to stimulation with CXCL8. In the Boyden chamber and under agarose assays both CXCL8-induced chemotaxis has been shown (Knall et al. 1997; Heit et al. 2002). In agreement with these experiments CXCL8–induced dose dependent migration was demonstrated in the non-gradient and gradient assays. This finding enhances the existing data as the cells can be visualised enabling differences in cell shape and movement to be noted. Chemokinesis can therefore be assessed independently of chemotaxis, leading to the conclusion that when stimulated with CXCL8 chemokinesis occurs in the non-gradient and chemotaxis occurs in the gradient assay. In addition, as both the Boyden chamber and under agarose assays are based on the quantification of cell numbers following an incubation period, use of the 3D collagen migration assay allows other perimeters to be assessed including velocity. In both the non-gradient and gradient assays, stimulation with CXCL8 caused a dose dependent increase in velocity. Further analysis of the gradient assay achieved by splitting the movie of the gel into four equal sections showed the presence of a gradient, as the percentage of migration was greater the nearer the cells were to the CXCL8. Although this observation was not significant, it does highlight the presence of a gradient, which cannot be observed using the Boyden chamber assay.

Treatment with GM-CSF-induced a dose dependent chemokinetic migration and dose dependent increase in velocity in both the non-gradient and gradient assay. GM-CSF has been reported to stimulate chemotaxis in the Boyden chamber assay (Gomez-Cambronero et al. 2003). In this previous study, dose response curves were determined for chemotaxis in response to GM-CSF, as well as fMLP and CXCL8 (Gomez-Cambronero et al. 2003). GM-CSF caused the lowest migration through the membrane

in contrast to migration induced by both fMLP and CXCL8, which caused almost double the amount of cells to migrate (Gomez-Cambronero et al. 2003). When comparing the migration in the gradient assay with GM-CSF and CXCL8, CXCL8 causes a higher percentage of migration than GM-CSF; taken together with the previous observations GM-CSF appears to induce a low level of migration in a gradient or chemotactic setting in comparison to other mediators. In addition, the percentage of migration in response to GM-CSF was also lower in the gradient assay compared to in the non-gradient assay. As the migration in both the assays is chemokinetic, it would be assumed that the cells would migrate to the same extent when treated with the same concentration. However, this was not the case. The change in the dose response curve in the gradient assay could be due to GM-CSF binding with the collagen upon entry into the gel, which would prevent the GM-CSF from diffusing effectively across the gel. Growth factors have been previously demonstrated to interact with extracellular matrix components; for example, the growth factor platelet derived growth factor (PDGF) was shown to specifically interact with collagen, modulating the activation and location of PDGF (Somasundaram and Schuppan 1996). The potential binding of GM-CSF to the collagen within the 3D assay would need to be explored, as this binding may prevent the GM-CSF from producing and maintaining an effective gradient as it is unable to diffuse across the gel. This would therefore decrease the percentage of neutrophils migrating, as the cells would not effectively receive the signal from the GM-CSF to migrate. Based on the above assumption it is likely that the migration observed in the gradient assay is controlled by the same mechanisms as migration in the non-gradient assay. Therefore, the only difference between the two is that the neutrophils were exposed to a much lower concentration in the gradient assay compared to the non-gradient assay.

Both CXCL8- and GM-CSF- induced migration in the gradient migration assay was shown to be reproducible, as the coefficient of variations when using the same donor, to create the same gel on different days and the same donor, to create 8 of the same gel over the course of the day were low. Therefore, we could be confident that the results obtained in the 3D migration assay were due to the stimulants used not the day or indeed time of day the cells were used.

Stimulation of neutrophils with a combination of GM-CSF and CXCL8 did not increase the percentage or directionality of the migrating cells suggesting that there is no synergy between GM-CSF and CXCL8, they instead work independently. As there is a hierarchy for some chemoattractants it could be worth determining whether neutrophils will preferentially migrate towards CXCL8 or GM-CSF and this could be determined using the under agarose assay. Using this method, the neutrophils could be added to the middle well, and CXCL8 and GM-CSF to the outer wells, an equal distance apart. The migration would then be determined by measuring the number of cells that migrate towards the mediators and if more cells migrated towards either chemoattractant, a hierarchy could be established.

This is the first study to show neutrophil migration in response to stimulation with mediators released from epithelial cells in the 3D migration assay. Airway epithelial cells release CXCL8 spontaneously and its release dramatically increases in response to rhinovirus infections or inflammatory stimuli including TNF-α and IL-1β (Striz et al. 1999; Hull et al. 2000). In the case of rhinovirus infections, the release of CXCL8 is thought to cause a significant neutrophilic response (Newcomb et al. 2007). The epithelial cells were treated with Poly-IC, which is used in-vitro to mimic the effects of viral stimulation through toll like receptor 3 (TLR3). Treatment of epithelial cells with poly-IC causes a variety of inflammatory mediators to be released, including CXCL8,

IL-4, IL-5 and GM-CSF. The release of these mediators would enable the neutrophils to migrate towards the poly-IC treated epithelial cells as was shown experimentally. Treatment of neutrophils with anti-CXCR1 and anti-CXCR2 in combination caused a significant reduction in the percentage of neutrophils migrating towards poly-IC treated epithelial cells. It did not however completely inhibit the migration. The antibodies block the binding of the CXCR1 and CXCR2 ligands, CXCL6 and CXCL8, and the CXCR2 ligands, CXCL1, CXCL2, CXCL3, CXCL5 and CXCL7 (Palmqvist et al. 2007). Therefore, stimulation of the epithelial cells with poly-IC could cause a combination of these seven chemokines to be released, which in turn stimulated neutrophil migration. However, as this only accounts for approximately 50% of the migration seen other inflammatory mediators, such as GM-CSF and interleukins, may also be responsible for inducing the migration of the neutrophils towards the epithelial cells and this could be further explored.

To summarise, the migration/recruitment of neutrophils occurs in the lung in conditions, where the lung becomes infected or damaged causing the epithelial cells to release pro-inflammatory cytokines. This in turn causes the recruitment of leucocytes particularly neutrophils into the damaged/infected tissue to clear the infection to be cleared.

In conclusion to this chapter, based on the results obtained from the increasing concentrations of CXCL8 and GM-CSF in the non-gradient and gradients assays the concentrations 100ng/ml CXCL8 and 50ng/ml GM-CSF were chosen for use in further experiments, as they elicited the best responses in both assays.

Chapter 4: Establishing the key signalling pathways involved in neutrophil migration

4.1 Introduction

Neutrophil migration is a multistep process, whereby the activation and deactivation of a variety of receptors, proteins and kinases enables the control of the actin cytoskeleton, allowing the cell to contract, detach and protrude forwards.

Previous studies using a variety of inhibitors have built a picture of the main signalling components involved in migration. The majority of these findings have been obtained using a Boyden or related transwell chamber and the chemoattractant fMLP. This chapter addresses two of the hypotheses of this thesis. Firstly, that growth factor and chemokine-mediated migration occur via different signal transduction pathways and secondly, that the signal transduction events controlling neutrophil migration are different in a 3D environment compared to 2D environment. Experiments were therefore carried out using well-described inhibitors to highlight the key pathways involved in both chemokinesis and chemotaxis. These include inhibitors of the MAPK family of proteins, a broad-spectrum inhibitor of PI3-Kinases and an inhibitor of the downstream pathway of Rho GTPase, all of which have been shown to contribute to the control of neutrophil migration.

Therefore, the aim of this chapter is to determine the key signal transduction events involved in CXCL8- and GM-CSF-induced migration in both the non-gradient and gradient assays.

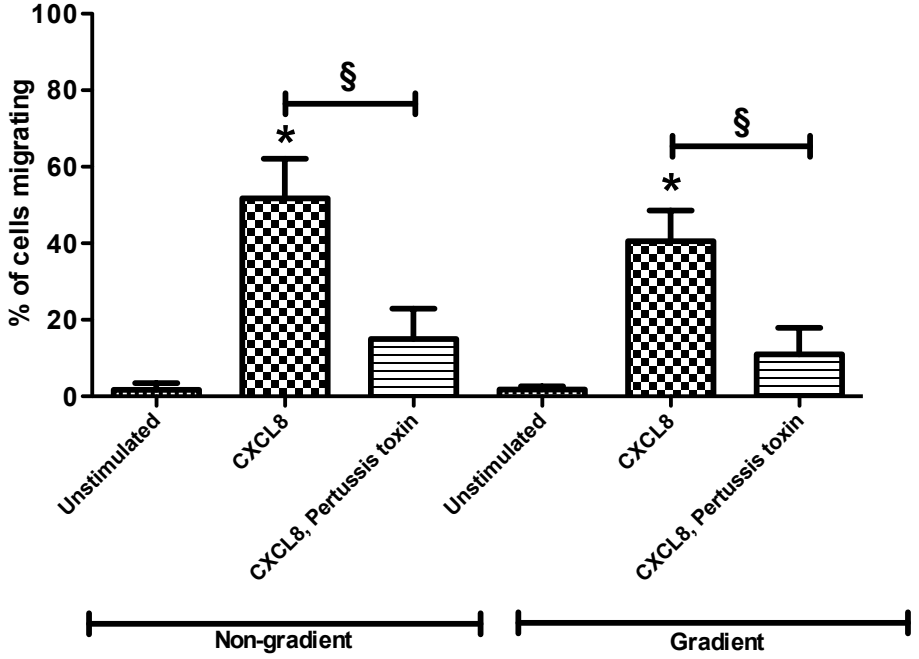
4.2 The effect of pertussis toxin on CXCL8- and GM-CSF-induced neutrophil migration

The inhibitor pertussis toxin was used to investigate the role of the GPCR in neutrophil migration. Pertussis toxin inhibits the Gα subunit of the GPCR preventing the activation of the downstream signals responsible migration. Unstimulated neutrophils failed to migrate; in contrast, stimulation with CXCL8 significantly increased the percentage of neutrophil migration to 52±10% in the non-gradient assay and 41±8% in gradient assay (figure 4.1a). The migration in both assays was markedly inhibited when cells were treated with pertussis toxin (100ng/ml, 2h pre-treatment) with cell migration of 15±8% in the non-gradient assay and 11±7% in the gradient assay (figure 4.1a).

Pertussis toxin pre-treatment had no effect on the directionality of the neutrophils still able to migrate, in both the non-gradient and gradient assays, in response to stimulation with CXCL8 as highlighted by the vector diagrams (figure 4.2).

In both the non-gradient and gradient migration assays GM-CSF significantly increased the proportion of cells migrating from approximately 2% to 63±3% in the non-gradient assay and 41±4% in the gradient assay (figure 4.1b). In contrast to CXCL8-induced migration, GM-CSF-induced neutrophil migration in both assays was unaffected by pre-treatment with pertussis toxin (figure 4.1b.).

a)



b)

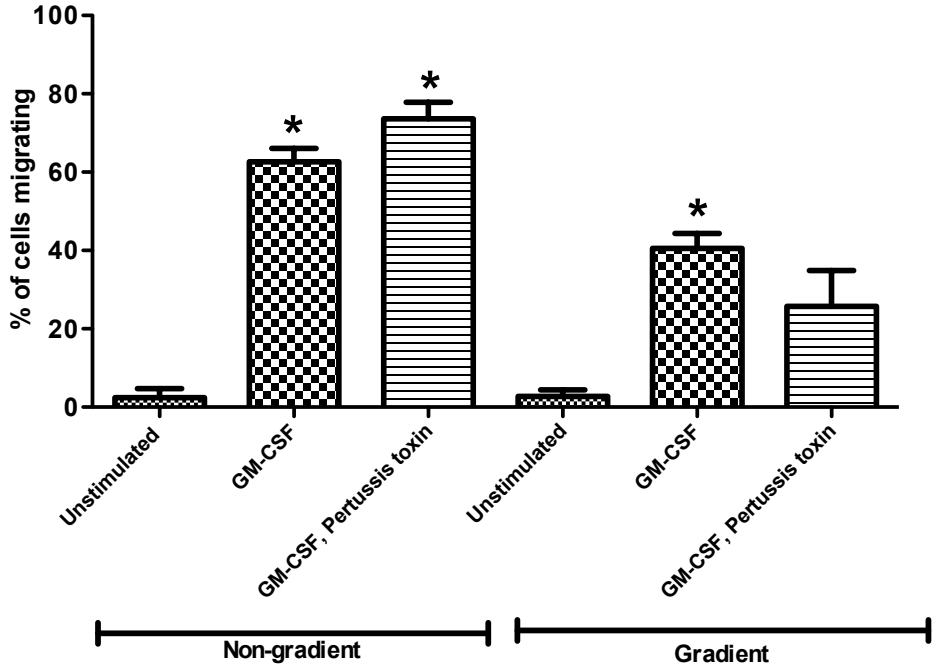
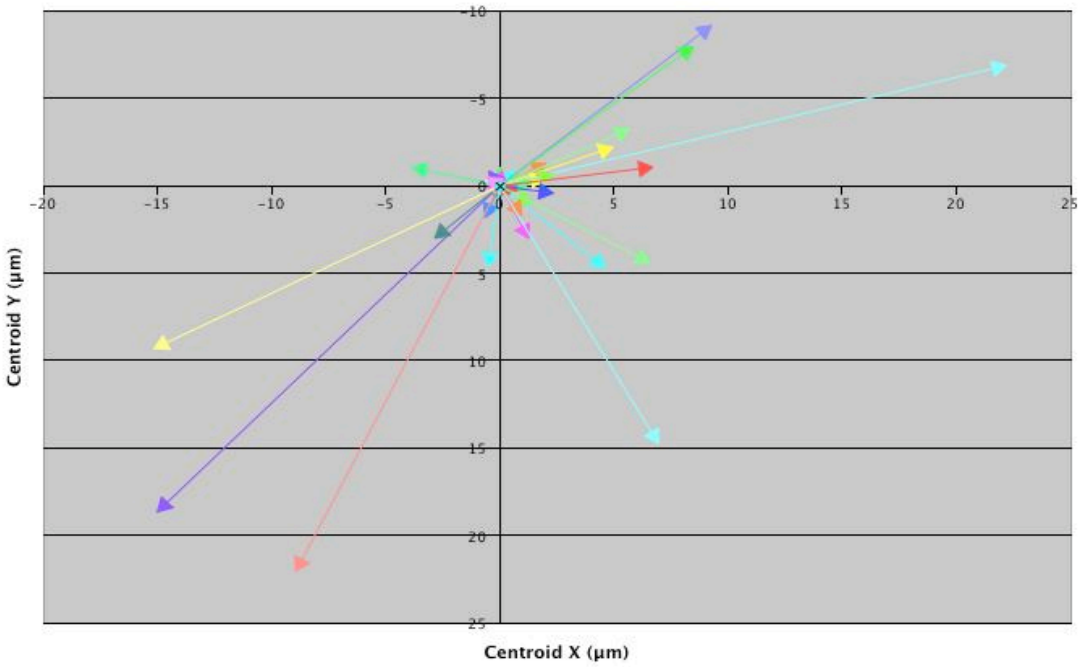


Figure 4.1. Pertussis toxin inhibits neutrophil migration induced by CXCL8 but not GM-CSF. Neutrophils were pre-treated for 2h with 100ng/ml pertussis toxin and then stimulated with a) 100ng/ml CXCL8 or b) 50ng/ml GM-CSF (b). Results shown are mean ±SEM (n=4) except for gradient CXCL8 where n=5 and non gradient GM-CSF where n=3. *Significantly greater than unstimulated cells, $p<0.01$. §Significantly inhibited by pertussis toxin, $p<0.05$.

a)



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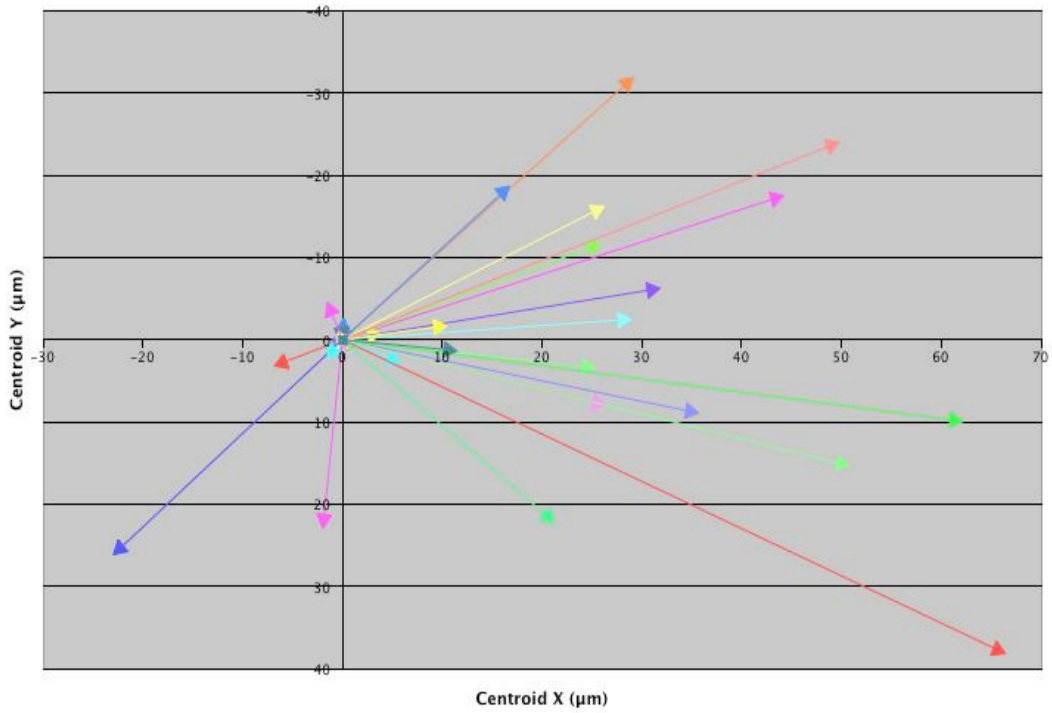


Figure 4.2 Vector diagrams illustrating the direction of migration for each neutrophil in one of the experiments carried out, following pre-treatment with pertussis toxin and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b). The diagrams shown represent the pattern seen for all repeated experiments.

4.3 The involvement of Mitogen Activated Protein Kinases in neutrophil migration

The Mitogen activated protein kinases (MAPKs), p38MAPK and ERK have both been suggested to play a role in cell migration. Therefore, using the inhibitors SB239063, SB203580 and PD98059 their role was determined in CXCL8- and GM-CSF-induced neutrophil migration.

The inhibitors SB239063 and SB203580 were used to evaluate the role of p38MAPK in migration. In both the non-gradient and gradient migration assays, CXCL8 significantly increased the proportion of cells migrating from 2% to approximately 80% (figure 4.3). The vehicle control for both inhibitors (0.2% DMSO) had no significant effect on neutrophil migration induced by CXCL8 in either the non-gradient or gradient assay (figure 4.3). In both assays, the treatment of cells with SB239063 and SB203580 (10μM, 30 min pre-treatment) had no effect on the proportion of cells migrating.

In both the non-gradient and gradient migration assays, GM-CSF significantly increased the percentage of cells migrating from 5% to approximately 80% in the non-gradient assay and over 60% in the gradient assay (figure 4.4). The vehicle control for both inhibitors (0.2% DMSO) had no significant effect on neutrophil migration induced by GM-CSF in either the non-gradient or gradient assay (figure 4.4). In both assays, treatment with SB239063 and SB203580 (10μM, 30 min pre-treatment) had no significant effect on migration in response to stimulation with 50ng/ml GM-CSF.

As both CXCL8- and GM-CSF-induced migration were unaffected by pre-treatment with the p38MAPK inhibitors, fMLP was used to determine if the inhibitors are effective. As, in the literature the inhibitor SB203580 has been shown to inhibit neutrophil migration stimulated by fMLP. fMLP had not been used prior to these experiments therefore the optimum concentration to use was determined. Neutrophils

were treated with increasing concentrations of fMLP from 0.01μM to 10μM in both the non-gradient and gradient assay (figure 4.5). In the non-gradient assay, neutrophils migrated dose dependently with all concentrations eliciting a strong migration. 1μM caused the highest migration with an average of 60±5% of cells migrating (figure 4.5). In the gradient assay, treatment with fMLP caused a dose dependent migration with all concentrations causing a significant response. 0.1μM evoked the highest percentage of migration with an average of 85±5% of cells migrating (figure 4.5). Based on these results migration following pre-treatment with the p38MAPK inhibitor SB203580 was assessed using fMLP at a concentration of 0.1μM.

In both the non-gradient and gradient migration assays stimulation with fMLP significantly increased the proportion of cells migrating from 4±1% to 59±10% in the non-gradient assay and 84±6% in the gradient assay (figure 4.6). Treatment with the p38MAPK inhibitor SB203580 or the vehicle control (0.2% DMSO) had no effect on fMLP-induced neutrophil migration (figure 4.6).

The inhibitor PD98059 was used to investigate the role of the MAPK ERK in neutrophil migration. The inhibitor acts upstream of ERK on the MAPK kinase MEK, which directly activates ERK. Stimulation with CXCL8 in both the non-gradient and gradient assay evoked a significant increase in migration from 1±1% to over 70% in both assays (figure 4.7a). The vehicle control for PD98059 (0.07% DMSO) had no significant effect on neutrophil migration induced by CXCL8 in both the non-gradient and gradient assay. Treatment with the inhibitor PD98059 almost completely abolished neutrophil migration in the non-gradient assay to 8±4% (50μM, 30 minutes pre-treatment). In contrast, treatment with the inhibitor in the gradient assay caused a

significant but only minor decrease in the percentage of cells migrating to 40±4% (figure 4.7a).

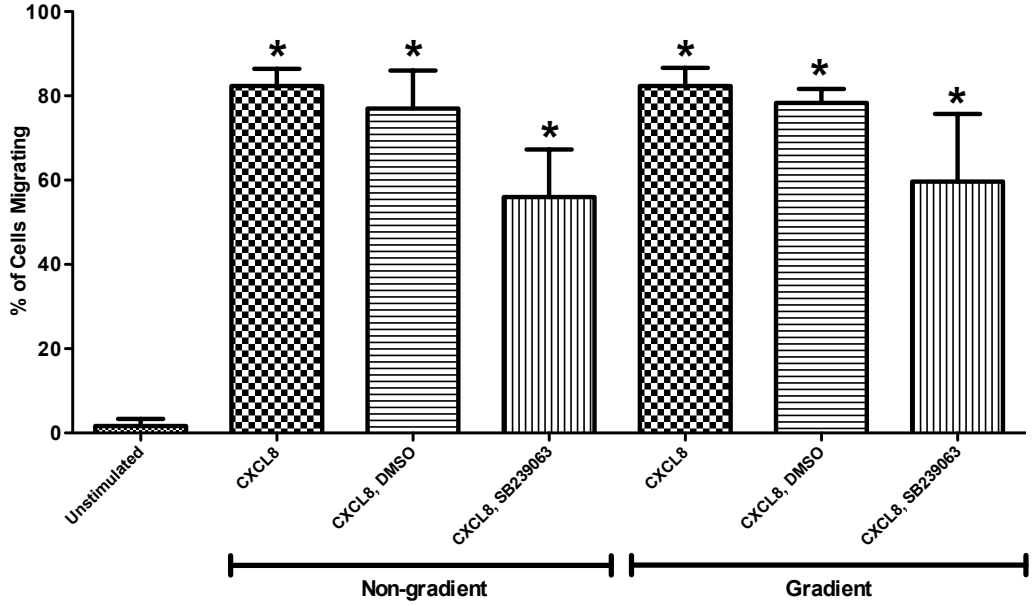
Pre-treatment with the PD98059 inhibitor had no effect on the directionality of migration (figure 4.8). The vector diagrams illustrate that following pre-treatment with PD98059, prior to stimulation with CXCL8, in the non-gradient assay the neutrophils responded in a chemokinetic manner (figure 4.8a). In the gradient assay, neutrophils maintained chemotactic migration in response to CXCL8 stimulation, even when exposed to the PD98059 inhibitor (figure 4.8b).

In both the non-gradient and gradient assays, stimulation with GM-CSF induced a significant increase in migration from 1±1% to 82±5% in the non-gradient assay and 50±11% in the gradient assay (figure 4.7b). The vehicle control for PD98059 had no significant effect on neutrophil migration induced by GM-CSF in both the non-gradient and gradients assay (figure 4.7b). Treatment with the inhibitor PD98059 (50μM, 30 minutes pre-treatment) caused a significant reduction in the proportion of cells migrating in the non-gradient (59±19%) and gradient assays (12±4%, figure 4.7b).

