Neutrophil Migration in the Healthy Elderly: Causes and Consequences for the Resolution of Inflammation

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Abstract

Neutrophils constitute the main immune defence against microbial invasion. When activated, they migrate towards the site of infection where they eliminate any foreign material in an effort to prevent wide-spread tissue damage and ultimately resolve infection. Previous work on neutrophil function in the elderly has highlighted a number of neutrophil effector functions, including phagocytosis, superoxide production and migration that exhibit decreased efficiency suggesting the potential for reduced pathogen clearance in older adults.

This thesis reveals a migratory phenotype distinctive of neutrophils isolated from healthy elderly donors (> 60 years) and characterised by a maintained speed of migration (chemokinesis) but with significantly reduced directional migration (chemotaxis) and overall migratory accuracy in response to a range of chemoattractants. This migratory phenotype was shown to be associated with a constitutive basal activation of PI3Kinase in neutrophils isolated from older donors and appears to be a causative factor as treatment of neutrophils with inhibitors selective for PI3Kinase-γ and –δ, was able to restore migratory dynamics. The 'old-migratory' phenotype was amenable to correction by pre-incubation with 1nM Simvastatin *in vitro* and a two-week prescription of 80mg/day Simvastatin *in vivo* in healthy older adults.

The ability of simvastatin to modulate migratory dynamics potentially provides a safe, cost effective intervention to reduce morbidity and mortality from infections in the elderly population.

To Mum and Dad

Thank you doesn’t even begin to cover it.

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Chapter 6: Clinical Trail: Can Simvastatin Improve Neutrophil Functions in the Healthy Elderly

List of Abbreviations

α1-AT	alpha1-Antitrypsin
α2M	α2-macroglobulin
AE	Adverse Events
ANOVA	Analysis of Variance
Arp2/3	Actin-Related Protein-2/3
ATP	Adenosine Triphosphate
ADCC	Antibody Dependent Cell Cytotoxicity
AEBSF	4-(2-Aminoethyl) Benzensulfonyl Fluoride Hydrochloride
ANCA	Anti-neutrophil Associated Auto-Antibody
BHT	Butylated hydroxytoluene
BLT1/2	Leukotriene-B_4 Receptor 1/2
BMI	Body Mass Index
BMP	bis (monoacylglycerol) phosphate
BPI	Bacterial Permeability Increasing Protein
BSA	Bovine Serum Albumin
Btk	Bruton's Tyrosine Kinase
χ^2	Chi^2
C	Cysteine
C/EBP	CCAAT/enhancer binding protein
C3	Complement Protein C3
C4	Complement Protein C4
C5a	Complement Protein 5a
C5aR	Complement Protein 5a Receptor
CAP	Community Acquired Pneumonia
Cer	Ceramide
CD	Cluster of differentiation

Cdc42	Cell Division Control Protein 42
CGD	Chronic Granulomatous Disease
CHO	Cholesterol
CK	Creatine Kinase
CL	Cardiolipin
CLP	Common Lymphoid Progenitor Cells
CMV	Cytomegalovirus
CN	Cyclic Neutropenia
COPD	Chronic Obstructive Pulmonary Disease
Cr	Creatinine
CR1	Complement Receptor-1
CR3	Complement Receptor-3
CRIB	Cell Division Control Protein 42/ Ras-related C3 botulinum toxin substrate Interactive Binding Domain
CRP	C-Reactive Protein
CTP	Citidine-triphosphate
DAG	Diacylglycerol
DCs	Dendritic Cells
DHEA	Dehydroepiandrosterone
DHEAs	Dehydroepiandrosterone-sulphate
DMPA	Dimyristoyl-phosphatidic Acid
DMPC	Dimyristoyl-phosphatidylcholine
DMPE	Dimyristoyl-phosphatidylethanolamine
DMPG	Dimyristoyl-phosphatidylglycerol
DMPS	Dimyristoyl-phosphatidylserine
DRFs	Diaphanous-Related Formins
DTT	Dithiothreitol

EBV	Epstein Barr Virus
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
E.coli	*Escherichia coli*
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme-linked-immuno-sorbent assay
ENA78	Epithelial neutrophil activating protein 78
ER	Endoplasmic Reticulum
ERG	Ergosterol
ERK	Extracellular signal-related kinase
ESI-MS	Electrospray Ionisation Mass Spectroscopy
ESL-1	E-Selectin Ligand-1
F-actin	Filamentous Actin
FEV	Forced Expiratory Volume
FITC	Fluorescein Isothiocyanate
fMLP	f-Met-Leu-Phe
FPP	Farnesyl Pyrophosphate
FPR1	fMLP Receptor-1
FVC	Forced Vital Capacity
GalCer	Galactosylceramide
GAP	GTPase Activating Protein
G-CSF	Granulocyte Colony Stimulating Factor
GDF	Guanosine-diphosphate Dissociation Factor
GDI	Guanosine-diphosphate Dissociation Inhibitor
GDP	Guanosine-diphosphate
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein

GGP	Geranylgeranyl pyrophosphate
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
GPCR	G-protein Coupled Receptor
GRK	G-protein-coupled Receptor Kinase
GROα	Growth Related Oncogene α
GSL	Glycopshingolipids
GTP	Guanosine-triphosphate
H2A	Histone 2A
H2B	Histone 2B
H_2O_2	Hydrogen Peroxide
Hb	Haemoglobin
HBSS	Hanks Balanced Salt Solution
HCl	Hydrochloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperasineethanesulfonic acid
HDL	High Density Lipoprotein
HIV1	Human Immunodeficiency Virus-1
HMG-CoA	5-hydroxy-3-methylglutaryl-coenzyme-A
HOCl	Hypochlorous Acid
HM Kinase	Hydrophobic Motif Kinase
HPA	Hypothalamic-Pituitary-Adrenal Axis
HRP	Horse-radish Peroxidase
HSC	Haematopoietic Stem Cell
HSP	Heat Shock Protein
ICAM1	Intercellular Adhesion Molecule-1
ICU	Intensive Care Unit
IDL	Intermediate Density Lipoprotein
IgA	Immunoglobulin-A

IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
IFNγ	Interferon-γ
IκK	I kappa – Beta Kinase
IL	Interleukin
iNKT Cell	Invariant Natural Killer Cell
iNOS	Inducible Nitric Oxide Synthase
INR	International Normalized Ratio
IP_3	Inositol-triphosphate 3
IQR	Interquartile Range
IRP	Immune Risk Phenotype
ISL	Inositol sphingolipid
JAM-A	Junctional Adhesion Molecule-A
JNK	Jun N-terminus Kinase
K	Potassium
KC	Keratinocyte Chemoattractant
KCO	Gas Transfer Coefficient
KLRG1	Killer Cell Lectin-like Receptor subfamily G member 1
LAD	Leukocyte adhesion Deficiency
LDL	Low Density Lipoprotein
LFA1	Leukocyte Function Associated Antigen-1
LFT	Liver Function Tests
LHPC	Low-substituted Hyrdoxypropyl Cellulose
LIMK	LIM Kinase
LoS	Length of Stay
LPA	Lysophosphatidic Acid
LPS	Lipopolysaccharide

LTB_4	Leukotriene-B_4
Mac1	Macrophage -1-antigen
MAPK	Mitogen Activated Protein Kinase
Mcl-1	Induced Myeloid Leukaemia Cell Differentiation Protein-1
MCP1	Monocyte Chemoattractant Protein -1
mDCs	Myeloid Dendritic Cells
MEK	Mitogen Activated Protein Kinase Kinase
MFI	Mean Fluorescence Intensity
MHC II	Major Histocompatibility Complex class II
MHRA	Medicines and Healthcare Products Regulatory Agency
MLCK	Myosin Light Chain Kinase
MOPS	3-[N-Morpholino] Propanesulfonic Acid
MPO	Myeloperoxidase
MRC	Medical Research Council
mTORC1/2	mammalian Target of Rapamycin Complex 1/2
Na	Sodium
Na_3VO_4	Sodium Orthovanadate
NAC	N-acetyl-L-cysteine
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine Dinucleotide Phosphate
NaF	Sodium Fluoride
NAP2	Neutrophil Activating Peptide 2
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor Kappa Beta
NHS	National Health Service
NK Cells	Natural Killer Cells