Phosphorylated-ERK was used to determine if the PD98059 inhibitor is capable of inhibiting ERK activation. The blot and densitometry results both show that stimulation of neutrophils with CXCL8 and GM-CSF increased the phosphorylation of ERK, although this was not significant (figure 4.9). Phosphorylation in response to treatment with GM-CSF was unaffected by treatment with the vehicle control for PD98059 (0.07% DMSO, figure 4.9). Treatment with the inhibitor PD98059 (50μM, 30 minutes pre-treatment) reduced the phosphorylation of ERK. However, this was not significant (figure 4.9). ERK was used as a control and showed that the loading of each well was

approximately the same so the effects seen with the Phosphorylated-ERK are due to differences in the stimulation and activation not in the quantity of cells loaded.

a)



b)

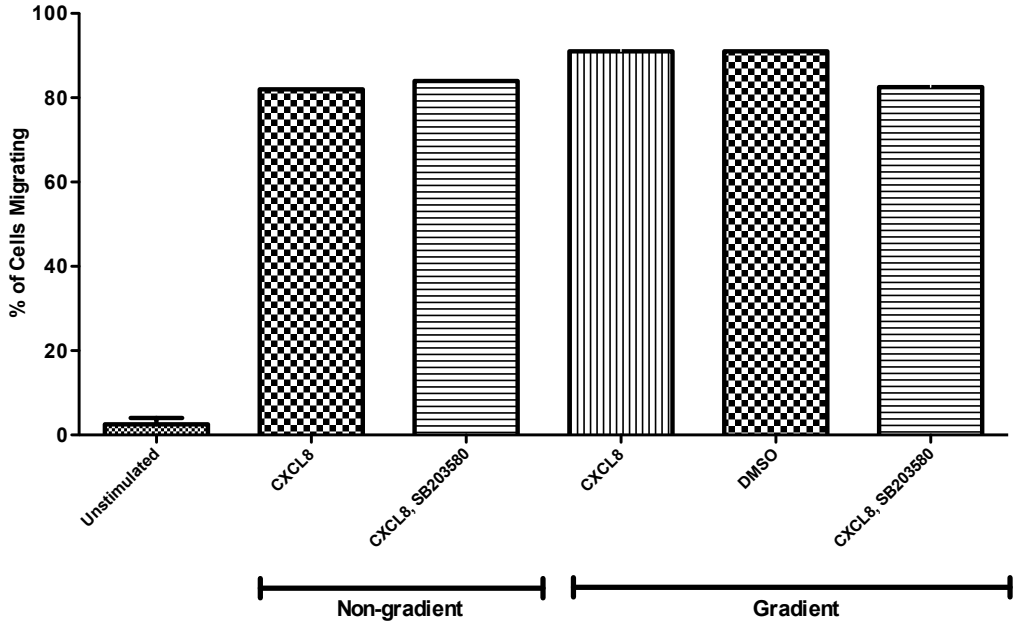
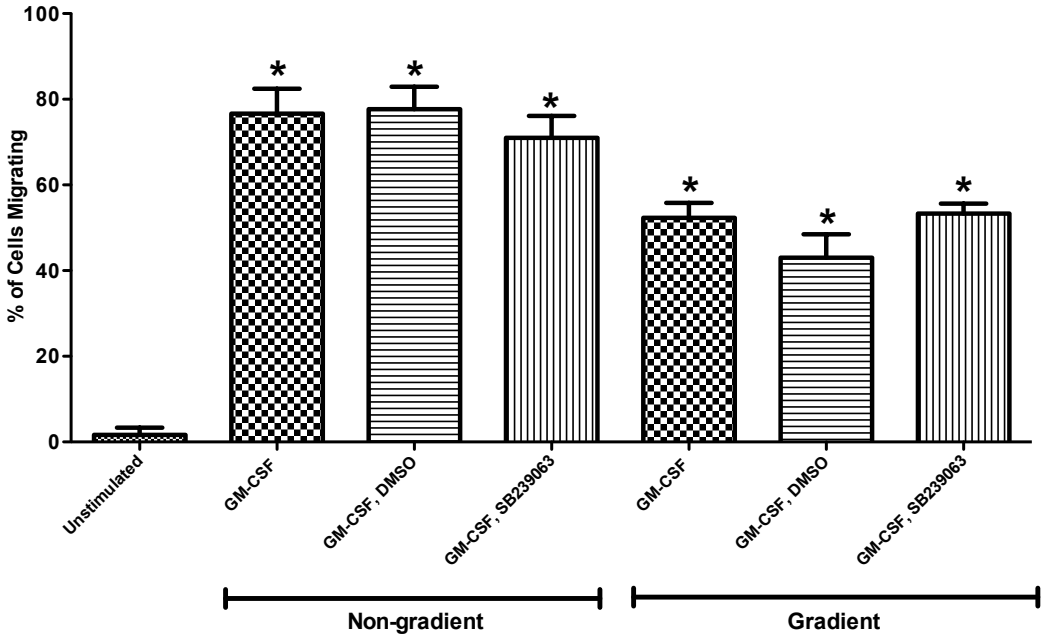


Figure 4.3. The p38MAPK inhibitors SB239063 and SB203580 have no effect on CXCL8-induced neutrophil migration. Neutrophils were treated for 30 min with 10μM of either SB239063 (a) or SB203580 (b) prior to stimulation with 100ng/ml CXCL8. Data are shown as mean ±SEM (n=3 for SB239063 and n=1 for SB203580). *Significantly greater than unstimulated cells, $p<0.01$.

a)



b)

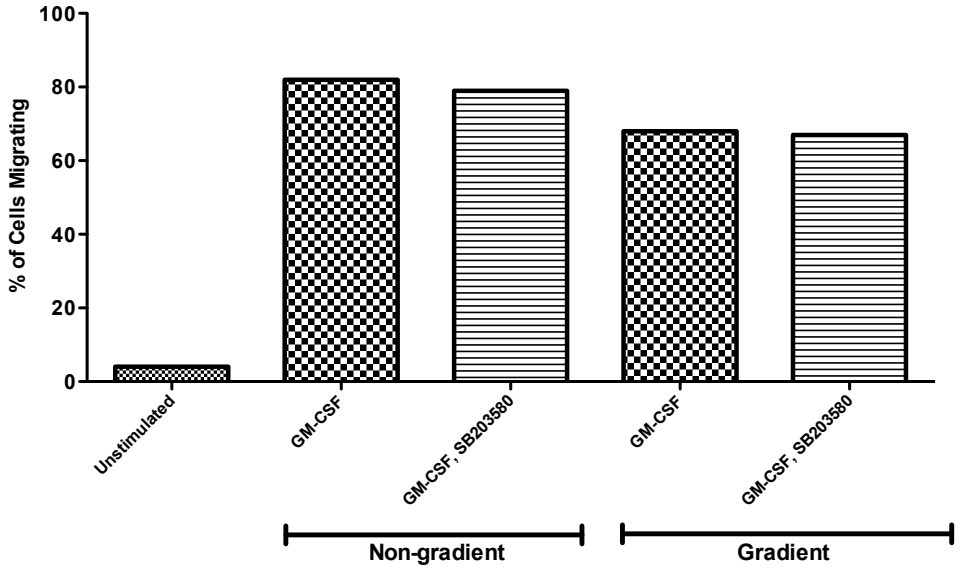


Figure 4.4. The p38MAPK inhibitors SB239063 and SB203580 have no effect on GM-CSF-induced neutrophil migration. Neutrophils were treated for 30 min with 10μM of either SB239063 (a) or SB203580 (b) prior to stimulation with 50ng/ml GM-CSF. Data are shown as mean ±SEM (n=3 for SB239063 and n=1 for SB203580). *Significantly greater than unstimulated cells, $p<0.001$.

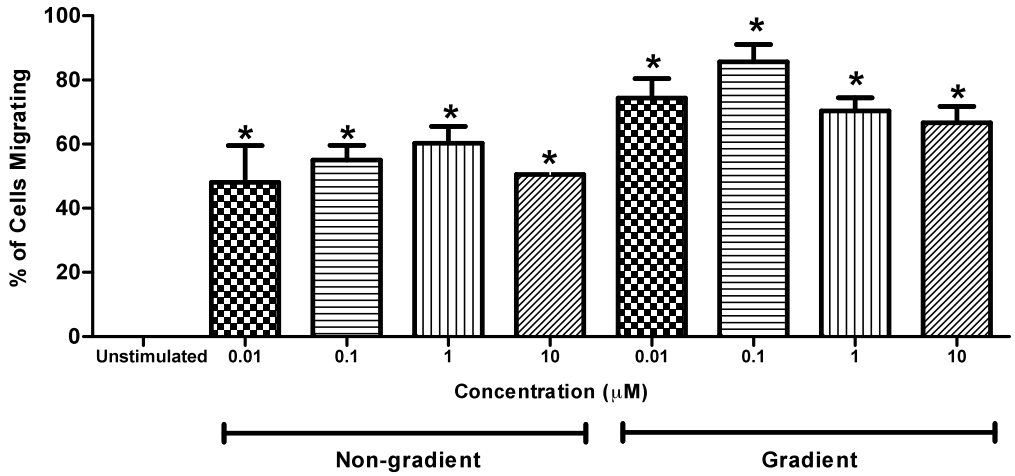


Figure 4.5. The percentage of neutrophil migration with increasing concentrations of fMLP in both the non-gradient and gradient assay. *Significantly greater as compared to unstimulated cells, $p<0.01$, $n=3$.

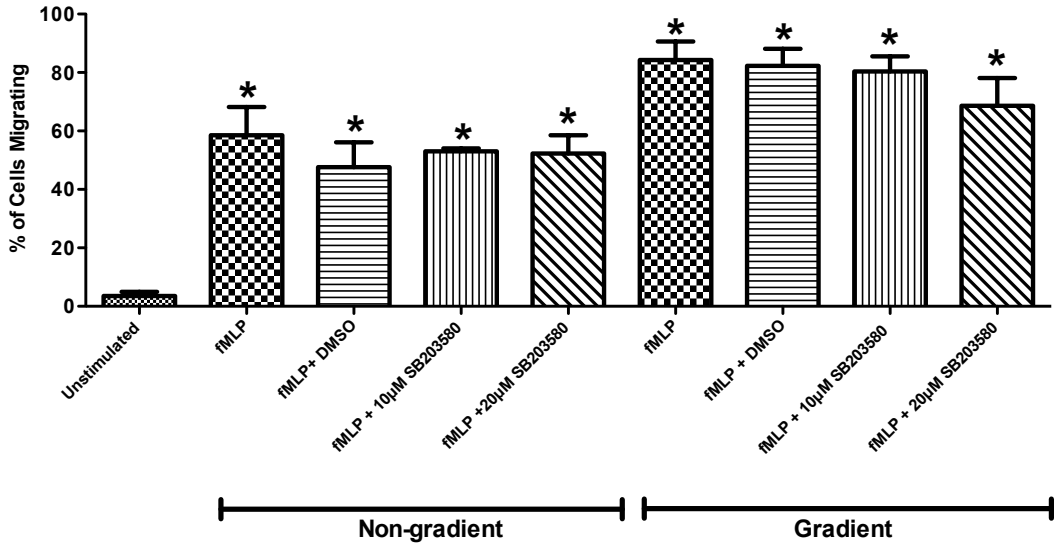


Figure 4.6. The p38MAPK inhibitor SB203580 has no effect on fMLP-induced neutrophil migration. Neutrophils were treated for 30 min with 10μM of SB203580 prior to stimulation with 0.1μM fMLP. Data are shown as mean ±SEM (n=3). *Significantly greater than unstimulated cells, $p<0.05$.

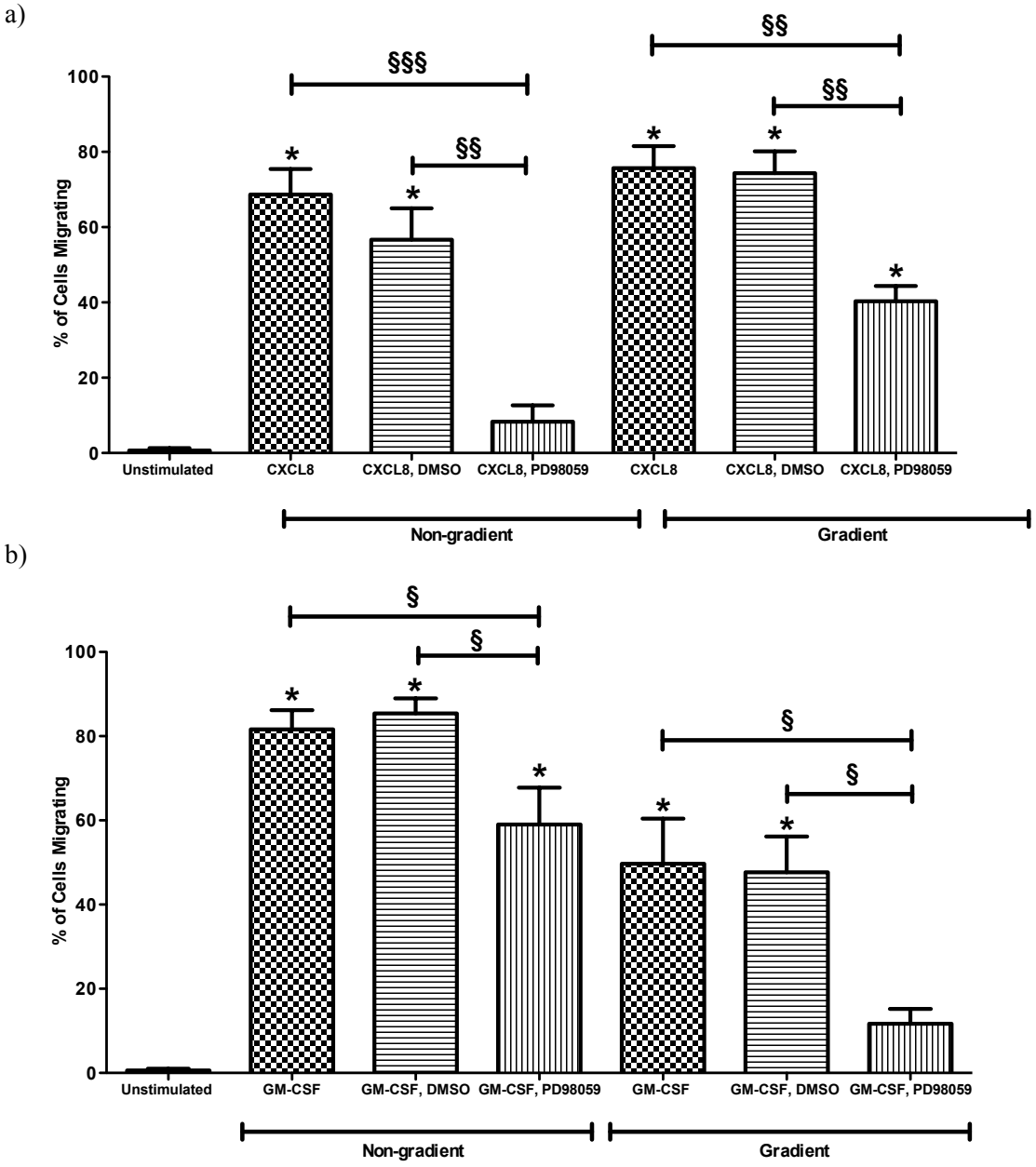
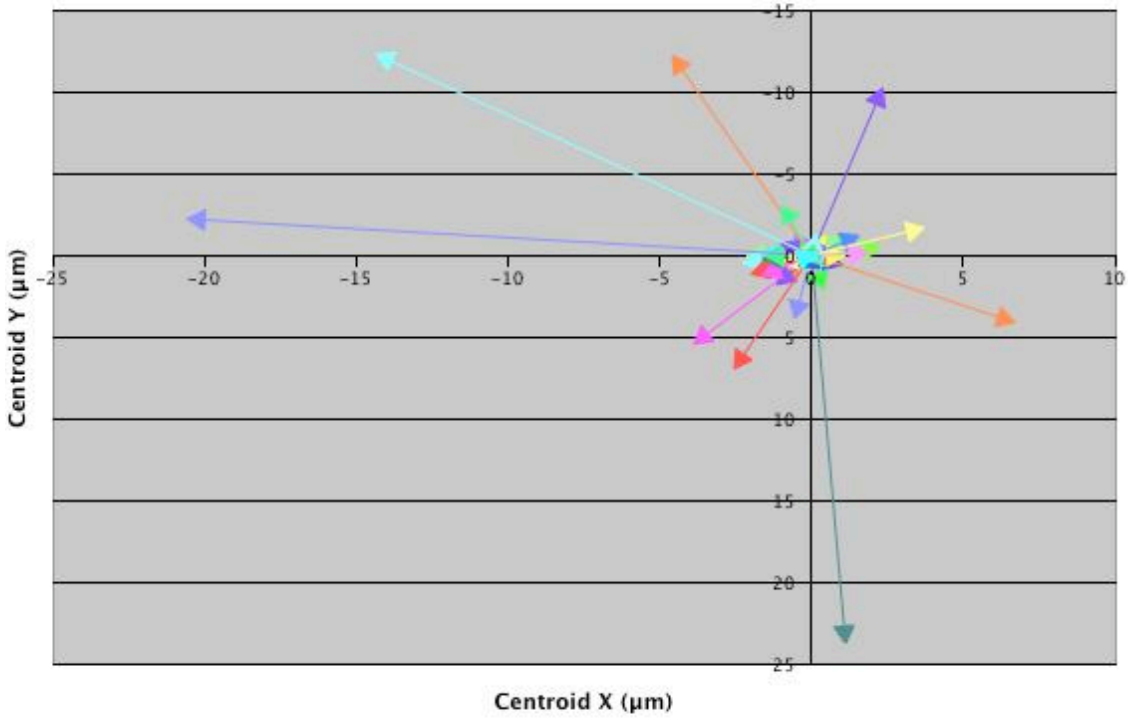


Figure 4.7. The MEK inhibitor PD98059 inhibits CXCL8 and GM-CSF-induced neutrophil migration. Neutrophils were pre-treated for 30 min with 50μM of PD98059 prior to the addition of 100ng/ml CXCL8 or 50ng/ml GM-CSF. Migration gels were then carried out. Results are shown as mean ±SEM (n=3), except for non-gradient GM-CSF where n=5. *Significantly greater as compared to unstimulated cells, $p<0.01$. §Significantly inhibited by PD98059 ($^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$).

a)



b)

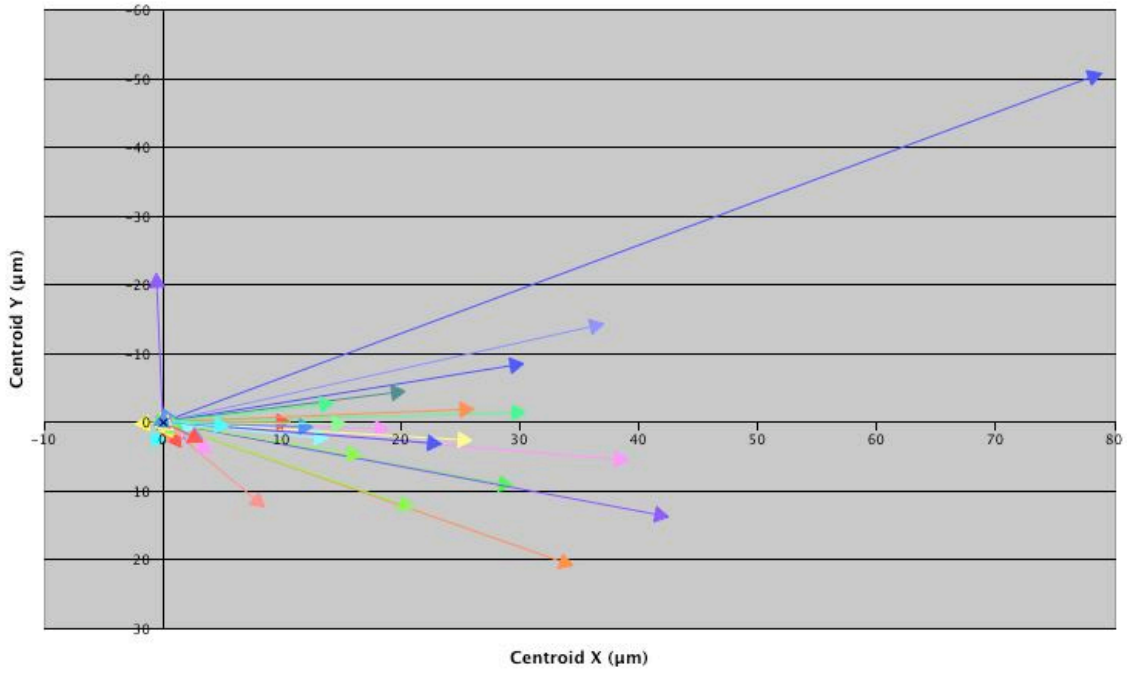
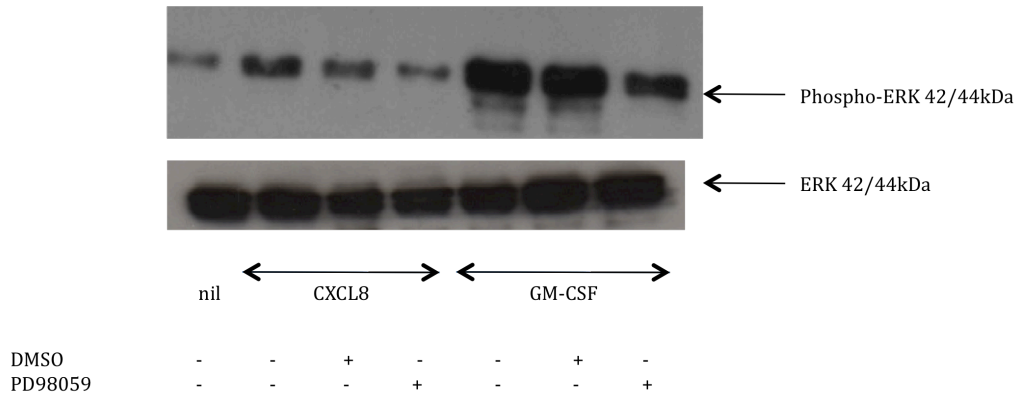


Figure 4.8. Vector diagrams illustrating the direction of migration for each neutrophil, following pre-treatment with PD98059 and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b). The diagrams shown represent the pattern seen for all repeated experiments.

a)



b)

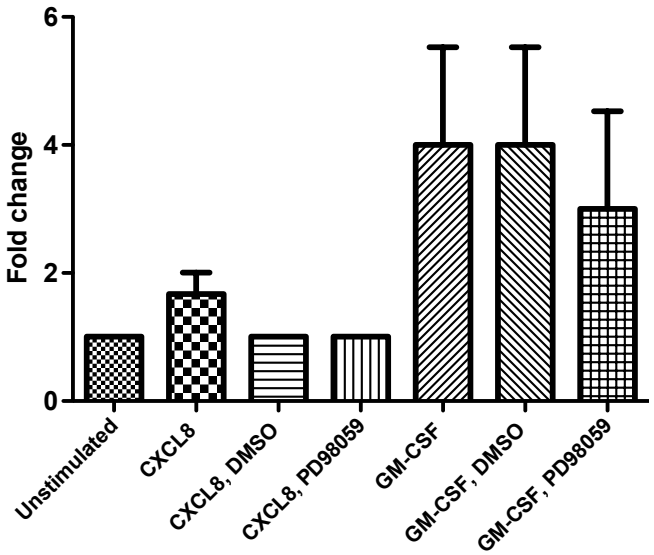


Figure 4.9. The MEK inhibitor PD98059 inhibits phosphorylation of ERK. Neutrophils were pre-treated for 30 min with 50μM of PD98059 prior to the addition of 100ng/ml CXCL8 or 50ng/ml GM-CSF, cells were then solubilised and western blots were carried out. a) shows an example of 1 of the 3 blots carried out, b) shows the densitometry on the 3 blots

4.4 The effect of Y27632 on CXCL8- and GM-CSF-induced neutrophil migration

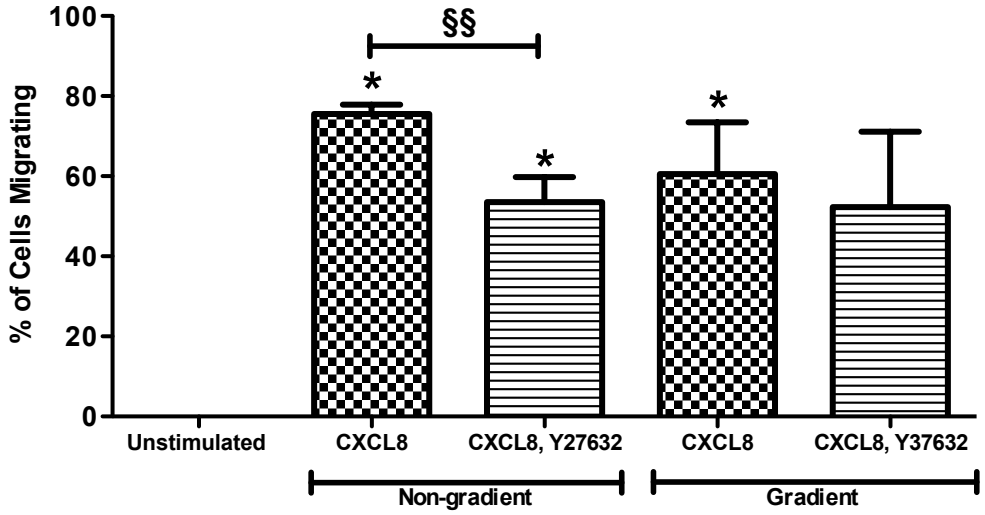
The inhibitor Y27632 was used to determine the role of ROCK in CXCL8- and GM-CSF-induced neutrophil migration.