NKCC	Natural Killer Cell Cytotoxicity
NL	Neutral Loss
O_2^-	Superoxide Radical
OH^-	Hydroxyl Radical
P	Precursor
PA	Phosphatidic Acid
PAF	Platelet Activating Factor
PAK	p21 Activated Kinase
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
pDCs	Plasmacytoid Dendritic Cells
PDK1	3-Phosphoinositide-Dependent Protein Kinase-1
PE	Phosphatidylethanolamine
PECAM1	Platelet/Endothelial Cell Adhesion Molecule-1
PG	Phosphatidylglycerol
PH	Pleckstrin Homology
PHLPP1/2	Pleckstrin Homology Domain leucine-rich repeat-containing protein phosphatase 1/2
PI	Phagocytic Index
PI(3)P	phosphatidylinositol-3-phosphate
PI(4)P	phosphatidylinositol-4-phosphate
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-triphosphate
$PI(3,4)P_2$	Phosphatidylinositol-3,4-bisphosphate
$PI(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate
PI3Kinase	Phosphatidylinositol-3-kinase
PKB	Protein Kinase B

PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear Neutrophils
PP2A	Protein Phosphatase 2A
PPoD	Predicted Probability of Death
PS	Phosphatidylserine
PSGL-1	P-Selectin Glycoprotein Ligand-1
PL	Phospholipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PR3	Proteinase-3
PRR	Pathogen Recognition Receptors
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinylidene Difluoride
PYH	Pack Year History
QEHB	Queen Elizabeth Hospital Birmingham
RA	Rheumatoid Arthritis
Rac	Ras-related C3 botulinum toxin substrate
RBD	Ras Binding Domain
RhoA	Ras homolog gene family member A
ROCK	Rho-associated Kinase
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
S1P	Sphingosine-1-phosphate
S.aureus	*Staphylococcus aureus*
SAE	Serions Adverse Events

SCAR	Suppressor of cAR
SCN	Severe Congenital Neutropenia
SD	Standard Deviation
SDF1	Stromal-cell Derived Factor-1
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
Ser	Serine
SHIP1	Phosphatidylinositol-3,4,5-triphosphate 5-phosphatase
SIRS	Systemic Inflammatory Response Syndrome
SLPI	Secretory Leukocyte Peptidase Inhibitor
SOD	Superoxide Dismutase
SM	Sphingomyelin
SPH	Sphingosine
TB	Tuberculosis
TBS	Tris-buffered Saline
TG	Triacylglycerol
TGFβ	Transforming Growth Factor-β
Thr	Threonine
TLC	Total Lung Capacity
TLR	Toll-like Receptor
TSH	Thyroid Stimulating Hormone
TNFα	Tumour Necrosis Factor-α
TREM-1	Triggering Receptor Expressed in Myeloid Cells
TST	Thyroid Function Tests
Tyr	Tyrosine
U&E	Urea and Electrolytes
Ur	Urea

VLDL	Very Low Density Lipoprotein
VLA	Very Late Antigen
VZV	Varicella Zoster Virus
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	WASP family Verprolin-homologous protein
WBC	White Blood Cell
WTCRF	Wellcome Trust Clinical Research Facility
ZAP	Zymosan Activated Plasma

CHAPTER 1

INTRODUCTION

1.0 Introduction

The demographics of our population are changing; since 2001 the number of males aged 75 and over has increased by 26% [1]. Current projections estimate the proportion of our population aged > 60 years will reach 21% by 2050, a forecast based on past proportions in which older adults constituted 8% of the population in 1950 increasing to 10% in 2000 [2]. By 2034 it is anticipated that 23% of the UK population will be over 65 years of age with 5% over 85 years [3], proportions that will exceed those aged ≤ 16 years by 5% [4]. Driving these changes is the continual increase in life expectancy seen in the Western world, currently at a rate of approximately 2 years per decade [5]. Importantly, increments in lifespan are not matched by improvements in healthspan, defined as the number of years the average person can expect to remain in good health [5] thereby reducing quality of life in our later years and increasing healthcare utilisation which is associated with significant economic costs. For example, in-patient care for the treatment of community acquired pneumonia totalled £383.7 million of which 65% of admissions were in the 64 years and over age group [6]. Furthermore, in 2007 – 2008, heath care costs associated with a retired household totalled approximately £5,200 per annum; this is compared to £2,800 for a non-retired household [7] while department of health estimates suggest the average cost for provision of both hospital and community healthcare for a single adult aged ≥85 years is approximately 3 times that of an adult aged 65 - 74 years [7].

The link between mortality and ageing was first described by Benjamin Gompertz in 1889 when trying to model mortality rates mathematically. He wrote "*…the power to oppose destruction loses equal proportions in equal times"* [8], meaning that the older we get the less likely we are to survive. 123 years later we can now partly attribute the increased morbidity observed in old age to a progressive age-related decline in immune function. This is evidenced in part by:

(1) An increased incidence of infectious diseases in the elderly population of both bacterial and viral origin. This includes influenza [9], community acquired [10,11] and nosocomial pneumonia [12], urinary tract infections [13,14], tuberculosis [15,16], periodontitis [17] and shingles [18], the latter arising from re-activation of the varicella zoster virus (VZV) in older adults.

(2) Poorer outcomes of the elderly from infectious diseases including *Streptococcus pneumoniae* and staphylococcal infections [19], influenza [20] and community acquired pneumonia [21] among others.

(3) Increased incidence of chronic inflammatory and autoimmune conditions including diabetes [22,23], cardiovascular disease [24,25] and rheumatoid arthritis [26,27] in the elderly population.

1.1 The Immune System

The immune system has evolved to protect the host from infection, disease and injury and must therefore have the ability to immediately respond to both new and returning pathogenic threats. To achieve this, the immune system operates in two component parts: the adaptive and the innate (see **Figure 1.1**), though it is now clear that there is continual communication between the two and this divide is artificial in reality.

The adaptive immune response refers to antigen-specific lymphocyte populations that respond to a large array of antigen epitopes and encompasses the subsequent development of immunological memory providing long-term protection against pathogens. It is also known as the acquired immune response and is mediated by clonal selection and expansion of T and B lymphocytes upon contact with antigen, with a fraction of cells retained after each infection to provide immune memory. However, as this arm of the immune response can take a number of days to reach an effective level of protection, initial immediate protection against invading pathogens is provided by the innate immune system, which is capable of responding in a matter of hours. This branch of the immune response is present in all individuals at all times, is not antigen specific and does not increase with repeated exposure to a given pathogen as the adaptive side does. The main cellular mediators of the innate response are: the granulocytes, which respond effectively to rapidly dividing bacteria, fungi and yeast; monocytes/macrophages, which play a role in antigen presentation to T cells and also in immunity to intracellular pathogens; Dendritic cells (DCs) which are the major antigen presenting cells; and natural killer (NK) cells which provide immunity to virally infected cells and cancer cells.

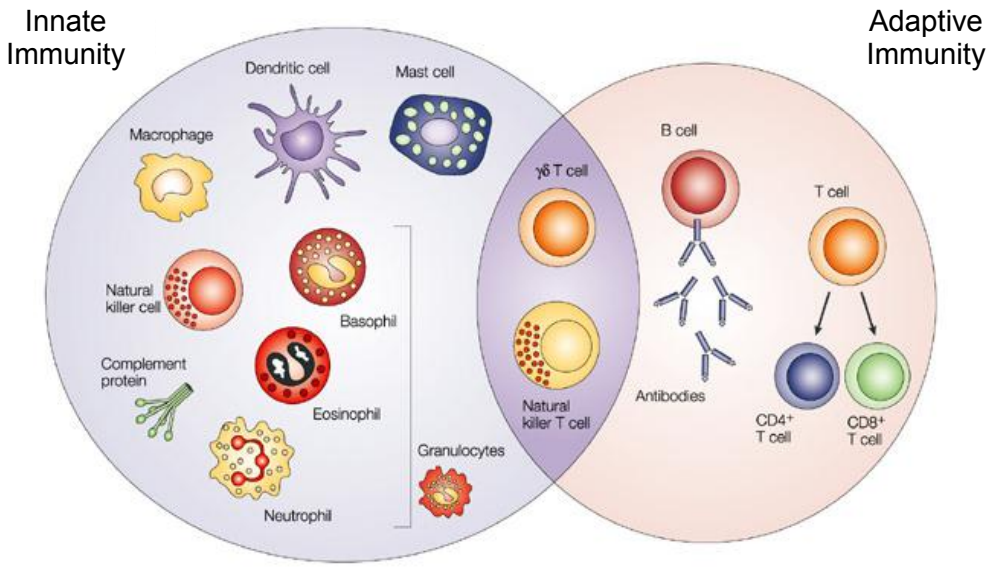


Figure 1.1 Mediators of the Innate and Adaptive Immune Response.

The innate immune compartment mediates the first response to infection providing immediate, non-specific protection. Components if the innate immune response include macrophages, natural killer cells, granulocytes (basophils, eosinophils and neutrophils), dendritic cells, mast cells and complement factors. Although, technically part of the innate compartment, dendritic cells also constitute a significant link between the innate and adaptive compartments in addition to cytotoxic lymphocytes such as γδ T cells and Natural Killer T cells. The adaptive immune compartment responds much slower, generating protection hours of days after activation however, this compartment is highly specific and generates immunological memory. The adaptive response is mediated by antibodies, B cells and CD4+ and CD8+ T cells.

Figure taken directly from [28]

1.2 Ageing and the Immune System

Ageing has been defined as a progressive, universal and intrinsic process that results in an increasing probability of death secondary to pathology [29], while Rossi *et al* [30] propose that ageing is characterized by a failure to maintain tissue homeostasis or return to normal homeostatic condition after exposure to stress or injury. The age-related decline or remodelling of the immune system was first described as immune-senescence by Makinodam and Kay [31] and manifests as an impairment of both cell-mediated and humoral immunity.

1.2.1 Adaptive Immune-senescence

Alterations in the adaptive immune compartment as a consequence of increasing age have been encompassed within the immune risk phenotype (IRP). This describes a set of immune parameters associated with poor immune function and increased mortality. The proportion of individuals within the population that satisfy the criteria of the IRP increases with advancing age [32].

The IRP was initially characterised by an altered ratio of $CD4^+$:$CD8^+$ cells (i.e. <1.0) with reduced proliferation to the T cell mitogen, Concanavalin A, [33]. An inverted $CD4^+$:$CD8^+$ ratio appears to be the result of both a reduction in the number of $CD4^+$ and an increase in $CD8^+$ cells. Immune senescence is also marked by atrophy of the thymus, which begins from puberty, and a decrease in the naive: memory ratio for T cells. The latter may be due to an attempt to maintain T cell homeostasis after thymic atrophy by expansion of the peripheral T cell pool [34,35]. Over subsequent years, the IRP has been expanded by various groups and now includes additional parameters including decreased telomerase activity and shortened telomeres in lymphocytes, cytomegalovirus (CMV) seropositivity, thymic atrophy and reduced $CD19^+$ B cells among others, forming what we now recognise as a more comprehensive IRP (see **Table 1.1**).

Cytomegalovirus (CMV) is a latent β-herpes virus present within 60-85% of the western population and approximately 80-90% of adults aged over 65 years [36]. CMV infection is thought to be a contributing factor to the development of the IRP and associated immune-senescence, and the driving force behind the accumulation of $CD3^{+}CD8^{+}CD28^{-}$ T cells [34]. CD28 is a co-stimulatory molecule required for full activation of T cells, of which reduced expression results in development of cellular anergy leading to limited activation and cytokine secretion. These cells have also been shown to be terminally differentiated [37] and exhibit an increased resistance to apoptosis [38]. In addition the terminally differentiated cells begin to express receptors normally associated with NK cells, such as NKG2D and killer cell lectin-like receptor subfamily G member-1 (KLRG1) [39], they also express cytotoxic granules [40]. As a result these senescent T cells are able to react more easily to the self-antigens recognised by the NK cell receptors, thus making older adults more prone to autoimmune disease. Taken together these alterations result in the accumulation of dysfunctional, autoimmune-prone, senescent T-cells that remain in the circulation, taking up immunological space, thus preventing formation of new immunological memory to novel pathogens and vaccines [41]. Older adults are thus more at risk from outbreaks of novel pathogens.

Table 1.1 Parameters of the Immune Risk Phenotype (IRP)

Parameter
$CD4^+$:$CD8^+$ Ratio ≤ 1
Thymic Atrophy
Decrease in the number of naïve ($CD8^+$ CD45RA $CCR7^+$) cell in the periphery
Reduced T cell proliferation (related to reduced IL2 secretion and sensitivity)
Accumulation of memory T cells
Reduced numbers of B cells ($CD19^+$)
CMV Seropositivity
Accumulation of CMV specific $CD3^+$ $CD8^+$ $CD28^-$ cells
Reduced Telomerase activity and subsequent telomere length

IL2, Interleukin-2; CMV, Cytomegalovirus

Natural ageing is also associated with the development of a chronic, systemic, sub-clinical inflammatory state termed inflamm-ageing [42]. This term, describes the increased levels of serum pro-inflammatory cytokines including Interleukin-6 (IL6), Tumour Necrosis Factor-α (TNFα) and acute phase proteins such as C-reactive Protein (CRP) [42-45]. It is also now recognised that ageing is accompanied by reduced levels of anti-inflammatory cytokines, notably interleukin-10 (IL10) [43] which contribute to inflamm-ageing. CMV was initially thought to be causally associated with the development of inflamm-ageing; however, recent studies have called this into question by demonstrating the development of inflamm-ageing in the absence of CMV infection [43]. Nevertheless, a number of candidates remain as potential drivers of inflamm-ageing including the presence of other latent viruses such as Epstein Barr Virus (EBV), increased adiposity (and the concomitant increase in adipokine production) [46,47] and a sedentary lifestyle [48]. It has also been proposed that chronic antigen exposure and subsequent immune exhaustion over the lifetime may contribute to inflamm-ageing [49].

Anti-inflamm-ageing is a term used to describe the activation of the hypothalamic-pituitary-adrenal (HPA) axis to produce a number of immune-modulatory hormones in response to pro-inflammatory cytokines [50,51] including IL6 and TNFα [52]. Most notably, hormones produced include cortisol, a well-characterised corticosteroid produced by the adrenal glands with powerful immunosuppressive effects [53] and dehydroepiandrosterone (DHEA), an immune enhancing hormone [54] which is found in the serum in its sulphated form dehydroepiandrosterone sulphate (DHEAs) [55]. An elegant balance exists between cortisol and DHEAs in regulating immune function however, in the elderly, this ratio shifts resulting in an increased cortisol:DHEAs ratio and a propensity for immune suppression [56]. This shift is driven by a gradual decline in the production of DHEAs and maintenance of cortisol levels across the life span, termed adrenopause [57]. Serum DHEAs concentrations peak around 24-30 years in males and 19-24years in females and decline thereafter to approximately 14%

of that maximal peak in males and 20% of maximum in females by the seventh decade [58,59].

Whether HPA activation is the result of chronic stress or an attempt to re-establish homeostasis as a result of the heightened pro-inflammatory state observed in the elderly remains to be determined. It is however, safe to conclude that the synergistic action of inflamm-ageing and anti-inflamm-ageing has consequences for immune function and may contribute to the senescent state observed in the elderly due to chronic low level stimulation of the immune system. Ultimately this renders these elderly individuals susceptible to infection, autoimmunity and disease. In addition low level systemic inflammation is now known to be a significant pathogenic factor in the major age-related conditions such as atherosclerosis [60], dementia [61] and sarcopenia [62].

Together, inflamm-ageing, the IRP and anti-inflamm-ageing document wide spread senescence within the adaptive and humoral compartments indicating significant immune re-modelling as we age. A major clinical consequence of this is the reduced ability of the elderly population to produce a protective antibody titre and maintain that titre over time following vaccination against a number of pathogens including diphtheria, tetanus, polio [63], pneumococcal pneumonia [64] and influenza [65]. After initial vaccination, booster injections are recommended every 10 years in the UK irrespective of the age of the recipient. Unfortunately, protective titres are rarely maintained over this time period, particularly adults aged over 60 years: 10 years post-vaccination, of 734 donors tested, 20% of recipients aged over 60 years no longer retained a protective titre. In fact, a significant proportion of recipients aged over 60 years (16%) were unable to maintain a protective titre just 5 years following vaccination [66]. In persons under the age of 60 years, antibody titres below the level that confers protection only occurred in <3% of subjects tested [66]. This study suggests that current vaccination strategies leave too many people over 60 years unprotected resulting in increased morbidity and mortality from many vaccine-preventable diseases. New strategies are needed to combat this phenomenon including an increased

frequency of vaccination and the development of new vaccines utilizing antigens and adjuvants specifically tailored toward the ageing immune system.