Stimulation with CXCL8 significantly increased the percentage of neutrophil migration from 0% to 76±2% in the non-gradient assay and 61±13% in the gradient assay (figure 4.10a). In the non-gradient assay, treatment of cells with Y27632 (10μM, 30 min pre-treatment) significantly reduced migration to 54±6% but had no significant effect on migration in the gradient assay.

Pre-treatment with Y27632 had no effect on the direction of migration demonstrated by neutrophils when stimulated with CXCL8 alone as highlighted by the vector diagrams. In the presence of Y27632, neutrophils maintained chemokinetic migration in the non-gradient assay (figure 4.11a) and chemotactic migration in the gradient assay (figure 4.11b).

In both the non-gradient and gradient migration assays GM-CSF significantly increased the proportion of cells migrating from 0% to 90±2% in the non-gradient assay and 47±7% in the gradient assay (figure 4.10b). Treatment of cells with Y27632 (10μM, 30 min pre-treatment) significantly inhibited migration in the non-gradient assay to 72±2% but had no effect on migration in the gradient assay.

a)



b)

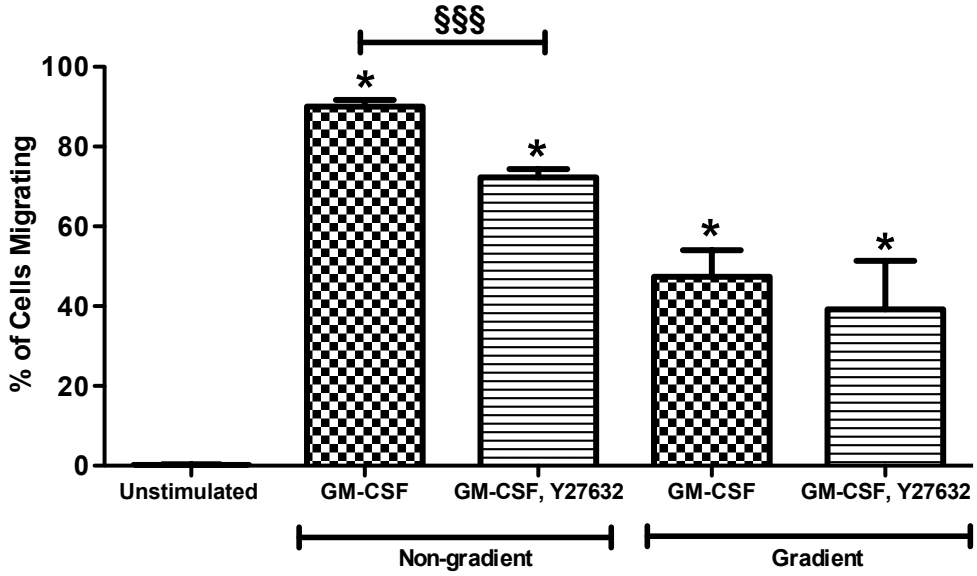
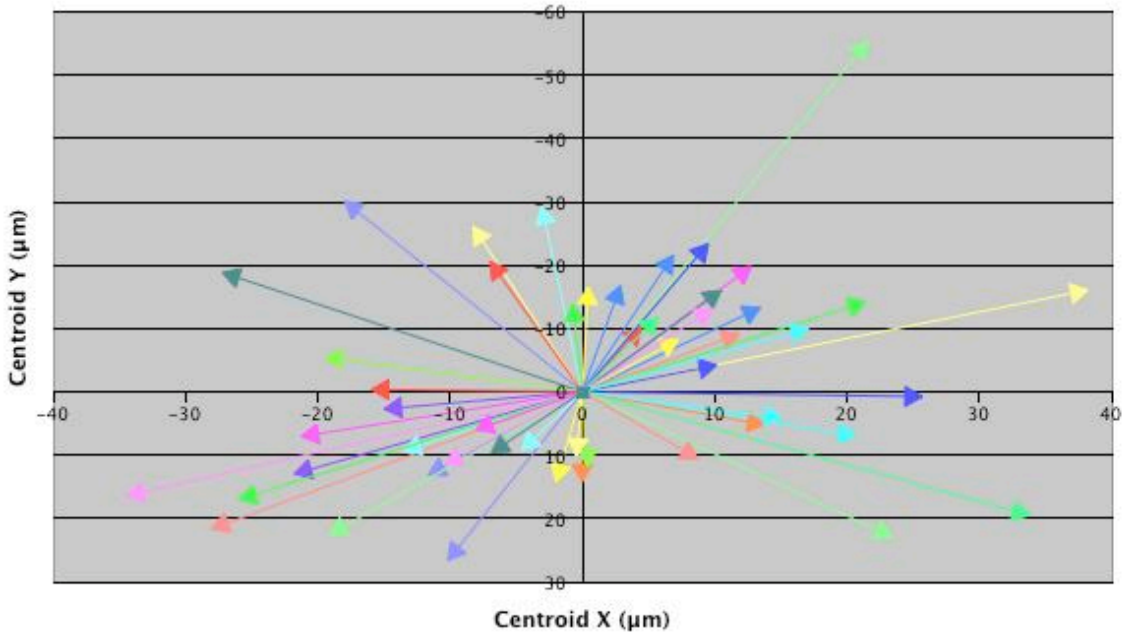


Figure 4.10. The ROCK inhibitor Y27632 inhibits non-gradient migration but not gradient migration in response to stimulation with CXCL8 and GM-CSF. Neutrophils prior to stimulation were treated for 30 min with 10μM Y27632. Cells were stimulated with 100ng/ml CXCL8 or 50ng/ml GM-CSF. Results are shown as mean ±SEM (n=4), except for gradient GM-CSF where n=5. *Significantly greater as compared to unstimulated cells, $p<0.05$. §Significantly inhibited by Y27632 ($^{§§}p<0.01$,$^{§§§}p<0.001$).

a)



b)

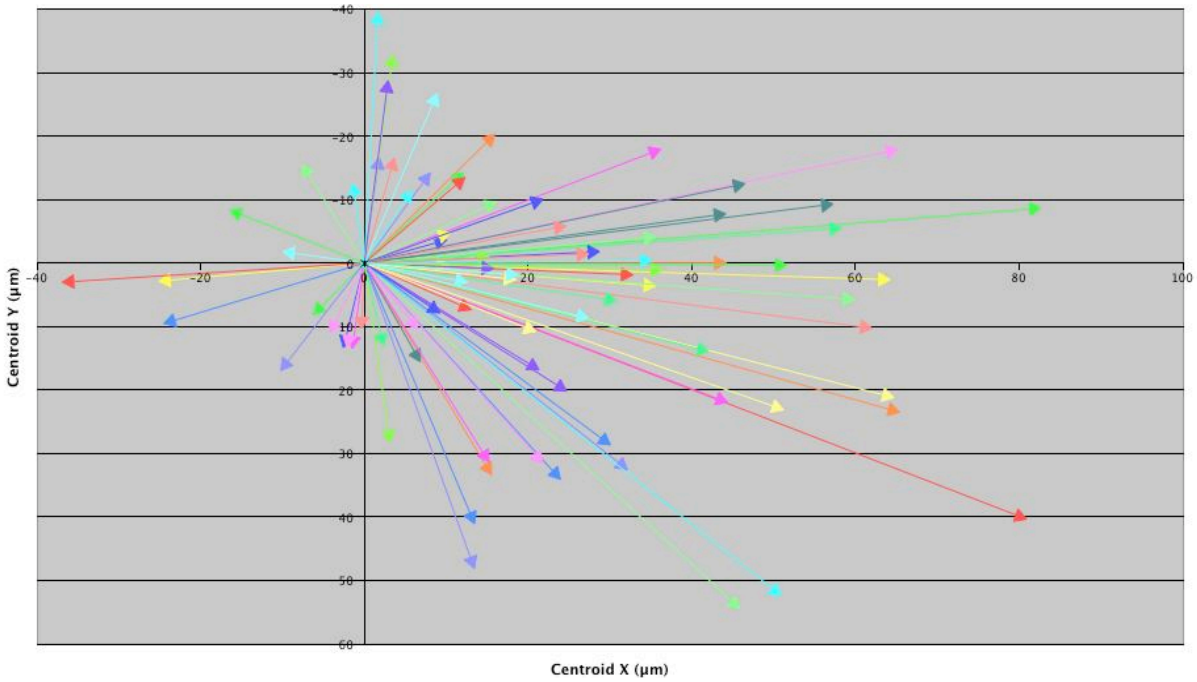


Figure 4.11. Vector diagrams illustrating the direction of migration for each neutrophil, following pre-treatment with Y27632 and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b). The diagrams shown represent the pattern seen for all repeated experiments.

4.5. Wortmannin inhibits neutrophil migration

The non-selective PI3-kinase inhibitor wortmannin was used to evaluate the role of PI3-kinases in CXCL8 and GM-CSF-induced neutrophil migration.

In both the non-gradient and gradient migration assays, CXCL8 significantly increased the proportion of cells migrating from 2% to approximately 70% (figure 4.12a). The vehicle control for wortmannin (0.3% DMSO) had no significant effect on neutrophil migration induced by CXCL8 in either the non-gradient (57±3%) or gradient assay (79±3%, figure 4.12a). In both assays, the treatment of cells with wortmannin (50nM, 30 min pre-treatment) significantly reduced migration to approximately 20%.

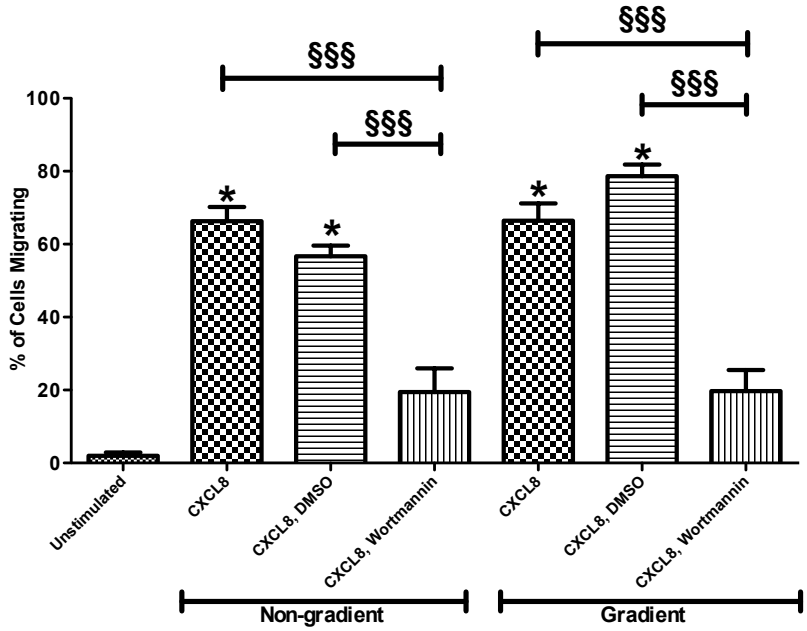
Although the percentage of cells migrating was reduced, the ability of the neutrophils that were still able to migrate to sense a chemoattractant gradient and migrate chemotactically in the gradient assay, in response to stimulation with CXCL8, was unaffected by pre-treatment with wortmannin (figure 4.13b). This was also the case for the neutrophils migrating chemokinetically in response to stimulation with CXCL8 in the non-gradient assay (figure 4.13a).

In both the non-gradient and gradient migration assays, GM-CSF significantly increased the percentage of cells migrating from 2±1% to 93±1% in the non-gradient assay in response to stimulation with 0.5ng/ml GM-CSF, 73±3% in the non-gradient assay in response to stimulation with 50ng/ml GM-CSF and 47±5% in the gradient assay (figure 4.12b). The vehicle control for wortmannin (0.3% DMSO) had no significant effect on neutrophil migration induced by GM-CSF, in either the non-gradient (77±4%, 65±2%) or gradient assay (56±4%, figure 4.12b). In the non-gradient assay, treatment of cells with wortmannin (50nM, 30 min pre-treatment) significantly reduced migration in response to stimulation with 0.5ng/ml GM-CSF to 10±7% but had

no significant effect on migration in response to stimulation with 50ng/ml GM-CSF. In the gradient assay, migration was significantly reduced to 24±6%.

The effect of wortmannin was examined in relation to migration in the Boyden chamber. Stimulation of neutrophils with CXCL8 increased the number of cells migrating from 8±2 cells/high powered field to 53±7 cells/high powered field (figure 4.14). Treatment of the neutrophils for 90 minutes with 50nM of wortmannin or the vehicle control for wortmannin (0.3% DMSO) prior to stimulation with CXCL8 had no effect on the cell number/HPF (figure 4.14). Treatment with 100nM of wortmannin caused a reduction in migration compared to the vehicle control (0.3% DMSO, from 53±7 cells/HPF to 40±3 cells/HPF, figure 4.14) however, this was not significant.

a)



b)

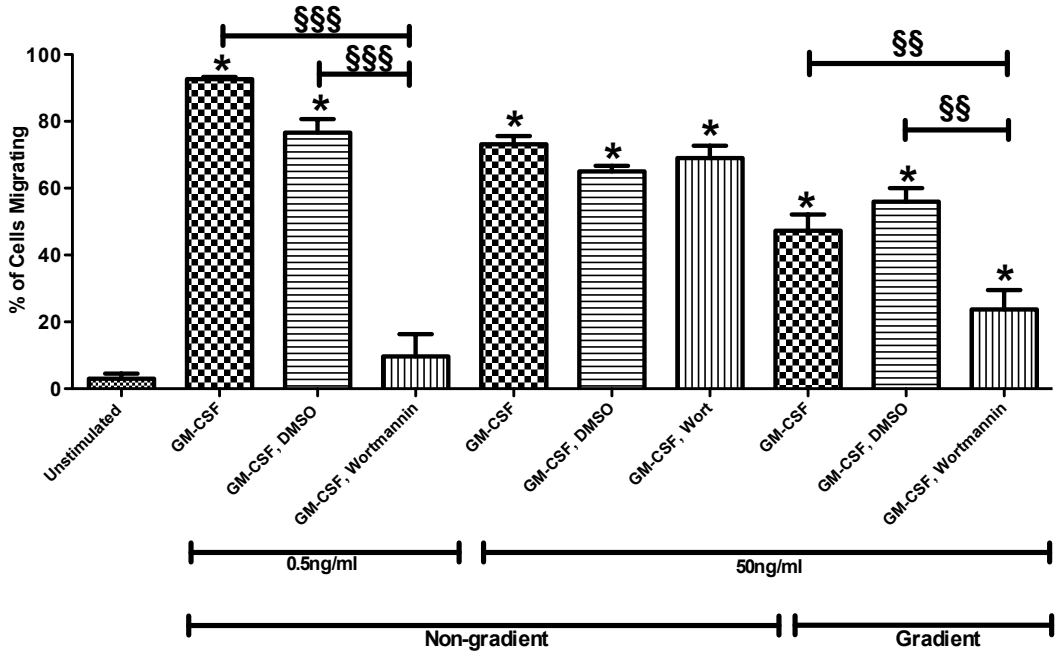
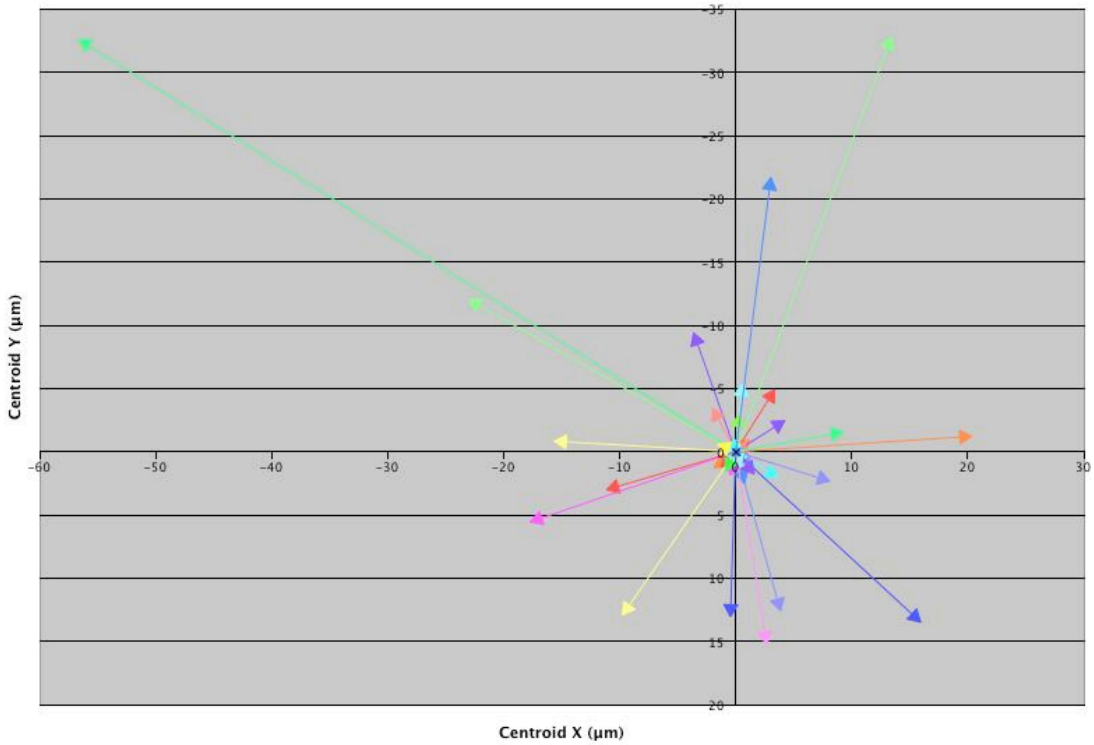


Figure 4.12. Wortmannin inhibits CXCL8- and GM-CSF-induced neutrophil migration. Neutrophils were pre-treated for 30 min with 50nM wortmannin and then stimulated with a) CXCL8 (100ng/ml) or b) GM-CSF (0.5ng/ml, 50ng/ml). Results are shown as mean ±SEM (n=7) except for DMSO controls where n=3. *Significantly greater as compared to unstimulated cells, $p<0.01$. §Significantly inhibited by Y27632 ($^{\S\S}p<0.01$,$^{\S\S\S}p<0.001$).

a)



b)

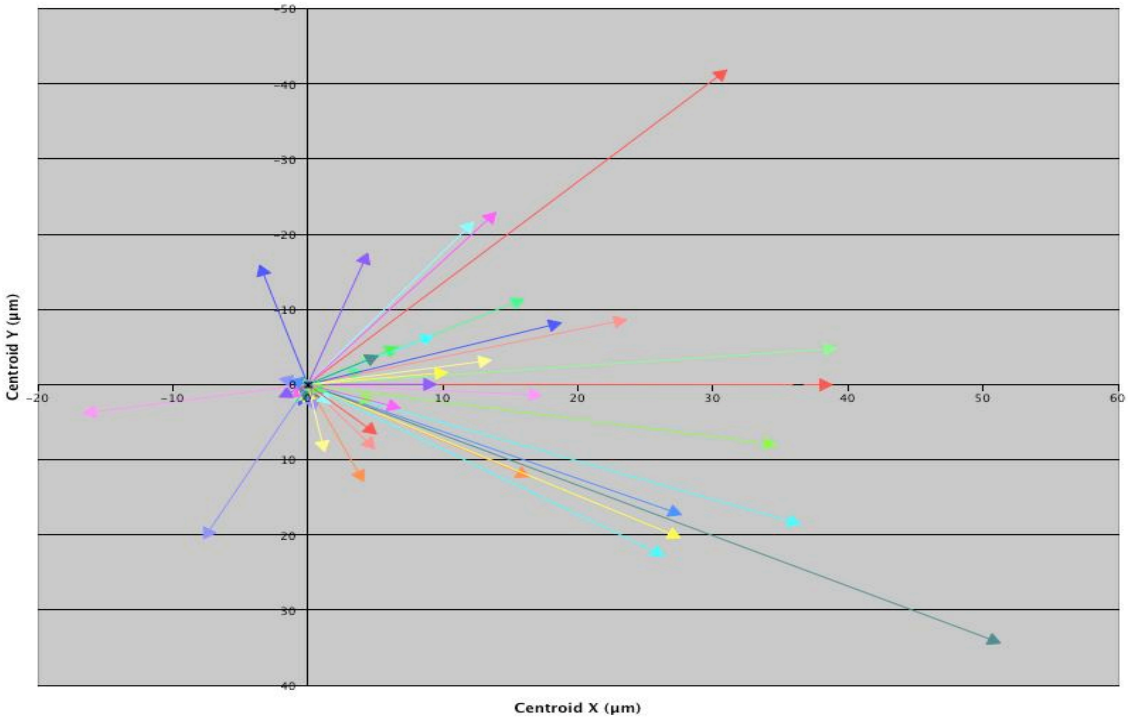


Figure 4.13. Vector diagrams illustrating the direction of migration for each neutrophil, following pre-treatment with wortmannin and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b). The diagrams shown represent the pattern seen for all repeated experiments.

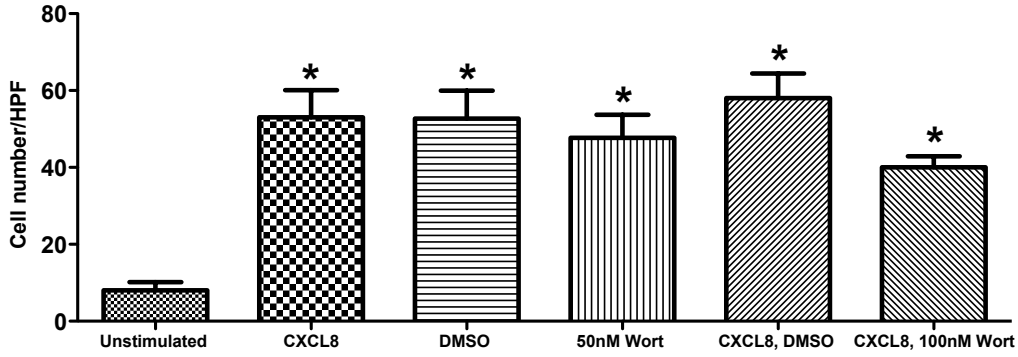


Figure 4.14. Effect of wortmannin on CXCL8-induced neutrophil migration in the Boyden chamber assay. Following pre-treatment the bottom wells of the Boyden chamber were filled with PBS or CXCL8 (100ng/ml). The membrane was placed on top as detailed in the methods section and pre-treated and control neutrophils were added to the upper wells. Results are shown as mean ±SEM (n=3). *Significantly greater as compared to unstimulated cells, $p<0.05$.

4.6 Discussion

Chemokine receptors belong to the GPCR family and are vital for the initiation of migration induced by a chemokine. CXCL8 can be recognised by the chemokine receptors CXCR1 and CXCR2 on the surface of a neutrophil, initiating cell migration. This was highlighted in the results as treatment of neutrophils with pertussis toxin prior to stimulation with CXCL8 in both the non-gradient and gradient assays almost completely abolished neutrophil migration. Pertussis toxin catalyzes the ADP-ribosylation of the Gα subunit, which prevents the G protein heterodimers from interacting with the receptor; this in turn blocks activation of the receptor and downstream effectors (Ribeiro-Neto and Rodbell 1989). GPCRs are not thought to control the chemotactic compass of the neutrophils as they are uniformly distributed along the plasma membrane (Servant et al. 1999; Merlot and Firtel 2003). This was shown in the vector diagrams of the migration in response to CXCL8. Pre-treatment with the inhibitor did not alter the normal migration pattern of the neutrophils still able to migrate in the non-gradient and gradient assay. Therefore, GPCRs are not responsible for controlling directional migration, but are required for the initiation of pathways at the front and rear of the cell.

Pertussis toxin failed to inhibit GM-CSF induced migration in both assays; this was expected as the GM-CSF receptor also known, as the Cluster of Differentiation 116 does not belong to the GPCR family. The receptor is a heterodimer consisting of an α chain (the binding site for GM-CSF), and a β chain, which activates signal transduction (Geijsen et al. 2001).

The MAPK pathway is an important signalling cascade activated in human neutrophils (Kutsuna et al. 2004). The MAPK subtypes MEK-ERK and MKK3/6-p38MAPK have

been suggested as the two subtypes responsible for human neutrophil functions. Although p38MAPK can be activated by stimulation with CXCL8, the p38MAPK inhibitor, SB203580, has been shown previously to only have a minor effect on chemotaxis to CXCL8 in both the Boyden chamber and under agarose assay (Knall et al. 1997; Heit et al. 2002; Heit et al. 2008). The results from our 3D collagen migration assay confirmed this, as CXCL8-induced migration was not significantly reduced in response to the p38MAPK inhibitors SB203580 and SB239063. Therefore, p38MAPK does not play a role in CXCL8-induced chemokinesis and chemotaxis. In the case of growth factor signalling, G-CSF was demonstrated previously to induce neutrophil migration in the Boyden chamber, although this migration was not inhibited with the p38MAPK inhibitor SB203580 (Nakamae-Akahori et al. 2006). In agreement with this GM-CSF-induced migration in our 3D migration assay was also not inhibited by pre-treatment with SB203580 and in addition pre-treatment with SB239063. Therefore, signalling via p38MAPK is not required for growth factor -induced chemokinesis.

In the under agarose migration assay fMLP-induced neutrophil migration is dependent on p38MAPK, as shown with the inhibitor SB203580 (Heit et al. 2002; Heit et al. 2008). As the SB203580 inhibitor failed to inhibit CXCL8- and GM-CSF-induced migration in our 3D migration assay, the effectiveness of the inhibitor was assessed using fMLP- stimulated migration. The inhibitor also failed to inhibit fMLP-induced migration in our 3D migration assay. As the fMLP experiments were carried out to provide a positive control further experimental work would need to be carried out to determine if the inhibitor was active. Firstly, as the dependence of p38MAPK for fMLP stimulated migration was shown by one group in the under agarose assay, the experiment could be repeated in our laboratory using the under agarose assay to determine if the results were reproducible and whether the difference seen is due to the

assay used. Secondly, western blotting with an anti-phosphorylated-p38MAPK antibody could be carried out. As, if the inhibitor significantly decreased the phosphorylation of p38MAPK it would confirm the inhibitor was active.

As CXCL8- and GM-CSF-induced migration occur independently of p38MAPK, we considered whether they are dependent on ERK signalling. The involvement of ERK in neutrophil migration has been disputed in previous studies, with migration shown to be both dependent and independent of ERK. To determine the role of ERK in cellular functions, inhibitors against the protein kinase kinase MEK are most commonly used. MEK signals directly upstream of ERK, meaning inhibition of MEK would prevent the activation of ERK and its downstream signalling pathway.

G-CSF-induced migration in the Boyden chamber and phosphorylation of ERK in western blots, have both been shown to be inhibited when cells were pre-treated with the MEK inhibitor PD98059 (Nakamae-Akahori et al. 2006). In contrast, in CXCL8-induced chemotaxis in the Boyden chamber, pre-treatment with PD98059 has been shown to have no effect on either migration. This lead to the conclusion that, CXCL8-induced migration occurs independently of ERK (Knall et al. 1997).

Using the 3D migration assay, we showed that CXCL8-induced migration was inhibited by PD98059. Chemokinesis in the non-gradient 3D assay was almost completely abolished by pre-treatment with the inhibitor. Whereas, in contrast, chemotaxis in the gradient assay saw a 50% reduction in migration, following pre-treatment with the inhibitor. The 50% that migrated in the presence of the inhibitor continued to migrate chemotactically in the gradient assay suggesting that ERK is does not control the directional migration of neutrophils. Taken together with the Boyden chamber results

presented in the previous study, there may be a differential requirement for ERK depending on whether migration is chemokinetic or chemotactic.

In the case of GM-CSF, a significant reduction in migration occurred in both assays, indicating the importance of ERK in chemokinesis.

CXCL8 and GM-CSF both caused the phosphorylation of ERK. However, from the densitometry results this was not significant. In the case of CXCL8, the phosphorylation was reduced with both the DMSO vehicle control and the MEK inhibitor PD98059. The phosphorylation shown with GM-CSF was not affected by the DMSO vehicle control but was reduced with the PD98059 inhibitor. The aim of the phosphorylated-ERK western blots was to determine the activity of the inhibitor and to show that ERK is downstream of MEK. However, the western blot results do not achieve this. Although the phosphorylated-ERK western blot was repeated three times with three different donor, the error bars were large so more repeats are necessary. In addition, the presence of DMSO appears to be affecting the phosphorylation of ERK. This could be a result of the length of time the neutrophils were exposed to the DMSO and/or the concentration of DMSO present. In these western blots, the neutrophils were exposed to the DMSO for 90 minutes to simulate the 3D migration assay experiments. However, in future western blots with a phosphorylated- Akt antibody, shown in chapter 5, the cells were only exposed to DMSO for 2 minutes and this did not appear to affect the phosphorylation when using the DMSO as vehicle control.

In summary, in the 3D migration assay, CXCL8-induced and GM-CSF-induced chemokinesis are dependent on ERK, whereas in contrast CXCL8-induced chemotaxis is only partially dependent on ERK.

The RhoA-ROCK pathway has been shown to be vital in the detachment of the uropod in neutrophils, with authors reporting a reduction in fMLP-induced neutrophil polarity, chemokinesis and chemotaxis in the absence of ROCK and Rho (Niggli 1999; Alblas et al. 2001). Our results suggest that involvement of the pathway may not be as necessary as previously thought. As, only a small albeit significant reduction in the percentage of migration occurred in the non-gradient assay when cells were treated with Y27632, an inhibitor of ROCK, prior to stimulation with CXCL8 or GM-CSF. In the gradient assay, the percentage and direction of migration induced by both CXCL8 and GM-CSF was not affected by treatment with Y27632. In agreement with these results, GM-CSF-induced migration in eosinophils has been shown to be only partially dependent on ROCK and independent of Rho (Muessel et al. 2008).

A recent study has highlighted a differential requirement for integrin and myosin in leucocyte migration, depending on whether the cells are moving on 2D substrates compared to 3D gels. In 2D migration, use of the inhibitor Y27632 blocks rear retraction resulting in the trailing edge tethering to the substrate, so although the leading edge of the cell protrudes forward it is held in place by the uropod (Lammermann et al. 2008). In contrast, when cells were treated with Y27632 in a 3D gel tethering did not occur suggesting that myosin is not important for migration (Lammermann et al. 2008). In 3D environments it is thought that the protrusion of the F-actin is sufficient to cause rapid cell migration and this is particularly the case when the cells encounter large pores in the gel (Lammermann et al. 2008). However, in narrow pores within a 3D gel, leucocytes need myosin to contract the rear of the cell to propel the cell forward (Lammermann et al. 2008). Therefore, leucocytes migrate via a ‘flowing and squeezing method’ (Lammermann at al. 2008). In the context of our neutrophil results, the migration within the 3D collagen gel is most likely flowing with the gel containing

larger pores, enabling the neutrophils to migrate without the need for myosin and its upstream effector ROCK.

PI3-Kinase has been shown to be an important component of the intracellular signalling pathways controlling neutrophil migration, with authors reporting a significant inhibition of migration, in response to pre-treatment with the pan-PI3-Kinase inhibitor wortmannin (Knall et al. 1997; Heit et al. 2002; Ferguson et al. 2007; Brkovic et al. 2007). In these studies pre-treatment with wortmannin substantially reduced the number of cells migrating in response to stimulation with CXCL8, whilst having no effect on the speed or direction of migrating neutrophils (Knall et al. 1997; Heit et al. 2002; Ferguson et al. 2007; Brkovic et al. 2007). In agreement with these previous studies, wortmannin caused significant inhibition of CXCL8-induced neutrophil migration in both the non-gradient and gradient assays. However, did not affect the directionality of the neutrophils that were still migrating in the presence of wortmannin, as migration remained chemokinetic in the non-gradient assay and chemotactic in the gradient assay. Taken together this is consistent with the view that PI3-Kinases are important components of chemokine-induced migration.

In our Boyden chamber assays however, there was no inhibition in migration in response to pre-treatment with wortmannin, at both 50nM and 100nM. Although 50nM was sufficient to cause a significant inhibition in our 3D collagen migration assay, in the literature where wortmannin was used in the Boyden chamber 100nM of wortmannin caused a significant reduction in migration (Knall et al. 1997). The difference between these experiments could be the length of time that the cells are pre-treated with wortmannin as to mimic our 3D collagen migration assays the cells were pre-treated with wortmannin for 90 minutes. As wortmannin is unstable in water, the dilution of wortmannin in PBS prior to use in the Boyden chamber may have impacted

on the ability of wortmannin to inhibit migration. Although in the literature presented wortmannin was used, LY294002 is widely used as a PI3-Kinase inhibitor instead of wortmannin, because it is much more stable in solution. Therefore, the use of a reduced incubation period or LY294002 instead of wortmannin could impact on the migration in the Boyden chamber. Furthermore, due to the aqueous instability of wortmannin, it may also be beneficial to use LY294002 to corroborate all the results with both CXCL8 and GM-CSF in the 3D migration assays.

In addition to inhibition of CXCL8-induced migration, wortmannin has also been shown to inhibit G-CSF stimulated migration, which as G-CSF is a growth factor could suggest a role of wortmannin in migration in response to GM-CSF stimulation (Nakamae-Akahori et al. 2006). Indeed, wortmannin strongly inhibited GM-CSF stimulated migration in the gradient assay and sub-optimal concentration (0.5ng/ml) migration in the non-gradient assay indicating a role for the PI3-Kinases in GM-CSF-induced migration. However, wortmannin had no effect on the migration, in the non-gradient assay, elicited by the optimum concentration of 50ng/ml.

As neutrophils share common signalling mechanisms with the amoeba *Dictyostelium discoideum,* many fundamental aspects of neutrophil migration have been derived from *Dictyostelium* (Parent 2003). Recent experimental work has shown, that in *Dictyostelium discoideum* migration in response to stimulation with cAMP at low concentrations, inhibition of either PI3-Kinase or phospholipase A2 (PLA2) inhibits migration (Van Haastert et al. 2007, Chen et al. 2007, Stephens et al. 2008, Veltman et al. 2008). However, at high concentrations both enzymes need to be inhibited to inhibit migration (Van Haastert et al. 2007, Chen et al. 2007, Stephens et al. 2008, Veltman et al. 2008). This has therefore led to the suggestion in *Dictyostelium discoideum* that PI3-Kinase and PLA2 are two redundant mediators of migration, as at high concentrations

of cAMP if one of the mediators is inhibited the cell can still migrate (Van Haastert et al. 2007, Chen et al. 2007, Stephens et al. 2008, Veltman et al. 2008). This could explain why migration is inhibited, in the presence of wortmannin, when cells are stimulated with the suboptimal concentration of GM-CSF (0.5ng/ml), but not inhibited when stimulated with the optimal concentration (50ng/ml) in the non-gradient assay. To test this theory inhibitors of PI3-Kinase and PLA2 could be used in combination prior to stimulation with 50ng/ml GM-CSF. If the migration is inhibited it would show, as is the case for *Dictyostelium discoideum,* that PI3-Kinase and PLA2 are redundant pathways in GM-CSF-induced migration. This could also be the case for CXCL8-induced migration, as a higher concentration than the 100ng/ml used in the experiments, could also prove insensitive to inhibition with wortmannin, this should therefore be examined.

In summary, the results of this chapter show that migration evoked by stimulation with CXCL8 is dependent on the GPCR. PI3-Kinase is vital for CXCL8-induced chemokinesis and chemotaxis and GM-CSF-induced chemokinesis. ERK is important for chemokinesis in response to stimulation with both CXCL8 and GM-CSF but there appears to be only a partial dependence on ERK in CXCL8-induced chemotaxis (Table 4.1). Therefore, as PI3-Kinase plays an essential role in all migration the role of each isoform will be further explored in chapter 5.

Mediator	Migration type	GPCR	P38MAPK	ERK	ROCK	PI3-Kinase
CXCL8	Chemokinesis (non-gradient assay)	+	-	+	**Partially dependent**	+
CXCL8	Chemotaxis (gradient assay)	+	-	**Partially dependent**	-	+
GM-CSF	Chemokinesis	-	-	+	**Partially dependent**	+

Table 4.1 Summary of the key signal transduction events involved in migration in response to stimulation with CXCL8 and GM-CSF. + Pathway involved, - pathway not involved, partially dependent inhibitor of pathway only partially inhibits migration.

Chapter 5: The involvement of class I Phosphoinositide 3-kinases in neutrophil migration

5.1 Introduction

As the use of wortmannin in chapter 4 highlighted an important role for the PI3-Kinases in neutrophil migration, this chapter will examine their role in more detail. Previous studies have suggested that the different class I PI3-Kinase isoforms may have differential roles in neutrophil chemokinesis and chemotaxis. The catalytic isoforms p110γ and p110δ have both been implicated in neutrophil migration. The p110γ isoform has been shown to affect the number of cells able to migrate (chemokinesis), but not the ability of cells to navigate once they are moving. Treatment with a p110γ selective inhibitor significantly reduced the proportion of cells migrating but had no effect on the speed or direction (Ferguson et al. 2007). Whereas, the p110δ isoform has been shown to affect the directional component of chemotaxis, but not random movement (chemokinesis; Sadhu et al. 2003).

As the different isoforms of the class I PI3-Kinases have been suggested to be responsible, in part, for the differences between chemokinesis and chemotaxis, this chapter will examine the different isoforms. This will explore the first hypothesis, that different signal transduction pathways control chemokinetic and chemotactic neutrophil migration. The second hypothesis that growth factor- and chemokine-mediated migration, occur via different signal transduction pathways, could also be a result of the involvement of different class I PI3-Kinase isoforms so will also be examined. In addition, the majority of the γ and δ specific work has been carried using the under-agarose or Boyden chamber migration methods. Therefore, in order to examine the third

hypothesis, that the signal transduction events controlling neutrophil migration are different in a 3D environment compared to a 2D environment, 3D migration assays and Boyden chambers will be studied. This will determine the involvement of the different isoforms of the class I PI3-Kinases and establish if the different isoforms have differential roles depending on the migration assay.

This chapter will examine the Class I PI3-Kinase isoforms α, β, δ and γ and their roles in CXCL8- and GM-CSF-induced neutrophil migration in both the 3D migration assay and Boyden chamber assay. PCR and western blot analysis will also be utilised to examine expression of the different isoforms and to examine the activity of the inhibitors.

5.2 Neutrophils express all the class I PI3-Kinase isoforms

RNA was extracted from unstimulated human neutrophils, primers specific to the class I PI3-Kinase catalytic isoforms α, β, δ and γ were designed and RT-PCR was used to determine their expression (figure 5.1). The isoforms α, δ and γ were expressed in all three experiments using different neutrophil donors. The β isoform was detected in one of the three experiments. From the experiment where all four isoforms were expressed the DNA was extracted from the gel and was sent for sequencing, which confirmed that each band was specific to the isoforms of interest. As the α, δ and γ were always detected their role in neutrophil migration was further investigated.

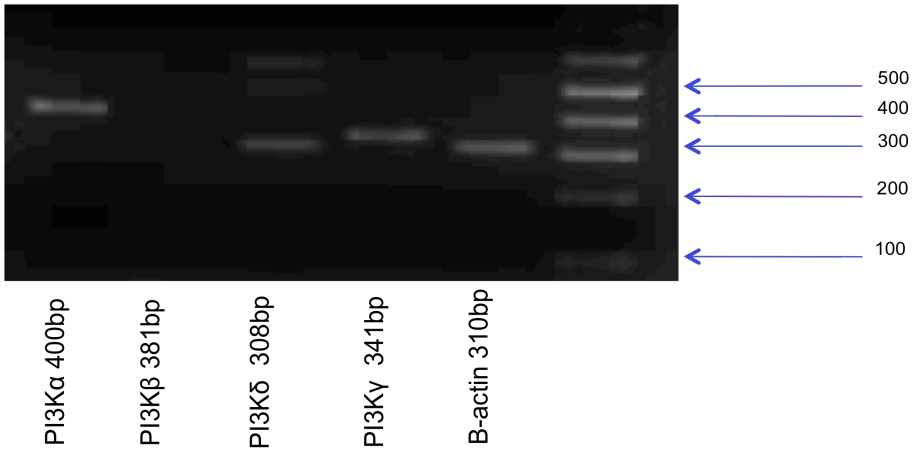


Figure 5.1. Neutrophils express mRNAs for the class I PI3-kinase catalytic subunits α, δ and γ. RNA was isolated from unstimulated neutrophils. RT-PCR was carried out using primers for each of the four classes I PI3-Kinase catalytic isoforms and β-actin as a control. Imagine shown is an example from one of the three experiments carried out.

5.3 The PI3-Kinase α selective inhibitor, PIK-75 inhibits CXCL8-induced neutrophil chemotaxis

The catalytic isoform PI3-Kinase α has not previously been studied in relation to neutrophil migration. As the PI3-Kinase α selective inhibitor, PIK-75 had not been used in neutrophils; firstly, the optimum concentration and incubation time were examined. The concentration and incubation time were studied using migration in the gradient assay in response to stimulation with CXCL8. In both the concentration and time course experiments unstimulated neutrophils failed to migrate; stimulation with CXCL8 increased migration to over 80%. The vehicle controls for the varying concentrations of PIK-75 and the 30 minute and 2 hour time points had no significant effect on neutrophil migration, each maintaining migration at approximately 80%. Pre-treatment for 30 minutes with 0.1μM of PIK-75 prior to stimulation with CXCL8 had no effect on migration. In contrast, pre-treatment with 1μM and 10μM PIK-75 significantly inhibited CXCL8 mediated neutrophil migration to approximately 50% (figure 5.2a). The time course experiments were carried out with 1μM of PIK-75. CXCL8 mediated migration was significantly reduced at both 30 minutes (54±6%) and two hours (30±6%; figure 5.2b). Although two hours caused the greatest reduction in migration a time of 30 minutes was used for all other PI3-Kinase inhibitor experiments, so was therefore used for the remainder of the PI3-Kinase α experiments.

In both the non-gradient and gradient migration assays, CXCL8 significantly increased the proportion of cells migrating from 2% to approximately 80% (figure 5.3a). The vehicle control for PIK-75 (0.4% DMSO) had no significant effect on neutrophil migration induced by CXCL8, in either the non-gradient or gradient assay (figure 5.3a). Treatment of cells with PIK-75 (1μM, 30 min pre-treatment) significantly reduced

CXCL8-induced migration in the gradient assay to 51±4%. However, had no effect on CXCL8-induced migration in the non-gradient assay (67±6%).

Treatment with PIK-75 had no effect on the direction of migration (figure 5.4). The vector diagrams illustrate that in the presence of PIK-75, the neutrophils that were still able to migrate continued to migrate chemokinetically in the non-gradient assay (figure 5.4a) and chemotactically in the gradient assay (figure 5.4b) when stimulated with CXCL8.

In both the non-gradient and gradient migration assays, GM-CSF significantly increased the percentage of cells migrating from 0±1% to 90±3% in the non-gradient assay in response to stimulation with 0.05ng/ml GM-CSF, 86±2% in the non-gradient assay in response to stimulation with 50ng/ml GM-CSF and 61±2% in the gradient assay (figure 5.3b). The vehicle control for PIK-75 (0.4% DMSO) had no significant effect on neutrophil migration, induced by GM-CSF, in either the non-gradient or gradient assay (figure 5.3b). In the both non-gradient and gradient assays, treatment of cells with PIK-75 (1μM, 30 min pre-treatment) had no significant effect on GM-CSF-induced migration (figure 5.3b).

The effect of the PI3-Kinase α selective inhibitor, PIK-75 on migration was examined in the Boyden chamber. Stimulation of neutrophils with CXCL8 increased the migration of cells across the membrane from 8±2 cells/HPF (unstimulated) to 45±8 cells/HPF (figure 5.5). The vehicle control for PIK-75 had no effect on the cell number/HPF, as compared to neutrophils stimulated with CXCL8 (0.4% DMSO, figure 5.4). Treatment of the neutrophils with 1μM of PIK-75 for 90 minutes, prior to stimulation with CXCL8 in the Boyden chamber, caused a significant reduction in the

cell number/HPF compared to the vehicle control (from 41±9 cells/ HPF to 24±6 cells/HPF, figure 5.5).

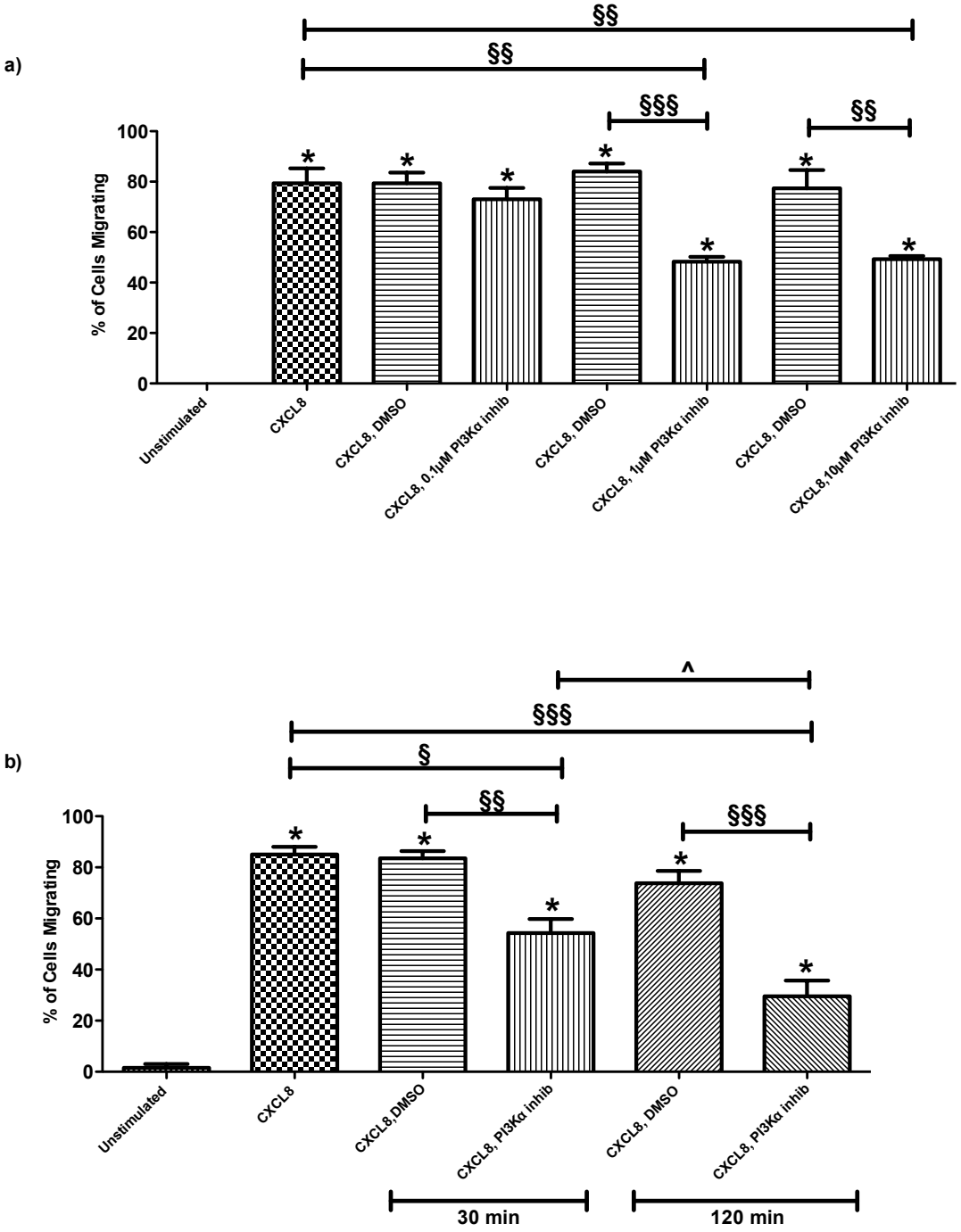


Figure 5.2. An antagonist of PI3-Kinase α inhibits CXCL8-induced chemotactic migration in a dose and time dependent manner in the gradient assay. a) Neutrophils were pre-treated for 30 min with varying concentrations (from 0.1μM to 10μM) of the PI3-Kinase α selective inhibitor PIK75, and then stimulated with 100ng/ml CXCL8. b) Neutrophils were pre-treated with 1μM of PIK-75 for 30 minutes or 2 hours, prior to construction of the migration gel and the addition of 100ng/ml of CXCL8. Results are shown as mean ±SEM; n=4, except for CXCL8, 1μM and the corresponding DMSO control where n=11. *Significantly greater as compared to unstimulated cells, $p<0.01$. §Significantly inhibited by PIK-75 ($^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$). ^Significant difference between two time periods, $p<0.01$.