1.2.2 Innate Immune-senescence

The innate immune compartment consists of a number of cell types working synergistically to provide near-immediate, non-specific protection from infection (see **Figure 1.1**). Monocytes fulfil this requirement two fold by possessing their own anti-microbial arsenal but also through differentiation into tissue resident macrophages, which patrol the tissues and alert the immune system to potential pathogenic threats, through secretion of cytokines and presentation of antigen-derived peptides. Macrophages are also professional phagocytes capable of phagocytosis and production of toxic metabolites. Neutrophils are the first cell type to be recruited to the site of infection where they mediate host protection in much the same was as macrophages as they are professional phagocytes themselves. NK cells exist to protect against viral infection, whilst DCs constitute the major link to initiation of the adaptive arm of the immune response through antigen presentation and cytokine secretion. **Table 1.2** details the age-related changes observed in this compartment with the notable exception of neutrophils of which their development and function will be discussed in detail in later sections as these cells are the focus of this thesis.

Table 1.2 Innate Immune-senescence

Cell Type		Effect of Ageing		Reference
Monocytes	Number	↔	Unchanged	[67,68]
	Composition	↓	Classical ($CD14^+$ $CD16^-$)	[69]
		↑	Non-Classical ($CD14^+$ $CD16^+$)	[67,69]
	Function	↓	Chemotaxis	[70]
Macrophages	Number	↓	Frequency (alveolar macrophages)	[71]
	Function	↓	Chemotaxis	[70]
		↔	Phagocytosis	
			Increased	[72,73]
			Unchanged	[74]
			Decreased	[75,76]
		↓	ROS Production	[77-79]
		↓	Cytokine Production	[80-83]
	Signalling	↓	MHC II, CD80 and CD86 Expression	[84,85]
		↓	TLR Signalling Impaired	[81,82]
pDCs	Number	↔	Unclear	
			Reduced	[86-89]
			Unchanged	[86-91]
	Function	↓	Cytokine Secretion	[86,87,89,92,93]
	Signalling	↓	TLR-7 and -9 Expression	[86,87]
		↓	TLR Signalling Impaired	[93]
mDCs	Function	↓	Stimulatory Capacity	[93-96]
		↓	IFNγ Production	[96,97]
NK Cells	Number	↑	Increased	[98-101]
	Composition	↑	Mature $CD57^+$ NK Cells	[99,100,102]
	Function	↓	NKCC	[101,103,104]
		↓	ADCC	[100,105,106]
		↓	Cytokine Production	[107-110]
	Signalling	↓	KLRG1 & NKG2A Expression	[98,100,111]
iNKT Cells	Number	↓	Reduced	[112]
	Function	↓	IFNγ Production	[112,113]

Immune-senescence of the cell-mediated arm of the innate immune response.

MHC II, Major Histocompatibility Complex class II; TLR Toll-like Receptor; pDCs, plasmacytoid Dendritic Cell; mDCs myeloid Dendritic Cell; IFNγ, Interferon-γ; iNKT Cell, Invariant Natural Killer T Cell; NK Cell, Natural Killer Cell, NKCC; Natural Killer Cell Cytotoxicity, ADCC, Antibody Dependent Cell Cytotoxicity, KLRG1, Killer Cell Lectin-like Receptor subfamily G member-1.

1.3 Neutrophil Immune-senescence

Neutrophils form our first line of defence against bacterial infection and are the first cells to be recruited to the site of infection. In times of inflammation and infection, neutrophils leave the circulation and migrate into the tissues where they act in concert with tissue resident macrophages to neutralize and eliminate potentially pathogenic material utilising a diverse range of anti-microbial machinery. The absolute requirement of neutrophils in host defence is demonstrated in patients with chronic granulomatous disease (CGD) (reviewed in [114]) or leukocyte adhesion deficiency (LAD) syndrome (reviewed in [115]) who are extremely susceptible to bacterial and fungal infections which are often fatal. Underlying these pathologies are mutations in genes encoding key components of a neutrophils anti-microbial arsenal resulting in loss of function and ultimately compromising host defences.

1.3.1 Neutrophil Development

Cells of the immune system are derived from, and are subsequently replenished throughout life by haematopoietic stem cells (HSCs) which reside within the bone marrow. During development, HSCs commit to either the myeloid (granulocytes, monocytes, megakaryocytes and erythrocytes) or lymphoid (T and B cells, NK cells) lineage and populate the immune system. Cells of the myeloid lineage leave the bone marrow as fully mature cells whereas T and B cells leave the bone marrow as immature cells, with maturation occurring in the thymus and lymphoid organs respectively. Neutrophil maturation occurs in six stages, each characterised by the presence or absence of cell surface markers, granule subsets and nuclear morphology (see **Figure 1.2**).

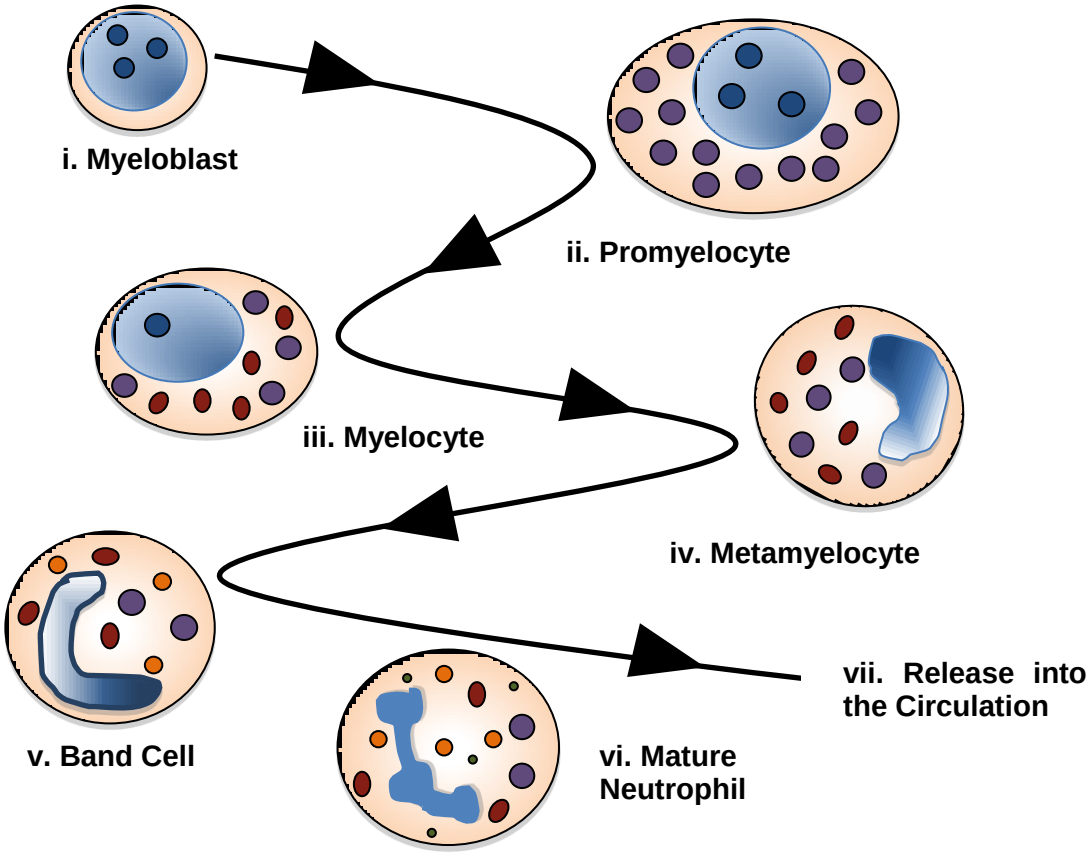


Figure 1.2 Neutrophil Bone Marrow Development.

Neutrophil development occurs in the bone marrow in 6 stages. (i) Myeloblast: an undifferentiated cell with a large nucleus and nucleolus, free from cytoplasmic granules; (ii) Promyelocyte: characterized by its large size, rounded nucleus and the first appearance of the azurophil granules in the cytoplasm; (iii) Myelocyte: large rounded nucleus with the first appearance of specific granules (iv) Metamyelocyte: exhibiting a kidney bean shaped nucleus with a mixed cytoplasmic granule population, at this stage in development, subsets lose their ability to proliferate becoming post-mitotic; (v) Band Cell: contains a condensed band shaped nucleus, and the first appearance of cytoplasmic gelatinase granules (vi) Mature Neutrophil: Nuclear constituents further condense forming the characteristic multi-lobed nucleus of peripheral blood neutrophils with the first appearance of cytoplasmic secretory vesicles (vii) Release into the blood stream.