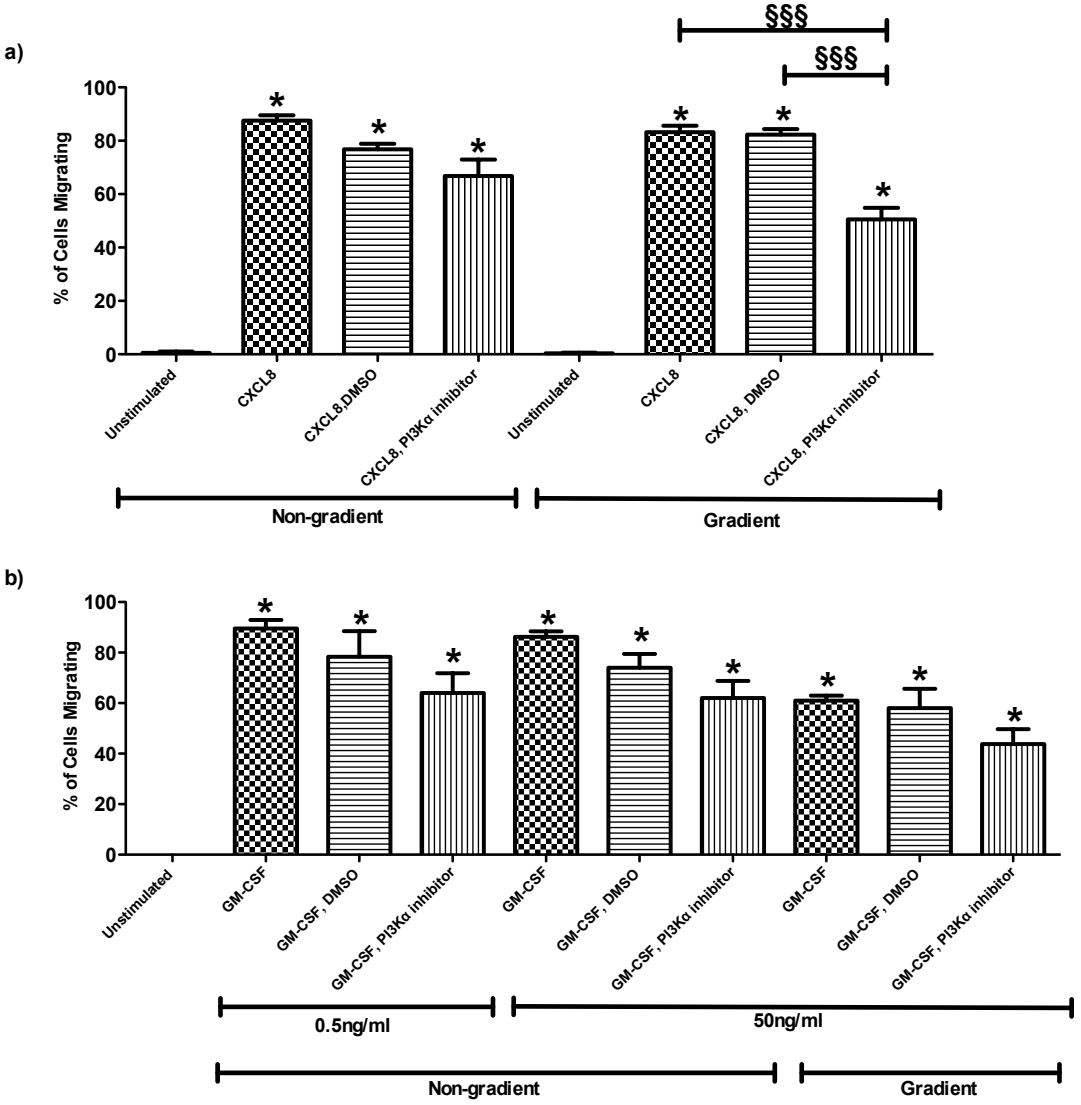
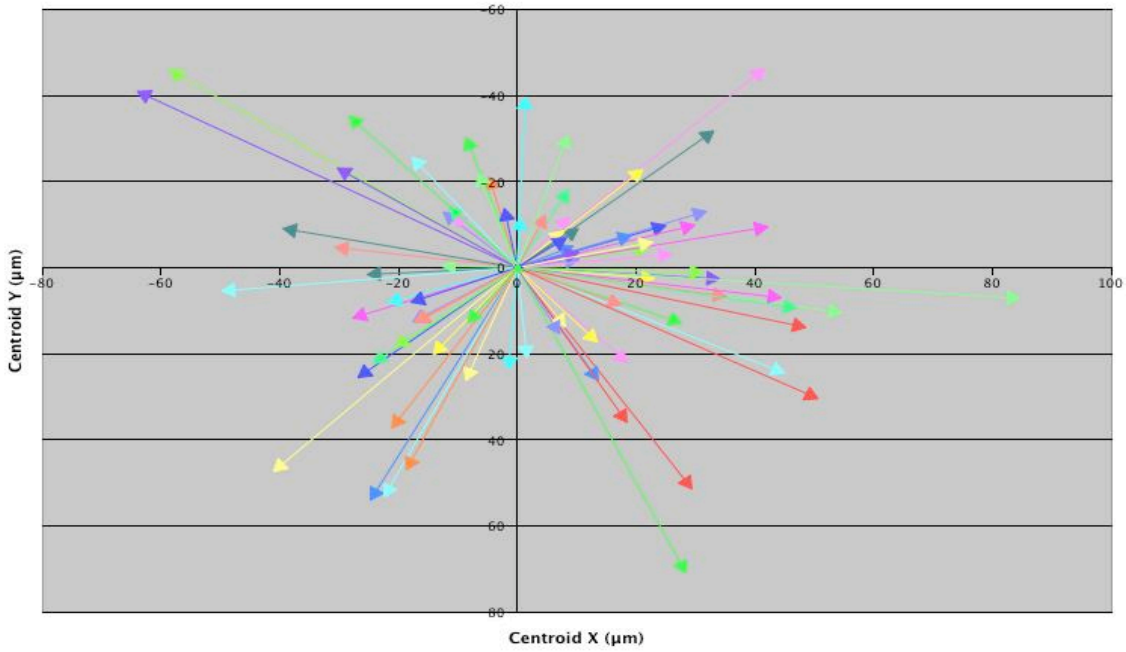


Figure 5.3. An antagonist of PI3-Kinase α inhibits CXCL8-induced chemotactic (gradient), but not CXCL8- and GM-CSF-induced chemokinetic migration. Neutrophils were pre-treated for 30 min with 1μM of the PI3-Kinase α selective inhibitor PIK75, and then stimulated with a) CXCL8 (100ng/ml) or b) GM-CSF (0.5ng/ml, 50ng/ml). Results are shown as mean ±SEM (n=4) except for gradient CXCL8 data sets where n=11. *Significantly greater as compared to unstimulated cells, $p<0.001$. §Significantly inhibited by PIK-75, $p<0.001$.

a)



b)

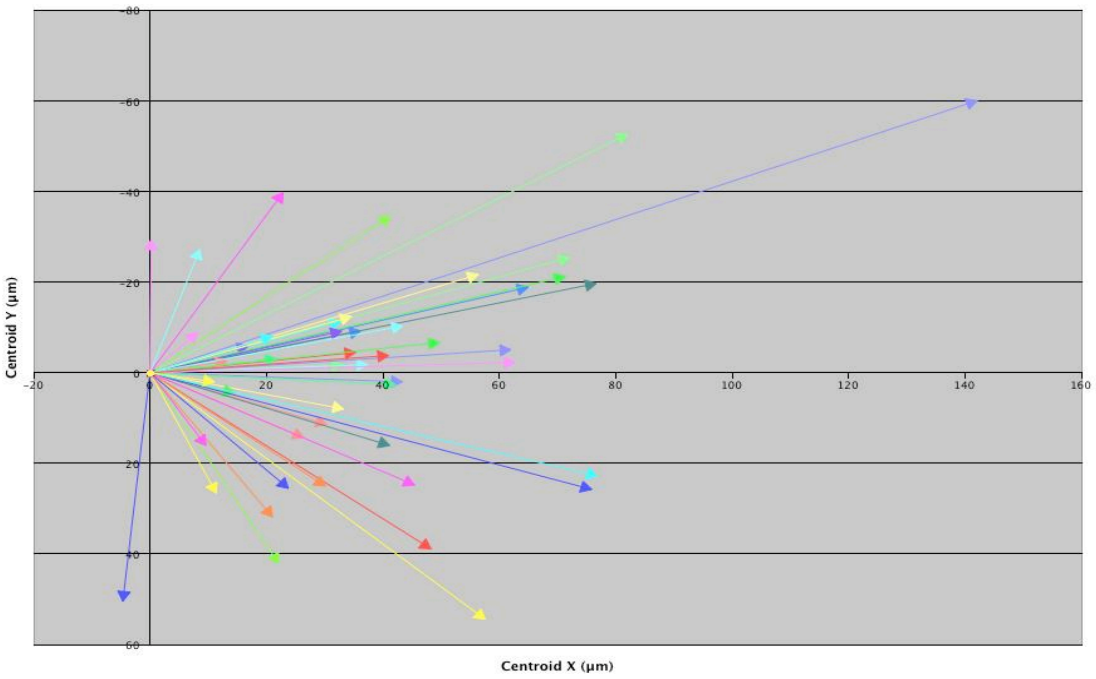


Figure 5.4. Vector diagrams, illustrating the direction of migration for each neutrophil, following pre-treatment with PIK-75 and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b). The diagrams shown represent the pattern seen for all repeated experiments.

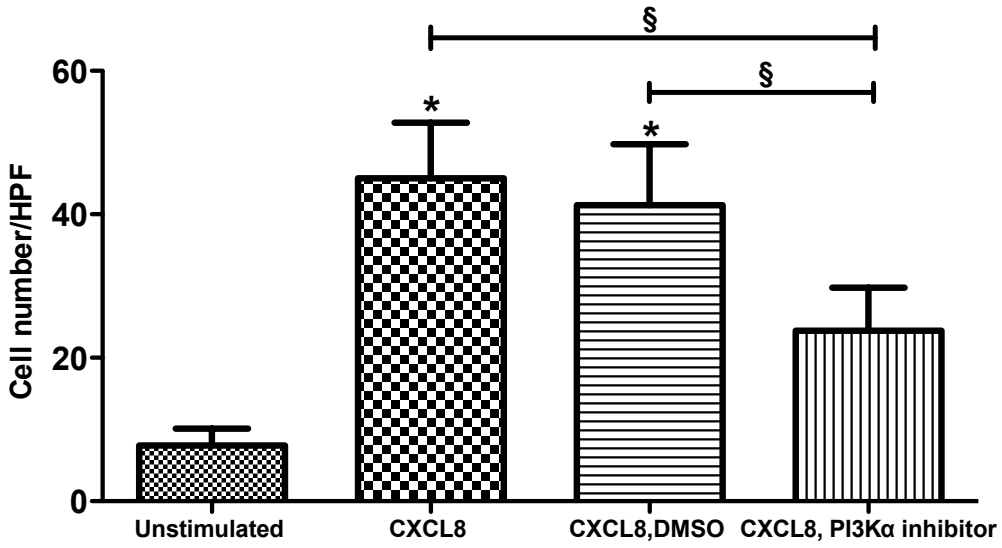


Figure 5.5. The effect of the PI3-Kinase α specific inhibitor PIK-75 on CXCL8-induced neutrophil migration in the Boyden chamber. Neutrophils were pre-treated with 1μM of PIK75 for 90 minutes. Following pre-treatment in the bottom wells of the Boyden chamber were filled with PBS or CXCL8. The membrane was placed on top as detailed in the methods section and pre-treated and control neutrophils were added to the upper wells. Results are shown as mean ±SEM, n=3. *Significantly greater as compared to unstimulated cells, $p<0.05$. §Significantly inhibited by PIK-75, $p<0.05$.

5.4 Neutrophil migration is dependent on PI3-Kinase δ

The PI3-Kinase δ selective inhibitor PIK-294 was used to examine the role of PI3-Kinase δ in neutrophil chemokinesis and chemotaxis. Unstimulated neutrophils were unable to migrate (figure 5.6). Stimulation with CXCL8 caused over 70% of cells to migrate in both the non-gradient and gradient assays (figure 5.6a). Treatment with the vehicle control for the PI3-Kinase δ selective inhibitor, PIK-294 (1% DMSO) prior to stimulation with CXCL8 had no effect on migration, causing 72±9% and 87±2% of cells to migrate in the non-gradient and gradient assays respectively. Treatment of cells with PIK-294 (1μM and 10μM, 30 min pre-treatment) prior to stimulation significantly reduced CXCL8-induced migration in both the non-gradient and gradient assays (figure 5.6a). Pre-treatment with 1μM inhibited migration to a greater extent in the non-gradient assay (46±8%), than in the gradient assay (65±5%). Pre-treatment with 10μM of PIK-294 almost abolished migration in both assays with fewer than 20% of cells migrating following treatment with the inhibitor.

The pre-treatment of cells with PIK-294 had no effect on the directionality of migration (figure 5.7). The vector diagrams highlight that in the presence of PIK-294 the neutrophils that are still able to respond and migrate to CXCL8 follow their normal pattern of migration (figure 5.7). I.e. in the non-gradient assay, the neutrophils migrated chemokinetically (figure 5.7a) and in the gradient assay, the neutrophils migrated chemotactically (figure 5.7b, c).

Stimulation with 0.5ng/ml GM-CSF in the non-gradient assay caused 92±2% of cells to migrate (figure 5.6b). The vehicle control for the inhibitor had no significant effect on the cell migration induced by GM-CSF (1% DMSO, 80±7%; figure 5.6b). Treatment of cells with PIK-294 (1μM and 10μM, 30 min pre-treatment) prior to stimulation with

GM-CSF caused a reduction in migration. However, this was only significant when cells were treated with 10μM PIK-294, which reduced the number of cells migrating to 11±6% (figure 5.6b).

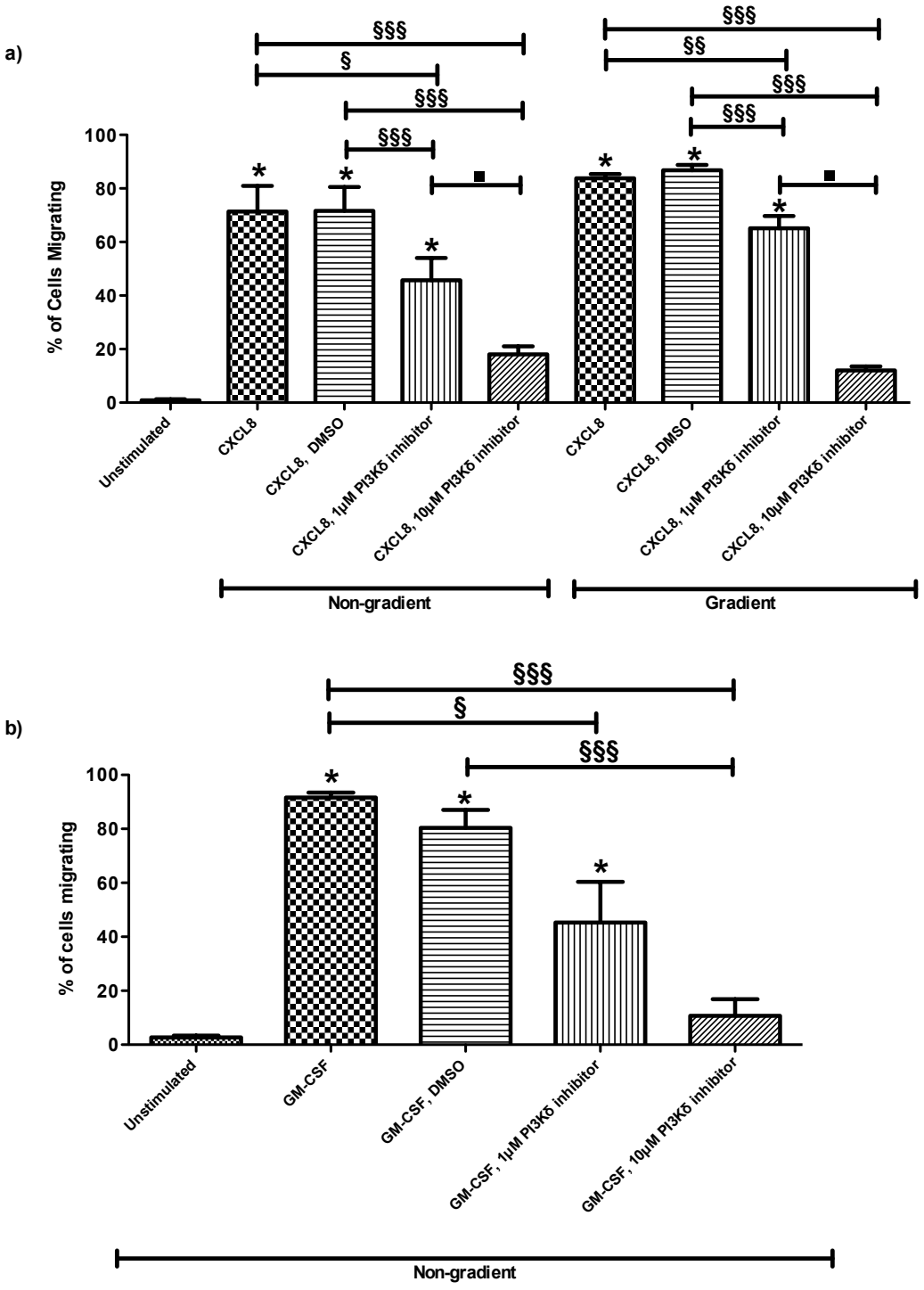
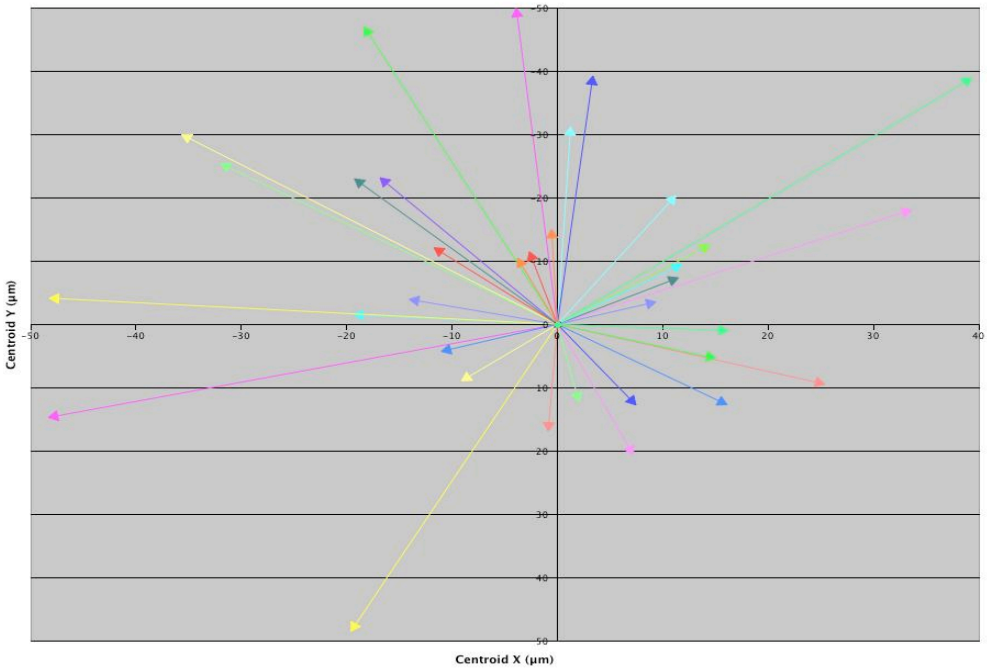


Figure 5.6. An antagonist of PI3-Kinase δ inhibits both chemotactic and chemokinetic migration mediated by CXCL8 and GM-CSF. Neutrophils were treated with 1μM and 10μM of the PI3-kinase δ selective inhibitor PIK-294 for 30 min prior to the addition of CXCL8 (100ng/ml, a) or 0.5ng/ml GM-CSF (b). Data shown are mean ±SEM (n=3) except for gradient DMSO and 1μM of PIK-75 where n=8. *Significantly greater as compared to unstimulated cells, $p<0.01$. §Significantly inhibited by PIK-294 ($^{§}p<0.05$, $^{§§}p<0.01$, $^{§§§}p<0.001$). ■ Significant difference between 1μM and 10μM of PIK-294, $p<0.001$.

a)



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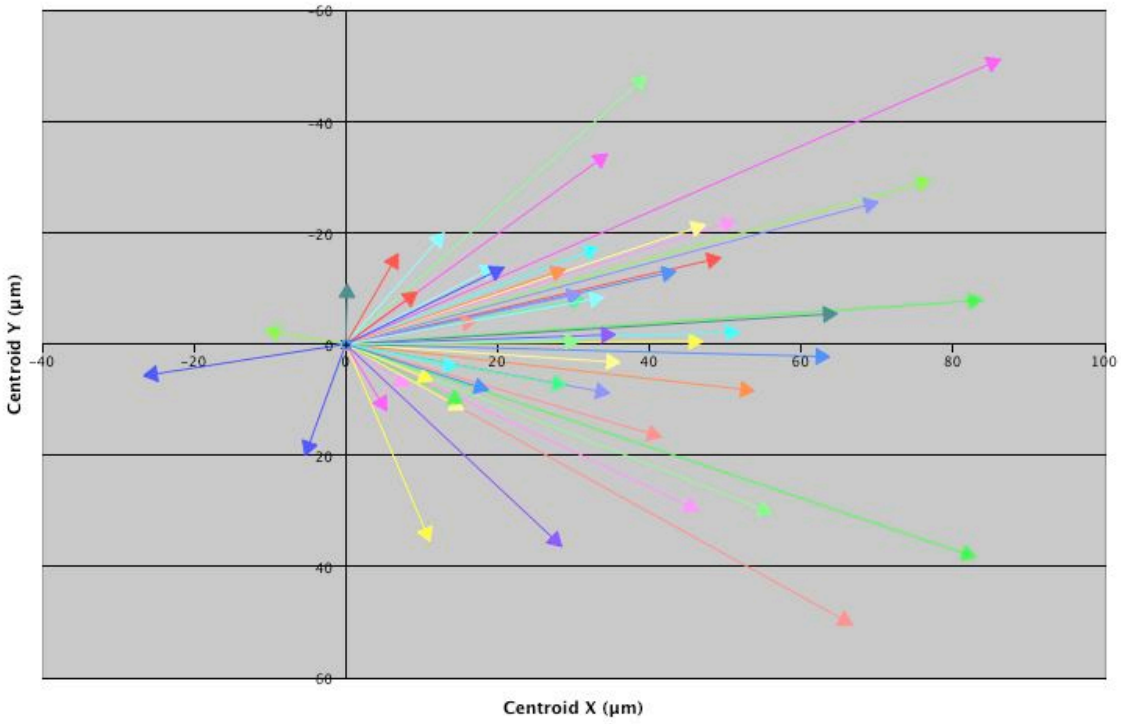


c)

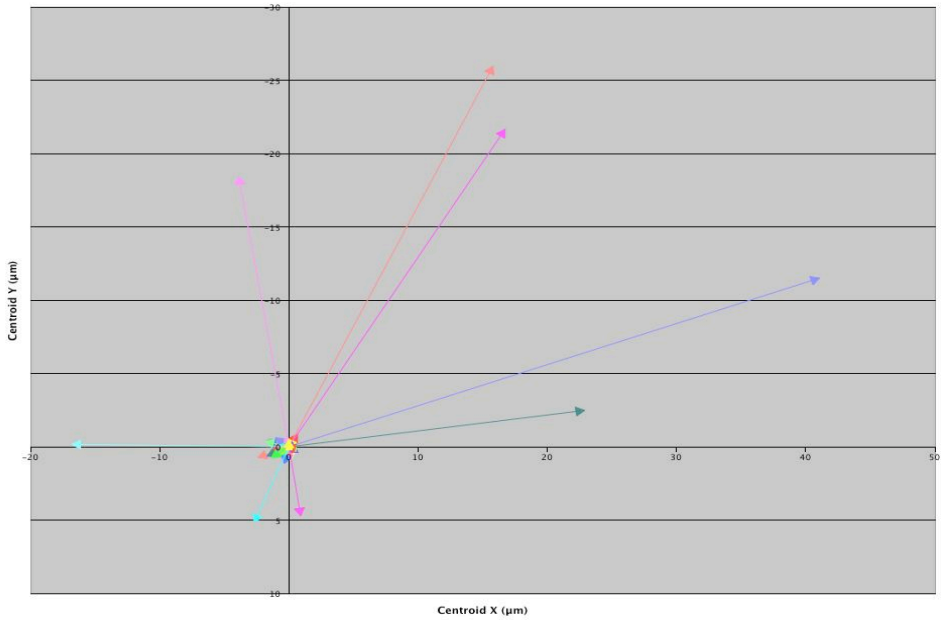


Figure 5.7. Vector diagrams, illustrating the direction of migration for each neutrophil, following pre-treatment with 1µM (a, b) or 10µM (c) PIK-294 and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b, c). The diagrams shown represent the pattern seen for all repeated experiments.