Under steady-state conditions, fully mature neutrophils are released into the circulation at a rate of approximately $5x10^{10}$ - $1x10^{11}$ cells/day [116] with numbers differing depending on age, gender, ethnicity and smoking status [117]. Adult caucasian females have approximately $7.4x10^{9}$ neutrophils/litre and caucasian adult males $7.2x10^{9}$ neutrophils/litre at any one time throughout the day [117]. Once in the circulation, neutrophils were widely thought to have a half-life of approximately 6-10 hours [116,118-122] due to the constitutive expression of the anti-apoptotic protein induced myeloid leukaemia cell differentiation protein-1 (Mcl-1), levels of which decline rapidly when cells are isolated from the blood and show a positive association with neutrophil survival [123]. However, neutrophil half-life has recently come under scrutiny with a new estimate, established using *in vivo* isotope labelling techniques, putting the half-life at 5.4 days [124]. During an infectious insult and in response to exposure to pro-inflammatory cytokines and bacterial products such as granulocyte/macrophage-colony stimulating factor (GM-CSF), Interleukin-1β (IL1β), Interferon-γ (IFNγ) and Lipopolysaccharide (LPS) [123,125], neutrophil output from the bone marrow increases approximately 10-fold [126] and the typical half-life of a neutrophil increases to 115 hours [125].

A number of factors are involved in the development of HSCs into mature neutrophils including differential expression of transcription factors, including CCAAT/enhancer binding protein (C/EBPs) and PU.1 [127], proteins including the small GTPase Ras-related C3 botulinum toxin substrate (Rac2) [128] and soluble factors such as granulocyte-colony stimulating factor (G-CSF), GM-CSF and interleukin-3 (IL3) [129,130]. Of these three factors, G-CSF seems to be the dominant factor in generating mature neutrophils as mice lacking the G-CSF gene ($G\text{-}CSF^{-/-}$) exhibit a 73% significant reduction in the number of mature neutrophils in the circulation compared to their wild type ($G\text{-}CSF^{+/+}$) counterparts [129]. Regulation of G-CSF levels within the bone marrow is also involved in the resolution of inflammation utilizing a feedback mechanism which also includes Interleukin-23 (IL23), and Interleukin-17 (IL17). Uptake of apoptotic neutrophils by tissue resident macrophages, a

process known as efferocytosis, induces a reduction in the secretion of IL23 by macrophages and DCs and IL17 by γδ T cells with an ensuing reduction in bone marrow G-CSF levels [131]. This results in reduced neutrophil release into the circulation and therefore restores homeostasis.

Within the hematopoietic stem cell compartment, a number of age-related changes have been observed. CD34, an adhesion molecule whose expression is gradually lost during development, is used to identify hematopoietic stem and progenitor cells present within the bone marrow and circulation. Numbers of $CD34^+$ cells in the bone marrow remain unchanged with age [130], however, increasing age negatively correlates with the number of circulating $CD34^+$ cells [132]. Within the circulation, $CD34^+$ cells contribute to vascular health due to their endothelial progenitor cell capacity, hence the reduced frequency of these cells associates with increased risk of cardiovascular disease [133].

With increasing age there is also a shift in the homeostatic control of HSC differentiation favouring progenitors of the myeloid lineage at the expense of common lymphoid progenitor cells (CLP) [30,134,135]. This imbalance can be traced back to the genomic level with 70% of lymphoid genes differentially expressed in aged HSCs were found to be down-regulated while 76% of differentially expressed myeloid genes were up-regulated [30]. Modulation in this manner appears not to result from modification of the genes themselves but instead from epigenetic dysregulation [136]. This phenomenon seems to have a greater impact on the adaptive immune response and although it is worth noting, it does not appear to have an effect on the number of late myeloid progenitors in the bone marrow [130,137] or mature neutrophils in the circulation [130,138].

Fully mature neutrophils can be found within the bone marrow, circulation and liver/spleen, with each compartment comprising 15%, 40% and 45% of the total marginated granulocyte pool [139]. If, and when, exposed to an inflammatory signal, neutrophils leave these sites and migrate through tissue to the site of infection where they mediate host defence by phagocytosing, eliminating and preventing further dissemination of pathogenic material.

1.3.2 Neutrophil Anti-microbial Functions

Neutrophils have three main mechanisms for killing microbial targets: phagocytosis followed by generation of reactive oxygen and nitrogen species; release of degradative granule contents; and extracellular killing via neutrophil extracellular traps. Intracellular mediators of neutrophil anti-microbial functions are fully developed upon release from the bone marrow and are stored throughout the life of a neutrophil ready to be employed as soon as the appropriate stimulus is received thus mediating host defence.

1.3.2.1 Neutrophil Degranulation

Upon egression from the bone marrow, neutrophils contain anti-microbial proteins and pro-inflammatory mediators utilized during an immune response in addition to a number of surface receptors and membrane proteins all of which are stored in four distinct granule and secretory vesicle subsets: the azurophil (primary), specific (secondary) and gelatinase (tertiary) granules and finally secretory vesicles, named according to their order of appearance during neutrophil development [140]. When the appropriate stimulus is received, granule release, known as degranulation, occurs in a hierarchical manner [141]. Neutrophils isolated from the inflammatory exudate obtained from a blister model of skin inflammation demonstrate 38.1% release of gelatinase, 21.9% of specific and 7% azurophil granule release with complete release of secretory vesicles [142]. Azurophil granules are thought to play a major role in the degradation of phagosomal contents [143] owing to the nature of anti-microbial and cytotoxic proteins such as neutrophil elastase, myeloperoxidase (MPO) and cathepsin G which may account for their limited extracellular release upon stimulation. Granule contents are determined by their presence or absence during the developmental process, which is known as the targeting by timing hypothesis [144]. Azurophil granules are identified by the presence of MPO [145] whereas the presence of gelatinase but absence of lactoferrin denotes gelatinase granules [146] (see **Table 1.3**). There are also a number of protein markers specific for different granule subsets, for example CD63, CD67 and annexin

I which identify the azurophil, specific and gelatinase granules respectively [147-149] while alkaline phosphatase and CD35 (CR1) have been used as markers of secretory vesicles [142].

Neutrophil functionality is mediated by synergistic granule release. For instance, proteins contained within all three granule subtypes are required for efficient production of reactive oxygen species [146]. In addition, mobilisation of secretory vesicles to the plasma membrane is thought to be the source of the adhesion complex CD11b/CD18 (Macrophage -1-antigen, Mac-1) which mediates firm adhesion upon the endothelium and allows extravasation from the circulation. CD11b (stored within the specific and gelatinase granules and the secretory vesicles) is also involved in recognition of complement protein C3bi and therefore mediates phagocytosis of opsonised material.

Acting in unison, granules and their contents are thus able to modify both the neutrophil surface phenotype, and the extracellular environment as well as being critical mediators of anti-microbial functions.

To date, there are few reports regarding the effect of ageing on neutrophil degranulation and the release of proteases. In 1990, MacGregor *et al* [150] found that the proportions of both β-glucuronidase in primary granules or vitamin B-12 binding protein in secondary granules was not affected by increased chronological age; nor did they find any change in the extent to which granules were released either spontaneously i.e. in the resting state, or following stimulation with fMLP [150]. This is supported by Dalboni *et al* [151] who demonstrated maintenance of elastase activity in neutrophils from old donors when compared with those from young donors in response to 10ng/ml IL8 [151].

Table 1.3 Neutrophil Granule Constituents

	Azurophil Granules	**Specific Granules**	**Gelatinase Granules**	**Secretory Vesicles**
Membrane	CD63	CD11b/CD18	CD11b/CD18	CD11b/CD18
		Cytochrome b_{558}	Cytochrome b_{558}	Cytochrome b_{558}
		fMLP-R	fMLP-R	fMLP-R
		G-protein α-subunit		CD16
		Leukolysin	Leukolysin	Leukolysin
		TNF-R		CD45
Cytosol	α1-antitrypsin	Collagenase	Acetyltransferase	Plasma Proteins
	BPI	Gelatinase	Gelatinase	
	Cathepsins	Lactoferrin		
	Defensins			
	Elastase			
	MPO			
	Proteinase-3			
	Lysozyme	Lysozyme	Lysozyme	

BPI, Bacterial Permeability Protein; MPO, Myeloperoxidase; fMLP-R, fMLP Receptor; TNF-R, TNF Receptor;

Adapted from [152]

1.3.2.2 Phagocytosis

Phagocytosis is the process by which foreign or altered-self material is recognised and internalised into a membrane bound vesicle via cytoskeletal re-modelling in a receptor-mediated manner. Following ingestion a complex network of intracellular signalling pathways are activated resulting in the degradation of ingested material [153]. These events include phagosome fusion with lytic granules, assembly of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH oxidase; see the following section for more detail) and production of pro- or anti-inflammatory cytokines. Depending on the type of material ingested, the secretory cytokine profile of these cells can be altered i.e. ingestion of pathogenic material initiates production of pro-inflammatory cytokines such as TNFα and IL8 [154] whereas ingestion of apoptotic cells induces secretion of anti-inflammatory cytokines such as transforming growth factor-β1 (TGFβ1), Prostaglandin E_2 [155] commencing the resolution of inflammation.

A diverse repertoire of receptors expressed on the neutrophil surface are capable of stimulating phagocytosis, these include but are not limited to, Fcγ Receptors (FCγRII [CD32] and FcγRIII [CD16]), which recognise antibody opsonised material and complement receptors (CR1 [CD35] and CR3 [CD11b]) which recognise complement opsonised material [156,157].

With ageing, a number of groups have observed a reduction in the phagocytic ability of neutrophils to a variety of stimuli including opsonised yeast [158-160]; *Staphylococcus aureus* (*S.aureus*) [161] and *Escherichia coli* (*E.coli*) [138,161].

Potential underlying mechanisms for reduced phagocytic ability in the elderly have been studied with a number of likely candidates put forward. Opsonisation marks material for destruction making phagocytosis more efficient. Major opsonins within the immune system are serum immunoglobulins and complement proteins. Serum concentrations of immunoglobulin-M (IgM) are significantly lowered with age whilst immunoglobulin-G (IgG) and immunoglobulin-A (IgA) concentrations remain unaltered [150]. Serum complement

proteins C3 and C4, show a 19% and 57% increase respectively in the elderly, however, values for both immunoglobulins and complement proteins remain within the normal clinical range [150] and are therefore unlikely to be driving reduced phagocytic function. In addition, surface expression of a number of phagocytic receptors remains unchanged with age including TLR2 and 4 [162], the fMLP receptor [163] and CR3 (CD11b) [138]. However, surface expression of FcγRIII (CD16) is significantly reduced on neutrophils from elderly donors and positively correlates with the observed reduction in phagocytic index [138]. At present, no studies have considered the effects of age on surface expression of other receptors involved in the phagocytosis of phagocytic material such as CD14 (an essential co-receptor for TLR4 in recognising lipopolysaccharide (LPS)), CD32 or CD35 and therefore defects in phagocytic ability cannot yet be fully attributed to reduced CD16 expression alone. However, phagocytosis of gram-positive bacteria such as *S.aureus*, is highly dependent on Fc receptors [164] and therefore may contribute to the increased incidence of *S.aureus* infection evident in the elderly population [165].

1.3.2.3 ROS Production

Reactive oxygen species (ROS) is an umbrella term that refers to a number of free radical oxidising species such as superoxide (O_2^-) and hydroxyl species (OH^-) as well as non-radical oxidising species such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) [166]. ROS are produced primarily through the action of the enzyme NADPH oxidase [167], which is assembled on the phagosome membrane during maturation [168]. NADPH oxidase transfers electrons from NADPH onto molecular oxygen to produce the radical O_2^- in a process known as the respiratory burst. This free radical is then metabolised by superoxide dismutase (SOD) to produce H_2O_2, which in itself is cytotoxic but can also be further metabolised by MPO, delivered to the phagosome through fusion with primary granules, to produce HOCl. In the basal state, the 5 subunits of NADPH oxidase reside independently, either membrane bound ($p22^{phox}$ and cytochrome b_{558}, also known as $gp91^{phox}$), or in the

cytosol ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) and are assembled on the phagosome or plasma membrane along with the small GTPase Rac2 in response to stimulation [169]. In addition, other GTPases such as Arf protein family members, particularly Arf6 have been shown to be required for activation of NADPH oxidase in a phospholipase-D (PLD) dependent manner [170].

Production of ROS are vital for maintaining the anti-microbial defences of neutrophils and macrophages, but also have a role in maintaining homeostasis in the absence of inflammation, acting as second messengers in a number of signalling pathways (such as tyrosine kinase activation [171], NF-κB [172] and Protein Kinase C (PKC) [173] activation) and as electron carriers in the electron transport chain.

The literature on the effect of age on the production of ROS is contradictory. A number of groups have reported a reduction in the levels of O_2^- produced in response to GM-CSF and fMLP stimulation [174-177] while other groups have reported enhanced ROS production (particularly H_2O_2) by neutrophils from elderly donors [178,179]. This discrepancy has been clarified recently with ROS production varying across stimuli, including the response to gram-positive or gram-negative bacteria. Neutrophils from elderly donors exhibit a significant reduction in ROS production in response to stimulation with *S.aureus* (gram-positive) compared to neutrophils from young donors, however, when stimulated with *E.coli* (gram-negative), neutrophils from old donors demonstrated a maintained ability to produce ROS when compared to young donors [161]. Wenisch *et al* [161] postulated this discrepancy is due to the differential effects of ageing on receptor surface expression and the related signal transduction pathways e.g. the CD14-dependent, calcium-independent pathways involved in ROS production in response to *E.coli* but not *S.aureus* [161].

1.3.2.4 Neutrophil Extracellular Traps (NETs)

In 2004 Brinkmann *et al* [180] described a novel neutrophil function that was believed to have extracellular anti-microbial properties persisting beyond the typical neutrophil lifespan. This

function was termed Neutrophil Extracellular Traps (NETs) and refers to the presence of neutrophil granule and nuclear constituents in an extracellular web-like structure made up of a chromatin backbone studded with cytoplasmic and granule proteins including histones (specifically H2A and H2B), MPO, serine proteases (neutrophil elastase, proteinase 3 and Cathepsin G), bacterial permeability increasing protein (BPI), lactoferrin and calprotectin [180-182]. Not only does the chromatin backbone provide the structural support for these anti-microbial proteins maintaining a high, localised concentration and potentially preventing collateral damage from these proteins, it also is hypothesised to limit microbial spread by physically trapping the bacteria and bringing them into direct contact with the anti-microbial proteins. A number of organisms that have been shown to bind to NETs include *S.aureus* [180], *Streptococcus pneumoniae* [183], *Salmonella typhimurium* [180] and the yeast *Candida albicans* [184]. NETs have also been shown to have both anti-fungal, mediating protection against fungal hyphae too large to be phagocytosed [184] and anti-viral activity against human immunodeficiency virus-1 (HIV1) mediating viral capture and subsequent elimination [185]. However, it is possible that the anti-viral activity of NETs is virus specific as NET formation is not required for protection against influenza infection [186].

Owing to their recent discovery, there is limited knowledge on the effect of increasing chronological age on NET production. Using a mouse model of severe skin infection, Tseng *et al* [187] demonstrated a reduced ability of neutrophils from aged mice to produce NETs in response to infectious stimuli with a concomitant increase in bacterial dissemination throughout the host [187]. As the production of reactive oxygen species has been shown to be essential in the formation of NETs [167], and since old age results in a stimuli-dependent reduction in ROS production [161] it is conceivable that NET production would decline with increasing age and may also be stimuli specific.

Of course the efficiency of these anti-microbial functions would be irrelevant should neutrophils be unable to migrate from the blood stream to the site of aggression be that of infection or injury. The ability to migrate, mechanisms controlling directional migration and

the effects of age on this process form the focus of this thesis and shall therefore be discussed in much greater detail than other neutrophil functions

1.4 Neutrophil Chemotaxis

Chemotaxis is the process of directed movement along a chemotactic gradient observed in many areas of cell biology and development including egress of immune cells from the bone marrow, neuronal development in the brain [188], leukocyte homing to the thymus [189], angiogenesis [190], re-epithelialisation mediating wound healing [191], metastasis [192] and immune defence against disease, injury and infection [193].