5.5 The PI3-Kinase γ selective inhibitor, AS-605240 inhibits chemokinetic migration

The PI3-Kinase γ selective inhibitor, AS-605240, was used to examine the role of PI3-Kinase γ in neutrophil chemokinesis and chemotaxis. A preliminary experiment was carried out using CXCL8-induced migration in non-gradient assay, to determine the time and concentration of the PI3-Kinase γ selective inhibitor, AS-605240 to use. Neutrophils were treated with either 1μM or 10μM of the inhibitor for 0, 15, and 30 minutes. Unstimulated neutrophils were unable to migrate, stimulation with CXCL8 caused 84% of cells to migrate and this was not affected by the vehicle control for AS-605240 (0.5% DMSO; figure 5.8). Treatment with the inhibitor prior to the addition of CXCL8 reduced the proportion of cells migrating in a concentration and time dependent manner, with 10μM of AS-605240 for 30 minutes causing the greatest inhibition, resulting in 16% of cells migrating (figure 5.8). This time and concentration was therefore chosen for use in the further experiments with the inhibitor.

In non-gradient and gradient assays, CXCL8 significantly increased the proportion of cells migrating from 3% to approximately 80% in both assays (figure 5.9a). The vehicle control (0.5% DMSO) had no significant effect on the migration in the non-gradient assay (77±4%) the effect was not examined in the gradient assay (figure 5.9a). In the non-gradient assay treatment of cells with the PI3-kinase γ selective inhibitor, AS-605240 (10μM, 30 min pre-treatment) significantly reduced cell migration to 29±4% (figure 5.9a). In contrast, in the gradient assay the inhibitor (10μM, 30 min pre-treatment) had no significant effect on migration (66±8%; figure 5.9a).

Pre-treatment of the neutrophils with AS-605240 did not affect the direction of CXCL8-induced migration (figure 5.10). As shown by the vector diagrams, the neutrophils that

are still able to migrate in the presence of AS-605240 maintain chemokinetic migration in the non-gradient assay (figure 5.10a) and chemotactic migration in the gradient assay (figure 5.10b).

In both the non-gradient and gradient migration assays, GM-CSF significantly increased the proportion of cells migrating from 2±1% to 93±1% in the non-gradient assay in response to stimulation with 0.05ng/ml GM-CSF, 79±6% in the non-gradient assay in response to stimulation with 50ng/ml GM-CSF and 63±4% in the gradient assay (figure 5.9b). The vehicle control for AS-605240 (0.5% DMSO) had no significant effect on neutrophil migration induced by GM-CSF, in either the non-gradient (77±4%, 55±4%) or gradient assay (53±11%, figure 5.9b). In the non-gradient assay, treatment of cells with AS-605240 (10μM, 30 min pre-treatment) significantly reduced migration in response to stimulation with 0.5ng/ml GM-CSF to 3±1%, but had no significant effect on migration in response to stimulation with 50ng/ml GM-CSF, in either the non-gradient (71±8%) or gradient assay (42±4%; figure 5.9b).

The effect of the PI3-Kinase γ selective inhibitor, AS-605240 on neutrophil migration in the Boyden chamber was also examined. Stimulation of neutrophils with CXCL8 increased the movement of cells from 6±2 cells/HPF (unstimulated) to 38±4 cells/HPF (figure 5.11). Treatment of the neutrophils with 10μM of AS-605240 or the vehicle control (0.5% DMSO) for 90 minutes, prior to stimulation with CXCL8, had no effect on the migration of cells, in comparison to the CXCL8 stimulated cells (figure 5.11).

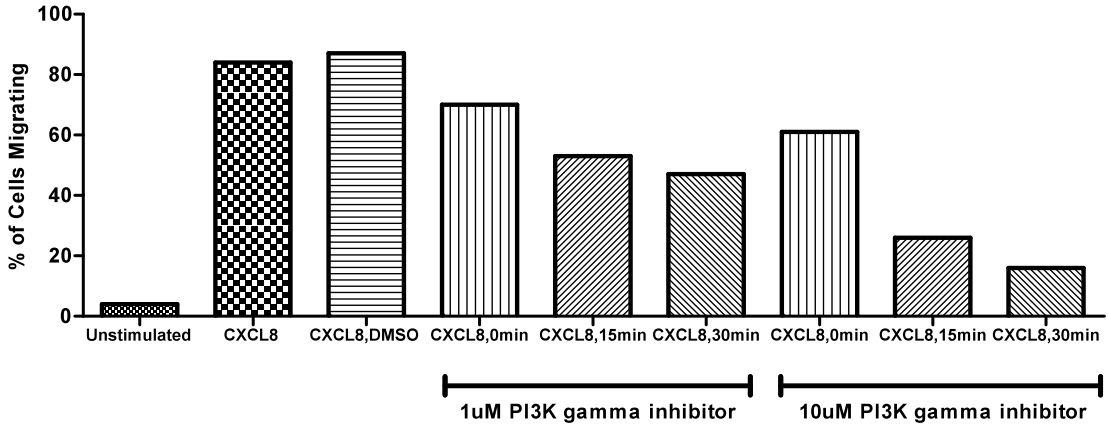


Figure 5.8. An antagonist of PI3-kinase γ inhibits CXCL8-induced chemokinetic migration in a dose and time dependent manner. Neutrophils were pre-treated with 1μM or 10μM of the PI3-Kinase γ selective inhibitor AS-605240 for 0, 15 and 30 minutes, and then stimulated with 100ng/ml CXCL8. Results are from one preliminary experiment.

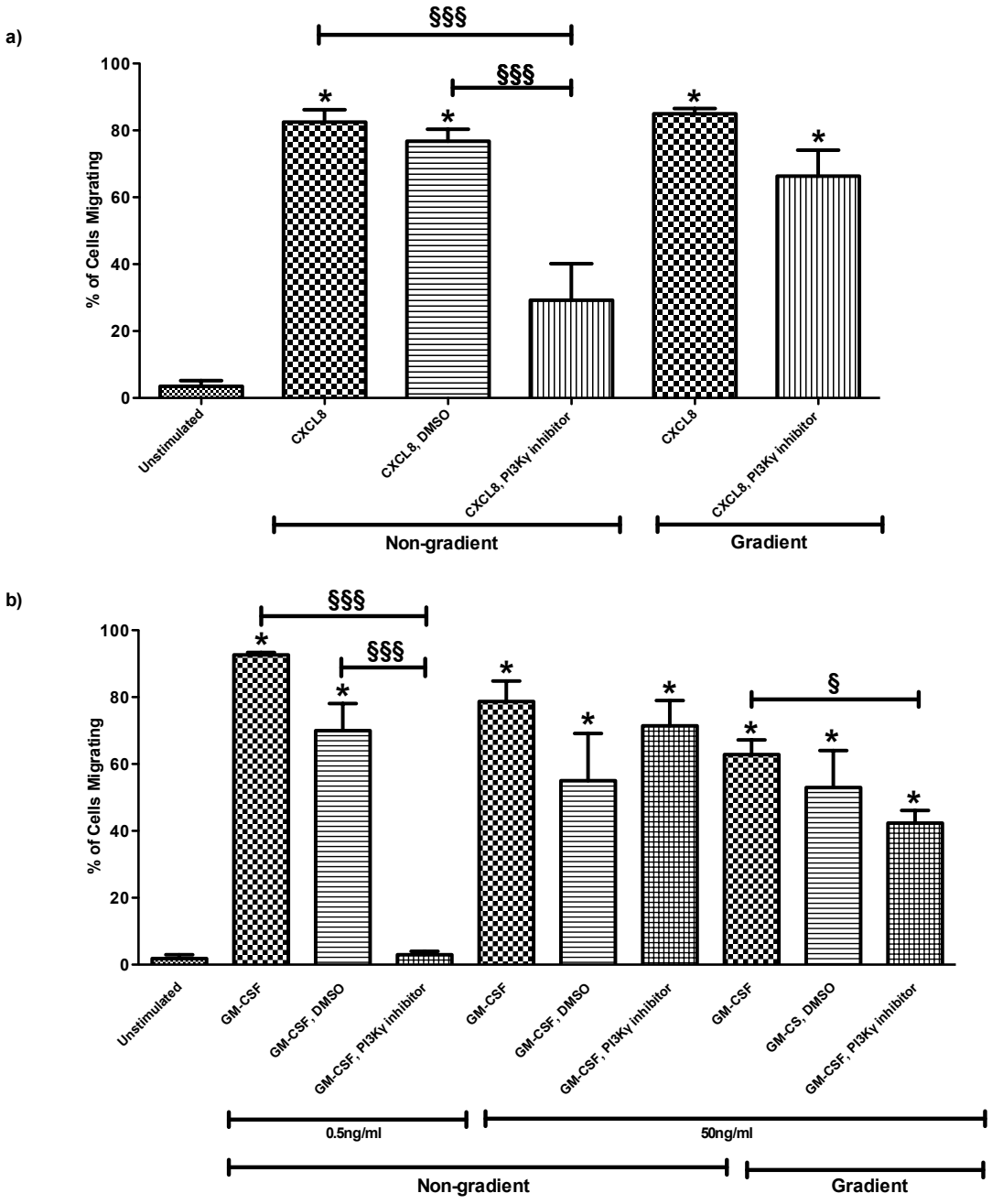
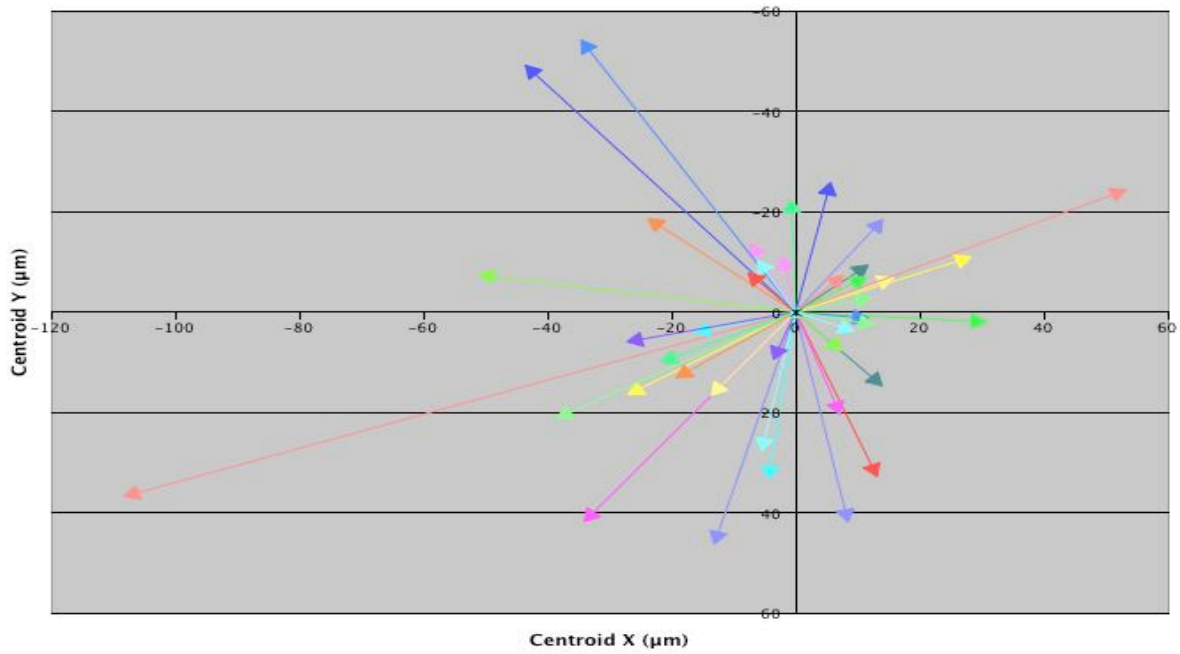


Figure 5.9. An antagonist of PI3-kinase γ inhibits CXCL8 mediated chemokinetic but not chemotactic migration and GM-CSF mediated chemokinetic migration at a sub-optimal concentration of GM-CSF. Neutrophils were treated with 10μM PI3Kγ selective inhibitor, AS-605240 for 30 min prior to the addition of CXCL8 (100ng/ml, a) or GM-CSF (0.5ng/ml, 50ng/ml, b). Data shown are mean ±SEM (n=3) except for non-gradient CXCL8 where n=4 and GM-CSF where n=6. *Significantly greater as compared to unstimulated cells, $p<0.01$. §Significantly inhibited by AS-605240 ($^{§}p<0.05$, $^{§§§}p<0.001$).

a)



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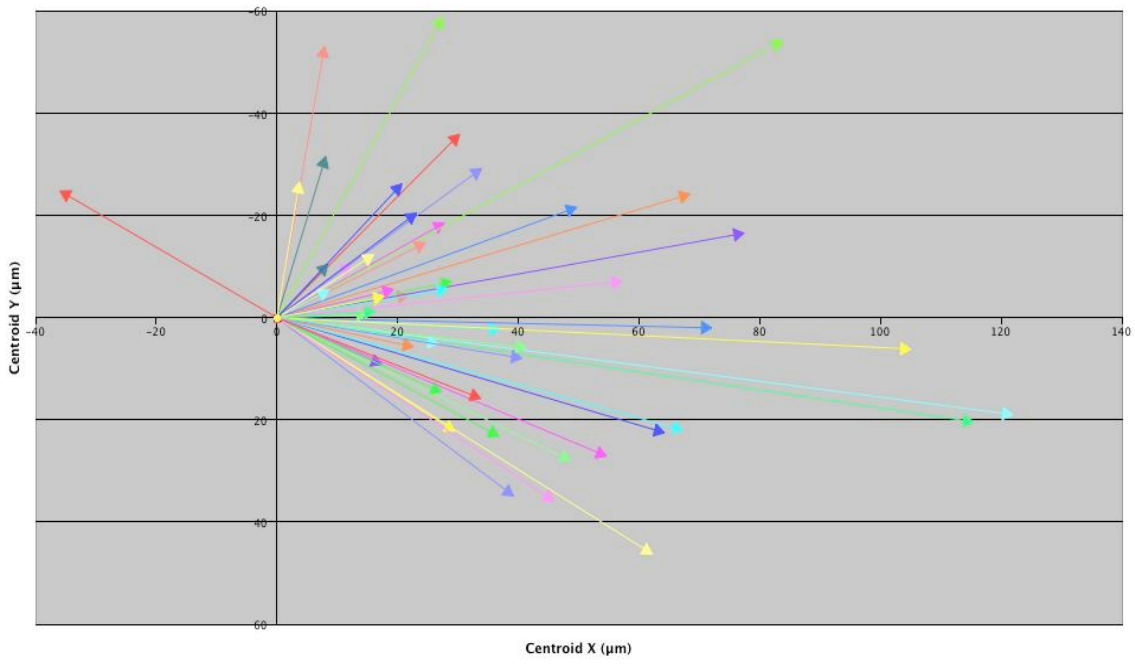


Figure 5.10. Vector diagrams, illustrating the direction of migration for each neutrophil, following pre-treatment with AS-605240 and stimulation with CXCL8. Cells were examined in the non-gradient (a) and gradient assays (b). The diagrams shown represent the pattern seen for all repeated experiments.

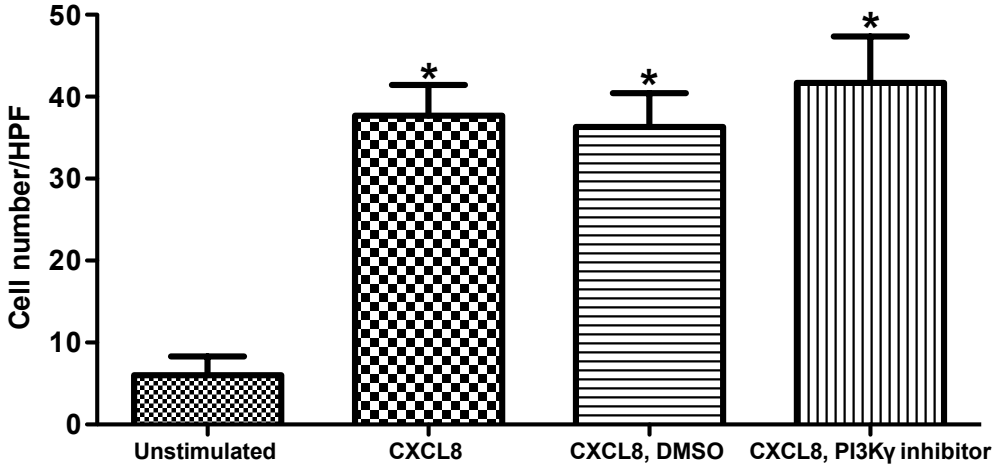


Figure 5.11. Effect of AS-605240 on neutrophil migration in the Boyden chamber. Neutrophils were pre-treated for 90 minutes with 10μM of AS-605240. Following pre-treatment the bottom wells of the Boyden chamber were filled with PBS or CXCL8. The membrane was placed on top as detailed in the methods section and pre-treated and control neutrophils were added to the upper wells. Results are shown as mean ±SEM (n=3). *Significantly greater as compared to unstimulated cells, $p<0.01$.

5.6 Pre-treatment of cells with the PI3-Kinase α selective inhibitor and PI3-Kinase δ selective inhibitor in combination increases inhibition of chemotaxis

As PI3-Kinase α and δ were both shown to play a role in chemotactic migration the effect of the two inhibitors when added in combination was examined. Unstimulated neutrophils were unable to migrate (1±1%), stimulation with CXCL8 in the gradient assay increased cell migration to 85±3% (figure 5.12). The vehicle control for the inhibitors (0.5%DMSO) had no significant effect on neutrophil migration (79±4%; figure 5.12). Neutrophils were treated with the PI3-Kinase α selective inhibitor, PIK-75 alone (2μM, 30min pre-treatment), the PI3-Kinase δ selective inhibitor, PIK-294 alone (2μM, 30min pre-treatment) or the inhibitors in combination (1μM of each inhibitor, 30min pre-treatment; figure 5.12). As previously demonstrated, treatment with both inhibitors alone significantly inhibited CXCL8-induced migration, this was again the case with PIK-75 reducing cell migration to 58±6% and PIK-294 reducing migration to 60±1% (figure 5.12). Treatment with the inhibitors in combination significantly increased the inhibition of neutrophil migration (39±8%), in comparison to the inhibitors alone (figure 5.12).

The addition of the two inhibitors did not affect the directionality of neutrophil migration. The neutrophils that were still able to migrate in the presence of both inhibitors continued to migrate in a chemotactic manner as demonstrated by the vector diagram (figure 5.13).

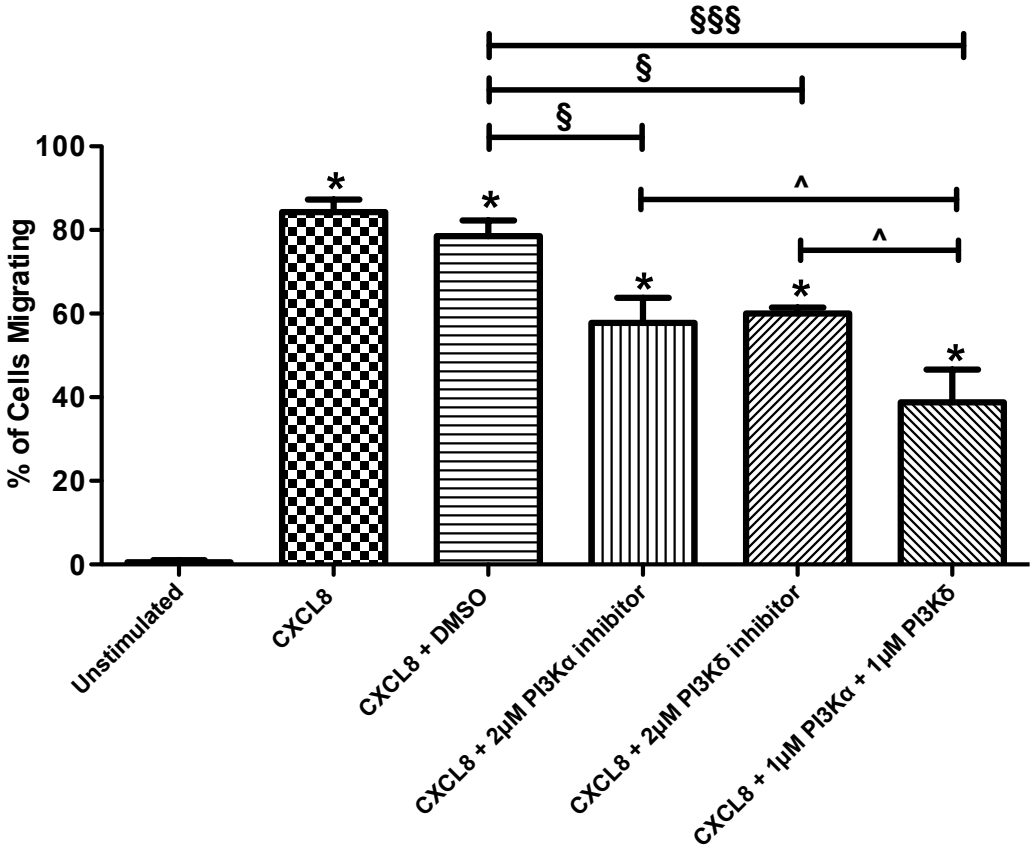


Figure 5.12. Antagonists of PI3-Kinase δ and α have additive effects on inhibition of CXCL8 mediated neutrophil chemotaxis. Neutrophils were pre-treated for 30 min with either 2μM of the PI3-Kinase α selective inhibitor, PIK-75, 2μM of the PI3-Kinase δ selective inhibitor, PIK-294 or 1μM of each and then stimulated with 100ng/ml CXCL8. Results are shown as mean ±SEM (n=4). *Significantly greater as compared to unstimulated cells, $p<0.001$. §Significantly inhibited by PIK-75 ($^{\S}p<0.05$, $^{\S\S\S}p<0.001$). ^Significant difference between inhibitor alone and in combination, $p<0.05$.

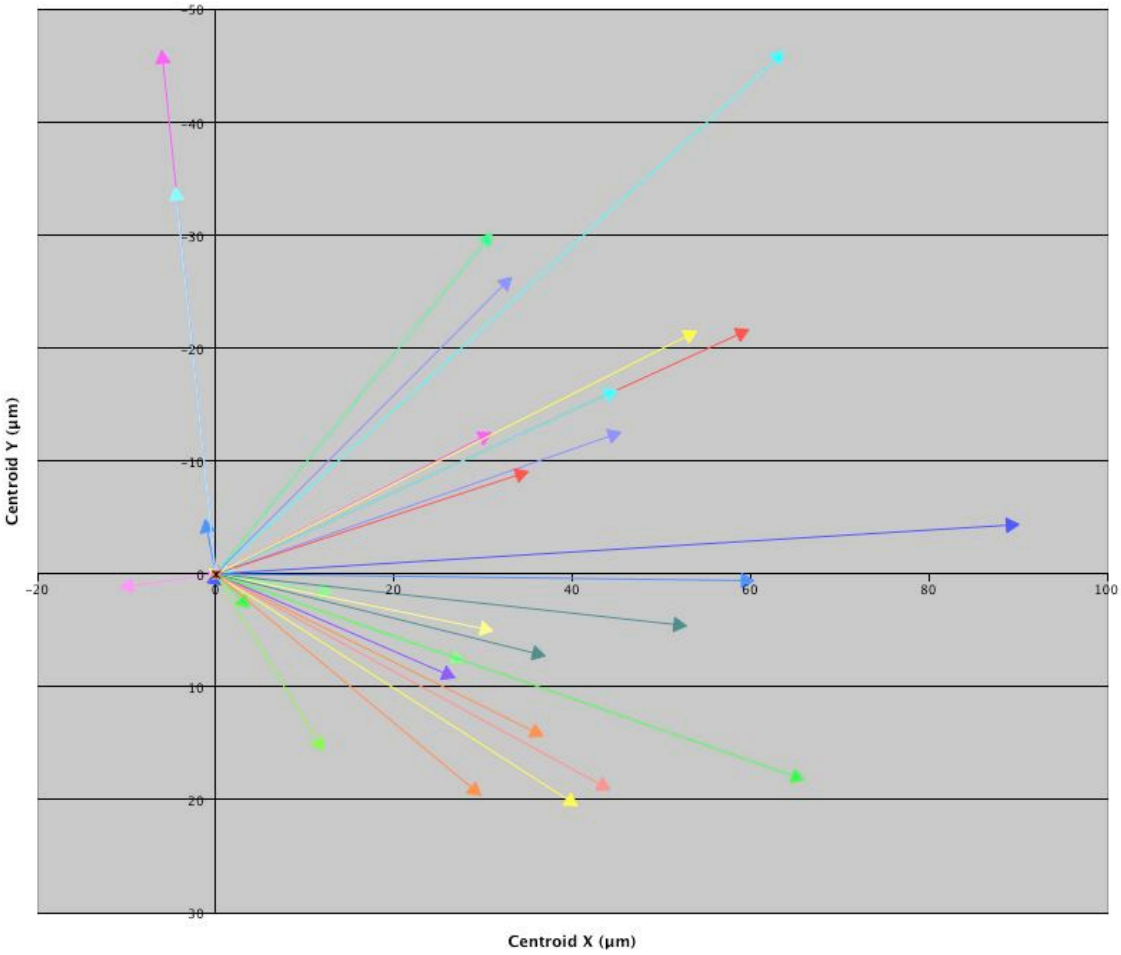


Figure 5.13. Vector diagram illustrating the direction of migration for each neutrophil, following pre-treatment with PIK-75 and PIK-294, and stimulation with CXCL8 in the gradient assay. The diagram shown represents the pattern seen for all repeated experiments.

5.7 CXCL8 and GM-CSF stimulate the phosphorylation of Akt in neutrophils

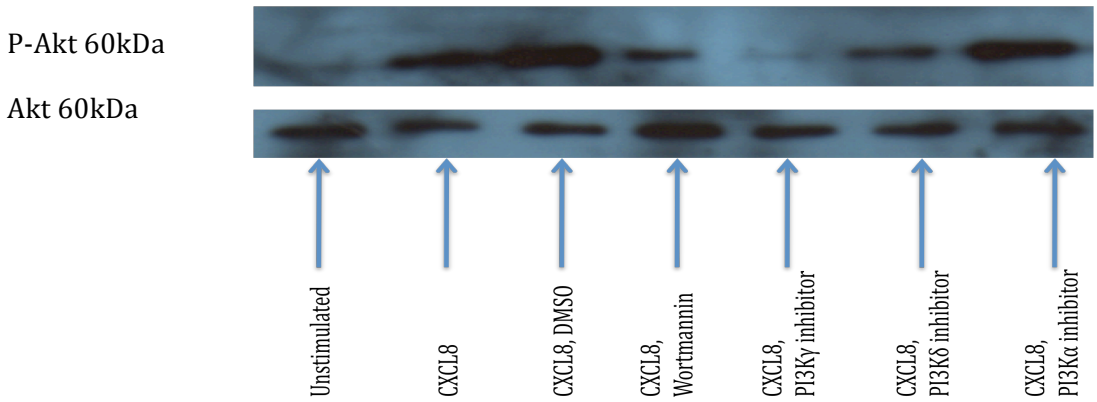
Phosphorylation of one of the downstream effectors of the PI3-Kinases can be examined to determine activation. In this case, the phosphorylation of Akt was studied.

Western blotting shows that stimulation with CXCL8 increased the phosphorylation of Akt, in comparison to control unstimulated neutrophils (figure 5.14a). This increase in phosphorylation was consistent over the course of three experiments, as indicated in the densitometry data shown in figure 5.14b. The vehicle control for the inhibitors (0.1% DMSO) had no effect on the phosphorylation of Akt in comparison to the CXCL8 stimulated cells. Treatment with the PI3-Kinase inhibitors wortmannin (50nM), PIK-75 (α, 1μM), PIK-294 (δ, 10μM) and AS-605240 (γ, 10μM) for 2 minutes reduced the phosphorylation of Akt in response to stimulation with CXCL8. Although the phosphorylation was reduced, only PIK-75 caused a significant reduction in phosphorylation, compared to the vehicle control (figure 5.14). Akt was used as a control and showed bands of equal size, indicating that the loading of each well was approximately the same. Therefore, the effects seen with the Phosphorylated-Akt are due to differences in the stimulation and activation not in the quantity of cells loaded (figure 5.14a).

Stimulation of neutrophils with GM-CSF induced the phosphorylation of Akt (figure 5.15a). This increase in phosphorylation was consistent in all three of the experiments, as shown in the densitometry data in figure 5.15b. The vehicle control for the inhibitors (% DMSO) had no effect on the phosphorylation of Akt. Treatment with the PI3-Kinase inhibitors wortmannin (50nM), PIK-294 (δ, 10μM) and AS-605240 (γ, 10μM) for 2 minutes reduced the phosphorylation of Akt, significantly, in the case of PIK-294.

Treatment with PIK-75 (α, 1μM) for 2 minutes had no effect on the phosphorylation of Akt compared to the vehicle control.

a)



b)

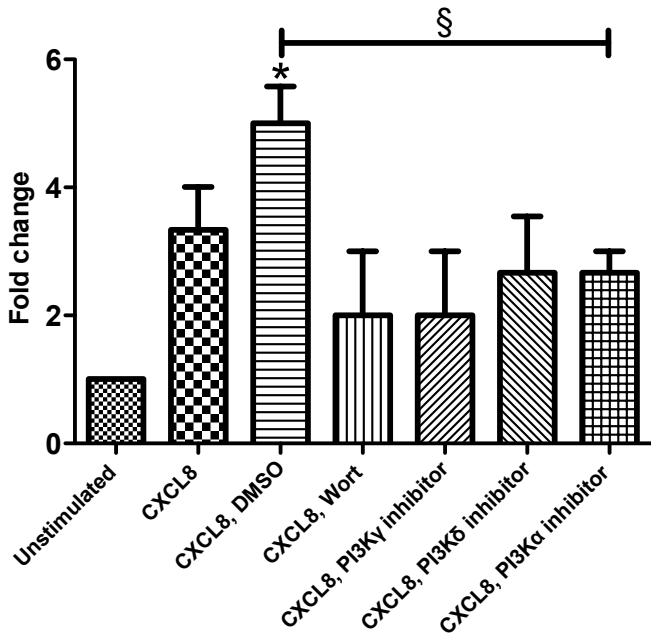
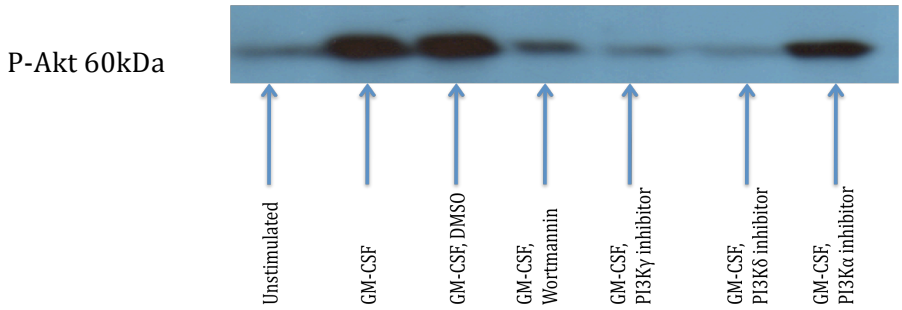


Figure 5.14. CXCL8 induces the phosphorylation of Akt. Neutrophils were treated with wortmannin (50nM) or the PI3-Kinase α (1μM), δ (10μM) and γ (10μM) selective inhibitors for 2 min in combination with 500ng/ml of CXCL8. Western blots were carried out using the antibodies Phosphorylated Akt and total Akt (a). Densitometry was performed on the western blot films (b). Results shown in b are mean ±SEM (n=3), *Significantly greater as compared to unstimulated cells, $p<0.05$. §Significantly inhibited, $^{§}p<0.05$.

a)



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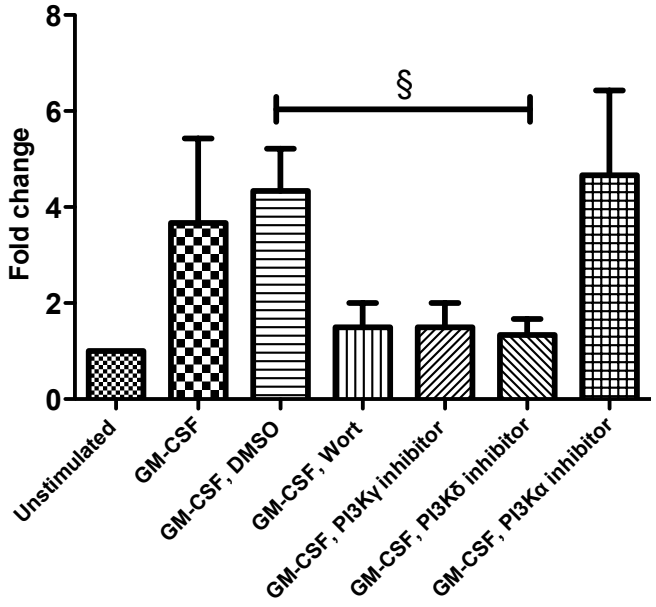

Figure 5.15. GM-CSF induces the phosphorylation of Akt. Neutrophils were treated with wortmannin (50nM) or the PI3-Kinase α (1μM), δ (10μM) and γ (10μM) selective inhibitors for 2 min in combination of 50ng/ml of GM-CSF. Western blots were carried out using the antibody Phosphorylated Akt (a). Densitometry was performed on the western blot films (b). Results shown in b are mean ±SEM (n=3), §Significantly inhibited, $^{§}p<0.05$.

5.8 A Potential downstream effector of the class I PI3-Kinases is the MAPK, ERK

In chapter 4 it was demonstrated that CXCL8 and GM-CSF both cause the phosphorylation of ERK. It has been suggested that ERK may be directly downstream of the class I PI3-Kinases. Therefore, the phosphorylation of ERK was examined using western blots and the PI3-Kinase inhibitors. As with the results in chapter 4, both CXCL8 and GM-CSF induce the phosphorylation of ERK (figure 5.16). This phosphorylation was unaffected by the vehicle control for the inhibitors (0.1% DMSO, figure 5.16). Neutrophils were treated with the PI3-Kinase inhibitors wortmannin (50nM), PIK-75 (α, 1μM), PIK-294 (δ, 10μM) and AS-605240 (γ, 10μM) for 2 minutes, in combination with stimulation from either CXCL8 or GM-CSF (figure 5.16). Treatment with the inhibitor AS-605240 reduced the phosphorylation of ERK when cells were stimulated with both CXCL8 and GM-CSF. In addition, treatment with the inhibitor PIK-294 reduced CXCL8-induced ERK phosphorylation (figure 5.16). Wortmannin and PIK-75 had no effect on CXCL8- and GM-CSF-induced ERK phosphorylation (figure 5.16).

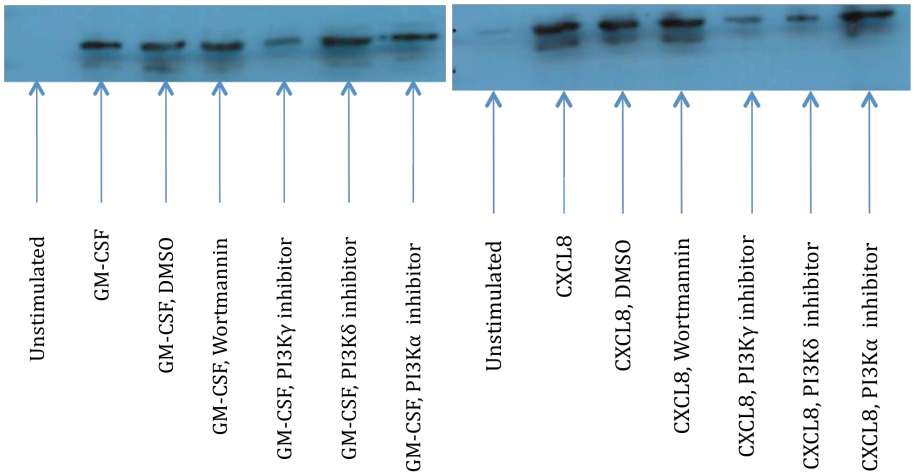

Figure 5.16. CXCL8 and GM-CSF- induce the phosphorylation of ERK. Neutrophils were treated with wortmannin (50nM) or the PI3-Kinase α (1μM), δ (10μM) and γ (10μM) selective inhibitors for 2 min in combination with 100ng/ml CXCL8 or 50ng/ml of GM-CSF. Western blots were carried out using the antibody Phosphorylated ERK. Blot is an example of three carried out.

5.9 Discussion

The class I PI3-Kinase catalytic subunits p110α and p110β are ubiquitously expressed, whereas in contrast the p110δ and p110γ isoforms are restricted to leucocytes in their expression (Vanhaesebroeck et al. 1997; Liu et al. 2007). Our results confirm this as PCR showed that p110α, p110δ and p110γ were expressed by neutrophils in all three of the experiments carried out. However, although thought to be ubiquitously expressed on all cells, p110β was only detected in one of the three experiments carried out. This could be due to only a small amount of RNA being present within the neutrophils or through contamination of another cell type such as eosinophils, which was not detected in two of the experiments. As p110δ and p110γ are both primarily expressed by leucocytes they could be the isoforms responsible for PI3-Kinase related functions within neutrophils and p110β may therefore not be required.

Although the class I PI3-Kinase isoforms have been well characterised the ability to study the involvement of each individual isoform is dependent on the creation of selective inhibitors and the knock out of genes, either through the generation of transgenic mice or human cell transfection. The PI3-Kinase isoforms α, δ and γ all have selective inhibitors. However, these have not been extensively studied in relation to migration of neutrophils, which means that the literature is limited particularly for the α and δ isoform inhibitors.

This is the first study to suggest the involvement of PI3-kinase α in human neutrophil chemotaxis, in response to stimulation with CXCL8. A role for the PI3-kinase α isoform in neutrophil migration has not previously been studied. The results showed that the PI3-Kinase α selective inhibitor, inhibited CXCL8-induced chemotaxis in both the 3D migration assay and the Boyden chamber. In both assays, migration was not

completely abolished. Therefore, PI3-Kinase α in addition to one or more of the other isoforms may be vital for chemotaxis. In contrast to chemotaxis, the results indicated that PI3-Kinase α is not involved in chemokinesis mediated by both CXCL8 and GM-CSF. As PI3-Kinase α is important for chemotaxis but not chemokinesis it would be assumed to play a role in controlling the neutrophil compass, which would enable the cells to move directionally towards the chemoattractant source. However, as shown by the vector diagrams the directionality of neutrophil migration was not affected by the PI3-Kinase α inhibitor. The neutrophils that were unaffected by the inhibitor maintained chemotactic migration in response to stimulation with CXCL8 in the gradient assay. Therefore, although the absence of PI3-Kinase α affects chemotactic migration it does not appear to control the directional compass. It may however be needed to activate a downstream pathway, which is needed for directionally sensing and migration.

The role of PI3-Kinase δ in migration has been previously described using the under agarose assay (Sadhu et al. 2003). The results of this study showed that pre-treatment of neutrophils with the PI3-Kinase δ selective inhibitor, IC87114 inhibited fMLP-induced chemotaxis but had no effect on chemokinesis. It was therefore concluded that PI3-Kinase δ was responsible for the directional orientation of neutrophils during migration (Sadhu et al. 2003). The 3D migration assay results confirm the involvement of PI3-Kinase δ in the chemotaxis of neutrophils. As, pre-treatment of the cells with the PI3-Kinase δ selective inhibitor PIK-294, which is related to the IC87114 inhibitor, significantly reduced migration in response to stimulation with CXCL8. However, the directional orientation of the neutrophils was unaffected by the PI3-Kinase δ selective inhibitor, as shown by the vector diagrams. In response to stimulation with CXCL8 in the gradient assay the neutrophils that were still able to migrate in the presence of the

inhibitor maintained chemotactic migration towards the attractant. Suggesting that PI3-Kinase δ may not be responsible for the directional orientation of neutrophils.

The 3D migration assay results also showed that PI3-Kinase δ was vital for both CXCL8- and GM-CSF-induced chemokinesis. The neutrophils were pre-treated with two concentrations of PIK-294 and interestingly the lower concentration of inhibitor (1μM) caused a greater reduction in CXCL8-induced chemokinesis compared to chemotaxis, the higher concentration (10μM) caused approximately the same reduction in both chemokinesis and chemotaxis. There are differences between the two studies, which could account for the different results. Firstly, Sadhu et al. (2003) used fMLP, whereas, our study used CXCL8 and GM-CSF and secondly, Sadhu et al. (2003) examined migration in the under agarose assay, whereas our study used the 3D collagen migration assay. As the results were conflicting in regards to the exact role of PI3-Kinase δ in neutrophil chemokinesis, this needs to be further explored.

Unlike the α and δ isoforms the involvement of PI3-Kinase γ in neutrophil migration has been studied in more detail in the literature. Inhibitor and knockout experiments with both human and mouse neutrophils have shown that migration is dependent on PI3-Kinase γ (Sasaki et al. 2000; Hannigan et al. 2002; Ferguson et al. 2007; Ferrandi et al. 2007; Reutershan et al. 2010). Rather than being involved in both chemokinesis and chemotaxis, it is thought that PI3-Kinase γ may only be important for chemokinesis. Ferguson et al. (2007) reported that pre-treatment with a PI3-Kinase γ selective inhibitor significantly reduced the number of cells migrating when stimulated with fMLP in a Dunn chamber, but had no effect on the migratory index, speed or direction. This lead to the conclusion that inhibition of PI3-Kinase γ results in impaired

chemokinesis but not the navigation of cells once they are moving (Ferguson et al. 2007).

In the non-gradient assay, in response to stimulation with CXCL8 neutrophil migration was substantially reduced when cells were pre-treated with the PI3-Kinase γ selective inhibitor, AS-605240. In contrast, chemotaxis in the gradient assay was unaffected by pre-treatment with AS-605240, prior to stimulation with CXCL8. The Boyden chamber assay also confirmed that PI3-Kinase γ is vital for chemokinesis but not chemotaxis, as migration in the Boyden chamber was unaffected by pre-treatment with AS-605240, prior to stimulation with CXCL8. Migration within the Boyden chamber is generally regarded as chemotactic. Therefore, as migration was not affected by the inhibitor, it confirms that PI3-Kinase γ is not required for chemotaxis.

In GM-CSF-induced migration, stimulation with 50ng/ml GM-CSF in both the non-gradient and gradient assays did not affect migration when cells were pre-treated with AS-605240. There did appear to be a trend for a reduction in migration in the gradient assay with the inhibitor, but this was not significant. In contrast, in the non-gradient assay when cells were stimulated with 0.5ng/ml GM-CSF, pre-treatment with AS-605240 abolished neutrophil chemokinesis. As was discussed in chapter 4 the difference between the inhibition seen with the suboptimal (0.5ng/ml) and optimal concentration (50ng/ml) of GM-CSF could be due to PI3-Kinase and PLA2 being two redundant mediators of migration.

As the PI3-Kinase α, δ and γ inhibitors are selective not specific there is potential for cross over with the inhibitors affecting more than one isoform. However, based on the IC_{50} values for the three PI3-Kinase inhibitors (Table 5.1) it is unlikely that the results seen are due to crossover of more than one isoform. The PI3-Kinase α inhibitor, PIK-75

antagonises PI3-Kinase γ at a 10 fold higher concentration and PI3-Kinase β and δ at a 100 fold higher concentration. Therefore, as no effect on gradient migration is seen with the PI3-Kinase γ selective inhibitor it is unlikely that the inhibition seen in the gradient assay is due to the inhibitor effecting more than one isoenzyme. The PI3-Kinase δ inhibitor, PIK-294 is thought to be 20-60 fold more potent than the related inhibitor IC87114. The IC87114 inhibitor antagonises the PI3-Kinase γ and α subunits at a 100 fold higher concentration, suggesting that the likelihood of isoform cross over is low. Finally the PI3-Kinase γ inhibitor antagonises PI3-Kinase α at a 10 fold higher concentration and PI3-Kinase δ at a 20 fold higher concentration. As no effect on chemokinetic migration was seen with the PI3-Kinase α selective inhibitor, it would suggest that the inhibitor specifically inhibited the γ isoform. However, based on the inhibitors IC_{50}s any effect can only be assumed. In order to be fully confident that the inhibitors only targeted the specific isoform of interest, gene silencing could be used to examine the effect. This possibility will be discussed in further detail in chapter 6.

Inhibitor	PI3-Kinase α	PI3-Kinase δ	PI3-Kinase γ	Published by
PIK-75	0.01μM	1μM	0.1μM	Knight et al. 2006
IC87114	100μM	0.1μM	100μM	Knight et al. 2006
AS-605240	60nM	300nM	8nM	Kong et al. 2008

Table 5.1 Published IC_{50} values for the PI3-Kinase selective inhibitors

Both chemokinesis and chemotaxis have been shown to be inhibited by more than one of the class I PI3-Kinase catalytic isoforms leading to the view that migration may be as a result of the interaction of different PI3-Kinase isoforms. In CXCL8-induced chemotaxis, when both the PI3-Kinase α and δ selective inhibitors were added in combination, the resultant migration was significantly reduced, in comparison to pre-treatment with the inhibitors alone. The migration was not completely abolished by treatment with the selective inhibitors in combination, which could be due to the low concentrations used of the inhibitors. Use of the two inhibitors together also did not affect the directionality of the neutrophils that were still able to migrate in the presence of the inhibitors. The cells continued to migrate chemotactically towards the chemoattractant source, suggesting that a combination of the two PI3-Kinase isoforms does not control the chemotactic compass.