Chemotaxis begins with extravasation, the process whereby neutrophils leave the blood stream by adhering to and then transmigrating through the vascular endothelium. In order to begin extravasating from the blood stream, the endothelium itself must first become activated. This occurs following exposure to pro-inflammatory cytokines such as TNFα and IL8 produced by tissue resident macrophages in response to infection [194-196]. This induces the expression of a number of adhesion markers on the endothelial surface e.g. P- and E- Selectin, which bind P-selectin glycoprotein ligand-1 (PSGL-1) and E-selectin ligand-1 (ESL-1) respectively on the neutrophil surface inducing transient tethering allowing the neutrophils to roll along the vasculature [197,198]. This allows neutrophil recognition of chemotactic signals inducing neutrophil activation and delivery of β1 and β2 integrins, particularly Mac1 (αMβ2; a heterodimer consisting of CD11b and CD18) and leukocyte function associated antigen-1 (LFA1, αLβ2; a heterodimer of CD11a and CD18) stored within the cytoplasmic granules to the cell surface [199,200]. Cellular activation results in a conformational change within β2 integrins [201] allowing recognition of intercellular adhesion molecule-1 (ICAM-1) expressed on the endothelium. Ligation of integrins with endothelial ligands induces firm adhesion and arrest of rolling neutrophils onto the vasculature [199]. Platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31), CD99 and junctional

adhesion molecule-A (JAM-A) then facilitate homing to cell junctions allowing migration through the endothelium in a process known as diapedesis [202].

Two modes of diapedesis through the endothelium have been demonstrated: paracellular and transcellular [203]. Paracellular migration describes migration through the tight junctions between adjacent endothelial cells while transcellular migration describes migration through a pore an individual endothelial cell [204]. These modes of migration are thought to arise following the formation of 'invadosome-like protrusions', actin-dependent protrusive structures that 'probe' the endothelium finding sites at which diapedesis can occur [204]. Interestingly, vascular permeability does not appear to be affected by either mode of diapedesis due to formation of dome-like structures by endothelial cells encapsulating the migrating neutrophil and therefore preventing increases in vascular permeability [205]. Overall, the mechanisms driving diapedesis remain elusive however, blocking of PECAM-1 using monoclonal antibodies results in neutrophils that are capable of locating endothelial junctions but are unable to initiate diapedesis and are therefore unable to migrate out of the vasculature [206] whilst ICAM-1 has been shown to facilitate diapedesis by forming 'cup-like' structures around migrating cells guiding diapedesis [207,208].

Once through the endothelium neutrophils begin the process of tissue migration also referred to as interstitial migration. Initiation of this process begins with the interaction between a chemoattractant and their receptors on neutrophils. Neutrophils are exquisitely sensitive to gradients of chemotactic molecules demonstrating accurate movement in very shallow gradients – a 2% change in chemoattractant concentration across the length of the cell is capable of inducing robust migration [209]. Various lipid mediators such as Leukotriene-B_4 (LTB_4) and platelet activating factor (PAF), complement proteins such as C5a and C3bi, inflammatory cytokines including IL8 and stromal cell-derived factor-1 (SDF1) and bacterial-derived proteins such as fMLP and LPS are all neutrophil chemoattractants.

Chemotactic cytokines, known as chemokines, are classified into 4 groups based in the position of the first two, highly conserved cysteine (C) residues within the chemokine

structure: CXC, CX_3C, CC and C where X denotes an amino acid residue other than cysteine [210]. The CXC chemokine IL8 (CXCL8) is a particularly potent chemokine produced in response to inflammation mediating neutrophil extravasation from the blood stream and the initial stages of interstitial migration. Given the complex nature of the immune system, it is entirely plausible that a migrating neutrophil may be surrounded by multiple chemoattractants at any given time and must therefore navigate towards the most appropriate signal. To do this, neutrophils respond to chemoattractants in a hierarchical manner taking preference for 'end-target' chemoattractants over 'intermediary' chemoattractants. Heit *et al* [211] categorised intermediary chemoattractants e.g. IL8 and LTB_4, as those that induce extravasation and bring neutrophils into the general vicinity of infection whereas 'end-target' chemoattractants are those produced directly by bacteria or through direct contact of immune components with bacteria e.g. fMLP and C5a and therefore hone directional migration to the site of infection. Therefore, not only do neutrophils respond to chemoattractants in a hierarchical manner [212] but chemoattractants themselves exist in distinct zones throughout the tissue guiding neutrophils directly and accurately to the site of infection or injury [213]. Production in this manner then requires neutrophils to ignore a particular chemoattractant once its source has been reached in order to continue toward the site of infection. This process is known as desensitisation and is achieved through internalisation of the chemoattractant receptor [211] a process involving G-protein-coupled receptor kinases (GRKs) and β-arrestin (reviewed in [214]).

Most chemoattractants, and all thus far identified chemokine receptors, are G-protein coupled receptors (GPCR) composed of 7-transmembrane domains (3 extracellular and 3 intracellular loops) coupled to heterotrimeric guanine nucleotide binding proteins (G-proteins) at the inner leaflet of the plasma membrane [210]. GPCRs constitute the largest superfamily of membrane receptors containing receptors for hormones, inflammatory mediators (both pro- and anti- inflammatory), lipids and calcium ions [215]. Known G-protein coupled chemoattractant receptors include CXCR1 (IL8-Ra) and CXCR2 (IL8-Rb) [210], FPR1 and 2

which recognise formylated peptides such as fMLP, C5a-receptor and BLT1 and 2 (LTB_4 receptors) allowing neutrophils to respond to both intermediary and end-point chemoattractants.

G-proteins consist of 3 subunits – α, β and γ that, in the quiescent state, exist as a single heterotrimeric protein with guanosine di-phosphate (GDP) associated to the α subunit [216]. Interaction of the GPCR with its ligand causes a conformational change allowing the exchange of GDP for GTP (guanosine-tri-phosphate) by the Gα-subunit and dissociation from the Gβγ-subunit [216,217]. This allows independent activation of a diverse and complex range of intracellular signalling pathways including PI3Kinase, PLC and small GTPases such as Ras- and Rac- GTPase (reviewed in [218]). Pathways activated by G-protein subunits are shown in **Table 1.4**. Heterogeneity between GPCR and the responses they elicit have been attributed to the combination of G-protein isoforms making up the complete protein. 4 classes of the α subunit have been identified – $G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12/13}$ which share 45-80% homogeneity whilst 5 β- and 12 γ- isoforms have been identified exhibiting 27-75% homegenity and a high rate of post-translational modification [219]. GPCR mediated signalling is antagonised through the intrinsic GTPase activating protein (GAP) activity of the Gα subunit hydrolysing GTP, restoring GDP to the nucleotide binding pocket. This causes the Gα subunit to re-associate with the βγ subunit once again rendering the complex inactive (reviewed in [218,219]).

Through coupling of membrane receptors to G-proteins, neutrophils are thus able to respond to a number of stimuli co-ordinating the relevant cellular response that provides both host protection against infections and mediates tissue repair upon injury or damage.

However, in order for effective migration to occur through tissues, we hypothesise that the release of granule-specific proteases during migration remodels the extracellular matrix (ECM) and facilitates recruitment to the site of infection. The major proteases involved in this process are elastase (EC 3.4.21.37), cathepsin G (EC 3.4.21.20) and proteinase 3 (EC 3.4.21.76). These serine proteases are stored within the azurophil granules of neutrophils

and possess both anti-microbial and fungistatic ability [220]. Small amounts of neutrophil elastase have also been observed within mast cells, basophils [221] and tissue resident macrophages [222].

Table 1.4 Function of Heterotrimeric G-protein Subunits

G-protein Subunit	Effect on Activity	Effectors
$G\alpha_s$	Enhances	Adenylyl cyclases
		Src Tyrosine Kinases
		GTPase of Tubulin
$G\alpha_i$	Reduces	Adenylyl cyclase
		Ca^{2+} Channels
	Enhances	Erk Activation
		K^+ Channels
		GTPase of Tubulin
		Src Tyrosine Kinases
$G\alpha_q$	Enhances	PLCβ
		K^+ Channels
		Bruton's Tyrosine Kinase
$G\alpha_{12/13}$	Enhances	PLD
		PLCε
		iNOS
		p115 Rho-GEF
		E-cadherin-mediated cell adhesion
		HSP90
Gβγ	Enhances	PLCβ
		PI3Kinase
		K^+ Channels
		P-Rex-1
		SRC Kinases
		GRK membrane recruitment
	Reduces	Adenylyl Cyclase I
		Ca^{2+} Channels

ERK, Extracellular Related Kinase; GTP, Guanine-tri-phosphate; PLC, Phospholipase C; PLD, Phospholipase D; iNOS inducible Nitric Oxide Synthase; GEF, Guanine Exchange Factor; HSP, Heat Shock Protein; PI3Kinase, Phosphatidylinositol-3-kinase; GRK, G-protein coupled Receptor Kinase.