As Akt lies downstream of PI3-Kinases, Akt phosphorylation was used to confirm that the PI3-Kinase inhibitors were active and capable of inhibiting the pathway. CXCL8 has been shown previously to induce Akt phosphorylation, which can be reduced by pre-treatment with wortmannin (Lane et al. 2006; Brkovic et al. 2007). In our experiments, CXCL8 induced Akt phosphorylation. This was reduced by pre-treatment with wortmannin and the PI3-kinase α, δ and γ selective inhibitors. However, only the PI3-Kinase α selective inhibitor caused significant inhibition. As with CXCL8, treatment with GM-CSF-induced the phosphorylation of Akt. This was reduced by pre-treatment with wortmannin and the PI3-kinase δ and γ selective inhibitors. However, only the PI3-kinase δ selective inhibitor caused significant migration. The PI3-Kinase α selective inhibitor had no effect on Akt phosphorylation induced by stimulation with GM-CSF. The role of Akt in neutrophil migration has recently been explored; a deletion

in Akt2 was shown to impair neutrophil migration (Chen et al. 2010). Akt2 could therefore be an important component in controlling neutrophil migration.

PI3-Kinase has been suggested to activate ERK via Rac and PAK. PAK phosphorylates MEK, which in turn activates ERK (Wennstrom and Downward 1999). Therefore, the phosphorylation of ERK was examined in neutrophils that were stimulated with CXCL8 and GM-CSF following treatment with the PI3-Kinase inhibitors. CXCL8 and GM-CSF stimulation both induced the phosphorylation of ERK. Pre-treatment of cells with the PI3-Kinase γ and δ inhibitors reduced the phosphorylation of ERK caused by stimulation with CXCL8 and pre-treatment with the PI3-Kinase γ inhibitor reduced GM-CSF induced phosphorylation of ERK. Therefore, ERK may lay downstream of the PI3-Kinase γ and δ isoforms.

In summary, the results of this chapter show that PI3-Kinase γ and δ are important for CXCL8- and GM-CSF- induced chemokinesis, whereas PI3-kinase α and δ are important for CXCL8- induced chemotaxis (table 5.2). It is likely that migration is due to two isoforms working in combination and that depending on the nature of the signal (non-gradient or gradient) there is a differential requirement for the α and γ isoforms.

Mediator	Migration type	PI3-Kinase α	PI3-Kinase δ	PI3-Kinase γ
CXCL8	Chemokinesis (non-gradient assay)	−	+	+
CXCL8	Chemotaxis (gradient assay)	+	+	−
GM-CSF	Chemokinesis	−	+	+

Table 5.2 Summary of the class I PI3-Kinase isoforms involved in migration in response to stimulation with CXCL8 and GM-CSF. + pathway involved, - pathway not involved.

Chapter 6: Discussion

6.1. General Discussion

Using the 3D collagen migration assay to visualise migration showed that CXCL8 and GM-CSF stimulate robust migration. CXCL8 mediated chemokinesis in the non-gradient assay and chemotaxis in the gradient assay, whereas, GM-CSF caused chemokinesis in both the non-gradient and gradient assays. Throughout the results, it was highlighted that the chemokinesis of GM-CSF in the gradient assay is controlled by the same mechanisms as in the non-gradient assay.

Initially thought that hematopoietic growth factors and chemokines mediate different signal transduction pathways, the chemokinetic migration in response to CXCL8 (figure 6.1) and GM-CSF (figure 6.2) saw a similar dependence on the signal transduction components tested. With the exception of the initiation of CXCL8 signalling via its GPCR (figure 6.1) and GM-CSF via its HGFR (figure 6.2).

In response to stimulation with CXCL8 or GM-CSF, the chemokinesis of neutrophils was dependent on the PI3-Kinases as shown by the use of the inhibitor wortmannin, more specifically the class I PI3-Kinase γ and δ isoforms and the MAPK, ERK. Chemokinesis induced by CXCL8 and GM-CSF was also partially dependent on ROCK.

In addition to highlighting a role for ERK in chemokinesis, the involvement of p38MAPK was excluded. Interestingly, ERK and p38MAPK are thought to have opposing roles in fMLP-induced neutrophil trafficking, with ERK activation inhibiting migration and p38MAPK activation promoting migration of human and mouse neutrophils as well as differentiated HL-60 cells on fibronectin coated cover slips (Liu

et al. 2012). The ‘stop’ and ‘go’ signalling between ERK and p38MAPK activation respectively, controls efficient migration to the site of infection (Liu et al. 2012). This is not the first time p38MAPK has been implicated in controlling neutrophil migration in response to stimulation with fMLP (Heit et al. 2002, Heit et al. 2008). However, CXCL8, GM-CSF and G-CSF-induced migration have all been shown to occur independently of p38MAPK, instead requiring ERK activation and signalling (Nakamae-Akahori et al. 2006). Therefore, in the case of CXCL8 and GM-CSF stimulated neutrophil migration the roles of p38MAPK and ERK may be reversed. This differential requirement for ERK/p38MAPK signalling could be important for signalling hierarchy, where following transmigration through the endothelium and migration into the infected tissue, neutrophils will preferentially migrate towards end target chemoattractants at the final site of infection such as fMLP, over intermediary chemoattractants including CXCL8.

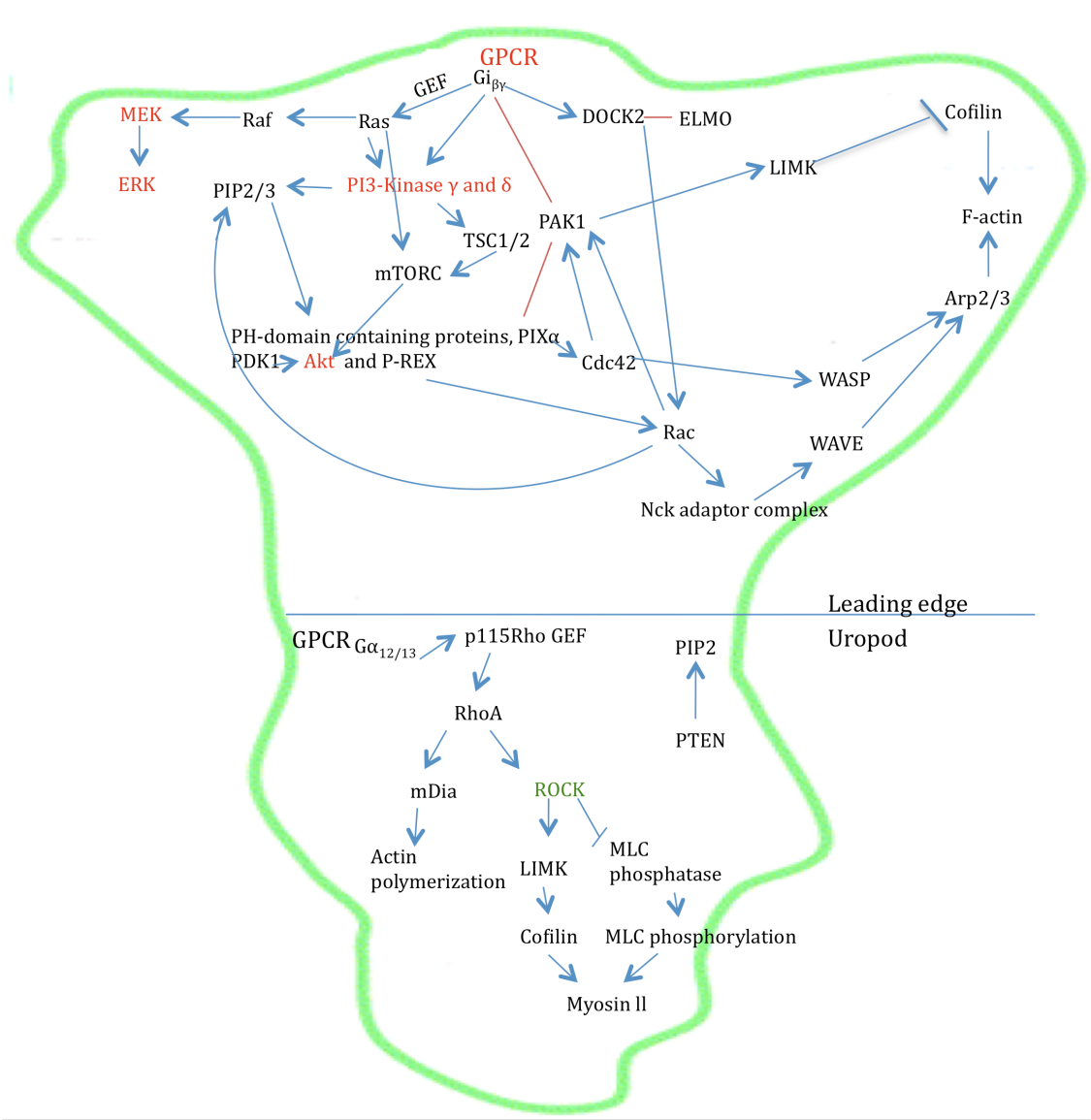

Figure 6.1 Diagram showing the pathways controlling CXCL8-induced neutrophil chemokinesis. Key: blue arrow represents activated by, blue line represents blocked by and red line represents binding, black-shown in literature, red- shown to be dependent from migration experiments and green- shown to be partially dependent on from migration experiments.

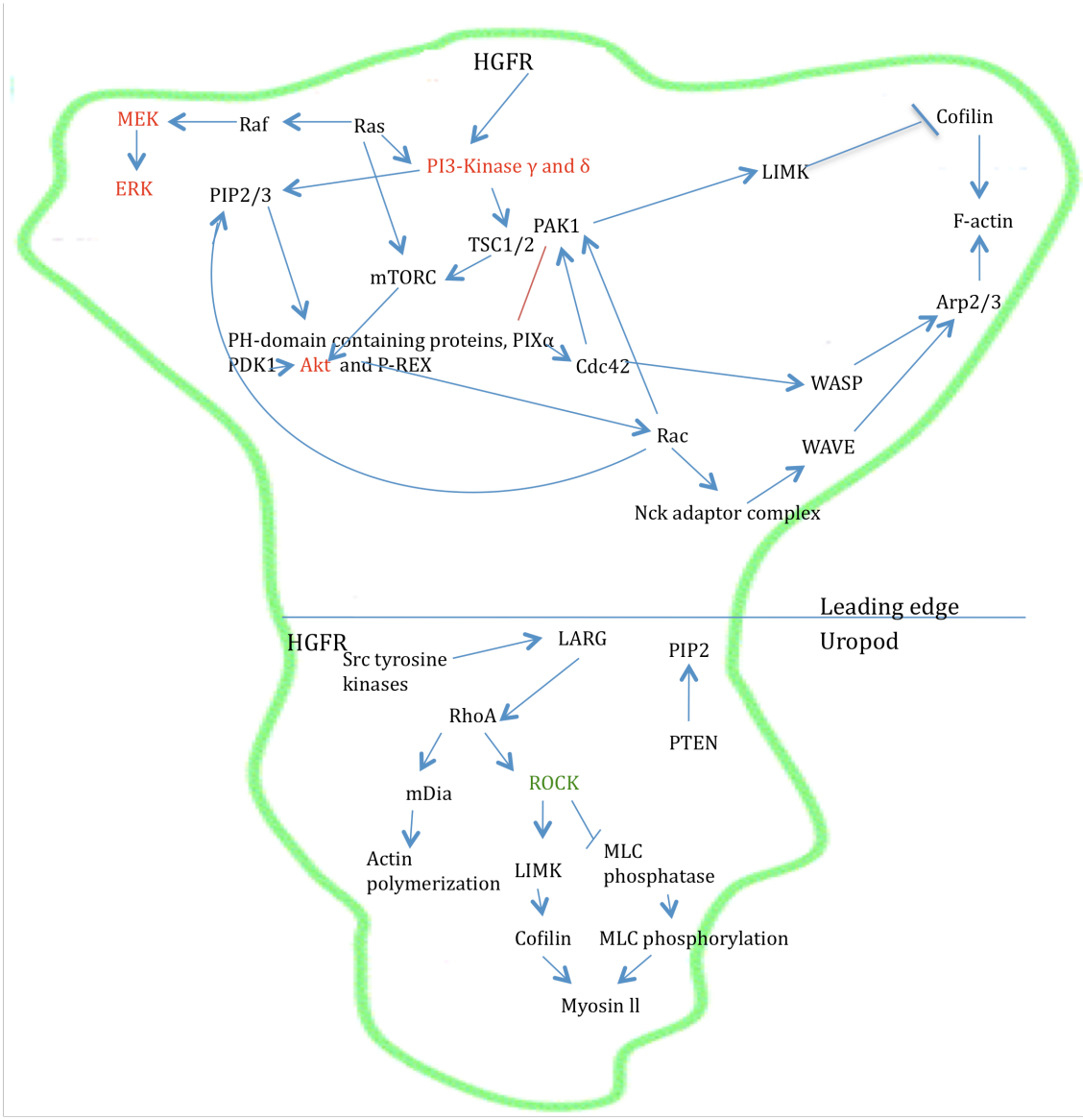

Figure 6.2 Diagram showing the pathways controlling GM-CSF-induced neutrophil chemokinesis. Key: blue arrow represents activated by, blue line represents blocked by and red line represents binding, black-shown in literature, red- shown to be dependent from migration experiments and green- shown to be partially dependent on from migration experiments.

Unlike chemokinesis, which was dependent on PI3-kinase γ and ERK, the chemotaxis of neutrophils in response to stimulation with CXCL8 was initiated by its GPCR. It was dependent on the PI3-Kinases, more specifically the class I PI3-Kinases α and δ isoforms and was only partially dependent on ERK (figure 6.3). Therefore, one theory for the difference between CXCL8-induced chemokinesis and chemotaxis could be due to the differential involvement of the PI3-Kinase α, δ and γ isoforms and ERK. This may not be the complete story as use of the PI3-Kinases α and δ inhibitors did not affect the directionality of the neutrophils still able to migrate in the gradient assay. Therefore, directionality could be due to other signal transduction pathways. Downstream of PI3-Kinases, Akt has been suggested as a mediator of the chemotactic compass, as cell polarisation towards fMLP and Akt phosphorylation in human neutrophils and HL-60 cells are reduced in the presence of two Akt inhibitors, Akt inhibitor and deguelin (Zou et al. 2012). Inhibition of Akt also decreases fMLP-induced F-actin polymerization and Rac activation (Zou et al. 2012). Of Akt's three isoforms, Akt2 is thought to be essential for neutrophil migration, as following fMLP stimulation of mouse neutrophils on fibrogen coated glass coverslips Akt2 translocates to the leading edge of polarised neutrophils (Chen et al. 2010). In contrast, in $Akt2^{-/-}$ neutrophils, Akt is unable to translocate and the cells are unable to move (Chen et al. 2010). Taken together as Akt is needed for cell polarisation, F-actin polymerisation and migration it may be a critical component of the chemotactic compass.

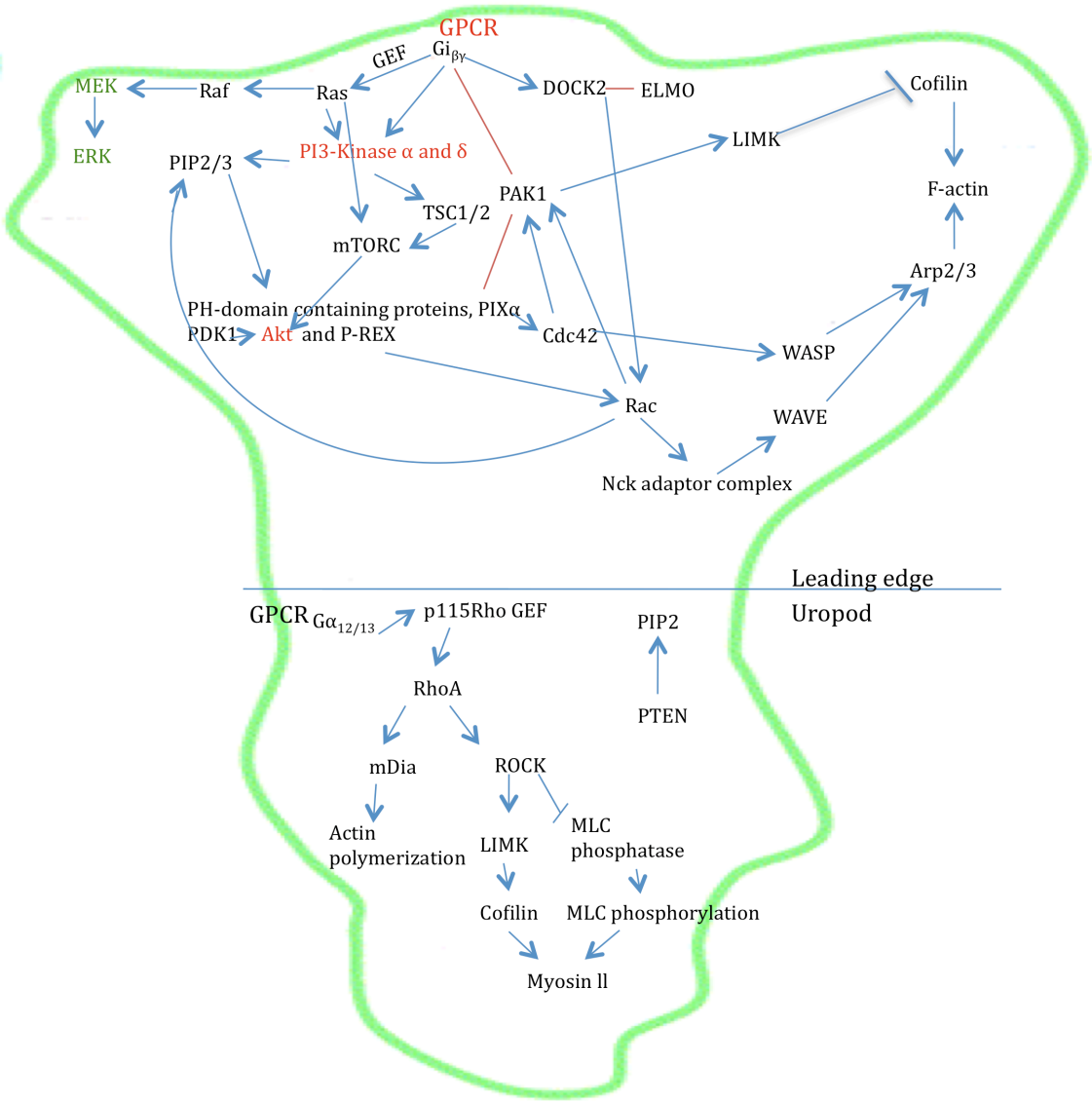

Figure 6.3 Diagram showing the pathways controlling CXCL8-induced neutrophil chemotaxis. Key: blue arrow represents activated by, blue line represents blocked by and red line represents binding, black-shown in literature, red- shown to be dependent from migration experiments and green- shown to be partially dependent on from migration experiments.

Throughout the results chapters migration in the 3D collagen assay was compared to migration using 2D migration assays particularly the Boyden chamber, carried out either within our laboratory or within the literature. The use of both assays confirmed the involvement of some of the components important in chemotaxis. Although the two assays showed similar results, the 3D migration assay has many benefits over the Boyden chamber. These include the visualisation of the cells as they migrate, the ability to clearly distinguish between chemokinesis and chemotaxis using the two different 3D assays, an easier and more efficient analysis and the ability to study other outputs including the total distance migrated within a set time period and the velocity of the cell.

Integrins had previously been thought to be vital for efficient neutrophil migration, however the use of 3D assays has shown this is not the case. When tethering and rolling in 2D shear-stress situations, such as during blood flow in the vessel, integrins are indispensible. However, upon entry into the tissue matrix, it is thought that integrins are not needed for neutrophil migration, shown using 3D migration assays (Koenderman et al. 2010, Lammermann et al. 2008). Therefore, the use of 2D and 3D assays could still prove beneficial in highlighting the components need to control migration depending on the *in-vivo* environment that would ordinarily be experienced by the neutrophil.

6.2. Critique

The majority of the results were obtained using commercially available inhibitors. Although most of the inhibitors used including wortmannin and pertussis toxin have been frequently used in the literature, the class I PI3-Kinase selective inhibitors are relatively new and therefore remain largely unpublished. As the inhibitors are only

selective, not specific, for the isoforms of interest it would have been beneficial to use transfection. This would silence the genes of interest to confirm the isoforms specific involvement in neutrophil migration. However, problems arose with this proposition, as it is well known that primary human neutrophils are very difficult to transfect. This is because neutrophils are relatively short-lived cells, which need to be used on the day they are obtained. This causes problems when trying to transfect cells as generally transfection reagents are incubated with neutrophils overnight.

The possibility of using the neutrophil like HL-60 cell lines was also discussed. However, use of this cell line is not ideal as they can only mimic the behaviour we would expect to see, and without the ability to compare the cell line to the primary neutrophils we would not be certain that the behaviour displayed by the cell line would be the same as the primary neutrophils.

Towards the end of the PhD, my laboratory did successfully transfect eosinophils and neutrophils using adenoviruses tagged with GFP. However, this was not assessed in relation to viability within the 3D collagen migration assay or with viral constructs capable of knocking down the specific isoforms of the class I PI3-Kinases.

6.3. Future studies

This PhD has only 'scratched the surface' on understanding neutrophil migration in a 3D collagen gel as migration. A significant amount of work could be carried out to further explore the other pathways highlighted in the Introduction, as the components and roles of many pathways are still being discovered.

The ability to further understand the specific components needed for the cell to migrate would be greatly enhanced if human neutrophils could be successfully transfected as mentioned above to knockout the components of interest. This would be especially beneficial as the generation of transgenic mice has lead to some substantial discoveries in the field, such as the involvement of PI3-Kinase γ (Reutershan et al. 2010). Therefore, the use of transfection for human cells could determine if the pathways are conserved amongst the two species. The exploration of this would be a priority.

The project only touched on the migration of neutrophils from healthy volunteers. A recent study has demonstrated that neutrophils from COPD patients moved at a greater speed but with a reduced accuracy in the presence of CXCL8, sputum and FMLP, compared to neutrophils from healthy volunteers (Sapey et al. 2011). Furthermore, the use of the broad-spectrum PI3-Kinase inhibitor LY294002 reduced the speed of neutrophil migration from patients with COPD, while returning the accuracy (Sapey et al. 2011). As there was no difference in the receptor surface expression, it was concluded that this difference was due to differenced in cell signalling (Sapey et al. 2011). Based on this, a comparison between neutrophils obtained from healthy individuals and those obtained from patients with inflammatory airway diseases such as COPD, cystic fibrosis and bronchiectasis, may provide a valuable insight into the signalling of neutrophils in patients with disease. While also determining if there are any differences between signalling in healthy and diseased state neutrophils.

Neutrophils were shown to migrate towards epithelial cells. In some respiratory diseases such as COPD and cystic fibrosis, damage occurs to the epithelial cells. This is usually as a result of inflammatory mediators and inflammatory cell influx. The damage releases further inflammatory mediators, which attract more neutrophils towards the site of infection. These mediators could be explored and assays involving bronchial and

nasal brushing could be carried out from patients with respiratory diseases. The pathways controlling migration could also be examined using the inhibitors presented in the Thesis to determine the effect on migration. In the treatment of inflammatory diseases where neutrophil influx causes adverse effects, inhibitors of the class I PI3-Kinase γ and δ isoforms could prove beneficial (Rommel 2011).

In addition, neutrophil migration could be compared to migration in eosinophils, as they are a related cell type. In the majority of patients with asthma, neutrophil numbers are normal. However, there is an increase in the number of eosinophils. If there is a difference in the signal transduction pathways controlling the migration and recruitment of eosinophils and neutrophils this could affect whether neutrophils or eosinophils are present in the lung.

The data presented in this Thesis extends the view that neutrophils can migrate in response to CXCL8 and GM-CSF. This migration is amongst other components, controlled by the involvement of the different isoforms of the Class I PI3-Kinases. This opens avenues for future work to further understand whether inhibition of the specific isoforms of the class I PI3-Kinases could be a potential beneficial therapy. The use of which could prevent neutrophil recruitment and migration, within the lung, of patients with respiratory diseases including asthma and COPD.

Chapter 7: References

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