Adapted from [218]

1.4.1 Serine Proteases as an aid to Migratory Processes

Upon neutrophil activation, serine proteases are released from the azurophil granules into either the extracellular space, although a proportion of this is retained within the plasma membrane [223-225], or released into the newly formed phagosome [226]. Importantly, serine proteases are also important mediators of non-oxidative host defence and act by facilitating transendothelial migration [223,227,228] and neutrophil detachment during migration [229]. Release of neutrophil elastase at the neutrophil leading edge mediates tissue digestion, allowing cells to move through dense tissue. The action of these proteases has also been associated with the progression of a number of chronic inflammatory diseases. For example, proteinase-3 (PR3) has been implicated in rheumatoid arthritis (RA) and anti-neutrophil associated autoantibody (ANCA) associated vasculitis [230] with an increased risk of relapse associated with increased surface expression of PR3 [231]. Elastase has also been implicated in disease pathogenesis, with an active role for this protease (usually acting in conjunction with other proteases) in the progression of chronic obstructive pulmonary disease (COPD) [232-235] and RA [236,237]. Mutations in the gene encoding neutrophil elastase, ELA2, have also been identified in 50% of patients diagnosed with severe congenital neutropenia (SCN), an inborn disorder of granulopoiesis due to arrest in the differentiation of granulocyte precursors, and in all cases of cyclic neutropenia (CN), a less severe form of granulopoiesis. Both SCN and CN leave individuals particularly susceptible to infection and have an increased propensity to develop acute myeloid leukaemia and myelodysplasia [238].

Although release of azurophil granules is relatively limited when compared to secretory vesicles or gelatinase granules, sequential release of single azurophil granules into the extracellular space has been shown to occur during neutrophil migration, each with concomitant protease activity [223,239]. Neutrophil elastase is released from granules at a concentration of 5.33mM [240], a concentration 150-1500 times greater than that of its endogenous inhibitors - α1-antitrypsin (α1-AT, 32.8μM) [239], secretory leukocyte peptidase

inhibitor (SLPI, 11μM) [241] and α2-macroglobulin (α2M, 3.5μM) [242] resulting in an area of obligate tissue damage at the point of release. Away from the point of release, the concentration of elastase decreases exponentially thus allowing inactivation of the protease once the elastase:inhibitor ratio has reached 1:1. At this point, protease activity is abolished by the action of local inhibitors, α1AT inhibiting free elastase in an irreversible manner and SLPI reversibly inhibiting membrane bound elastase [225], however this does not arise until a localised area of protease mediated tissue destruction has occurred, a phenomenon termed 'quantum proteolysis'.

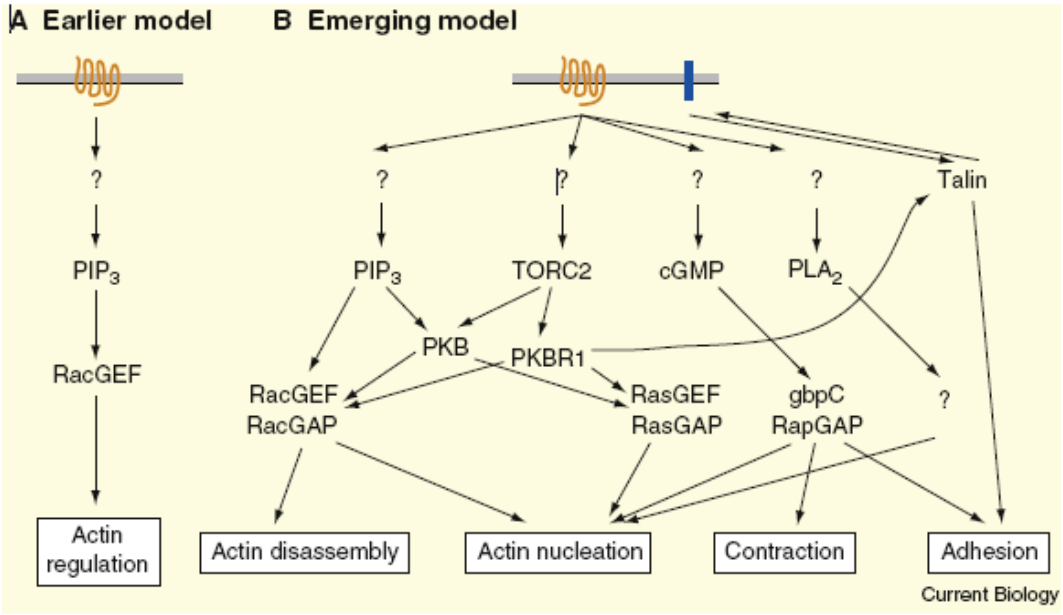
Endogenous substrates for these proteases encompasses a number of extracellular matrix components including elastin [243], fibronectin [244] and collagen [245] however out of the three serine proteases, neutrophil elastase is the most potent due its ability to degrade the vast majority of extracellular matrix components (reviewed in [246]). This makes neutrophil elastase a particularly powerful protease at mediating large-scale tissue damage, particularly during interstitial migration. Consequences of matrix degradation are undoubtedly negative when prolonged, for example in the context chronic inflammatory diseases or in the absence of protease inhibitors e.g. α1-AT deficiency. However, matrix degradation when adequately regulated, may also facilitate interstitial migration by exposing extracellular matrix components and strengthening host defences. Interstitial migration can occur in the absence of the β2 integrins [247], with adhesion instead mediated through the action of β1 integins such as very late antigen-6 (VLA-6, α6β2) and -9 (VAL-9, α9β2), expression of which is up-regulated following neutrophil activation e.g. in response to fMLP and transmigration following interaction with PECAM-1 [247,248]. β1 integrin ligands include a variety of extracellular matrix proteins including laminin [249], collagen [250] and fibronectin [251].

1.4.2 Signalling pathways involved in the regulation of migration

In order for migration to occur, neutrophils adopt a polarized phenotype involving the formation of a leading edge and uropod tail as a function of general motility [252]. Within this

morphology, the leading edge demonstrates enhanced sensitivity allowing directional sensing of the chemotactic gradient [253]. There are a significant number of signalling pathways involved in the generation of directional migration however it is important to remember they do not act in isolation and instead exist as a complex signalling network involving both positive and negative regulation, see **Figure 1.3**.

Figure 1.3 A Diagrammatic Representation of the Complex Network of Signalling Pathways Thought to be involved in the Regulation of Migration to Date



Taken directly from [254]

1.4.2.1 PI3Kinase

The phosphotidylinositol-3-kinase (PI3Kinase) family consists of conserved enzymes present in mammalian cells that phosphorylate the D3-position of the inositol ring of phosphoinositide lipids. This produces a number of inositol lipid second messengers in response to a number of extracellular signals including cytokines, integrin engagement and FcγR ligation. The PI3Kinase family, through association with pleckstrin homology (PH) domains present in target proteins, recruits a number of signalling molecules to the plasma membrane thus amplifying the signal.

The PI3Kinase family is divided into 3 classes based on their lipid products and the identity of the subunits present in each protein complex. Class I PI3Kinases, the most studied of the PI3Kinase subfamilies, are heterodimeric proteins consisting of both a regulatory and a catalytic subunit which phosphorylates phosphatidylinositol - 4,5 - bisphosphate ($PI(4,5)P_2$) to produce phosphatidylinositol - 3,4,5 - triphosphate (PIP_3), the only triphosphate inositol lipid present in mammalian species; Class II PI3Kinases exist as monomers and Class III as heterodimers both of which phosphorylate phosphatidylinositol (PI) to form phosphatidylinositol-3-phosphate (PI(3)P) (see **Table 1.5**).

Production of multiple lipid products places PI3Kinase at the centre of multiple signalling networks eliciting a role for this enzyme family in a multitude of cellular functions including migration, proliferation, cell survival and endocytosis. Human neutrophils are known to express all of the above mentioned isoforms [255], however, it is members of the class I family that are fundamental to the control of migration mainly due to their ability to be activated by a wide variety of cell surface receptors.

Class I PI3Kinase is sub-divided into class IA and class IB based on the structure of its component subunits: class IA contains 3 isoforms consisting of either a p85 or p55 regulatory subunit coupled to a p110α, β or δ catalytic subunit; the single isoform making up class IB PI3Kinase consists of a p101 regulatory subunit and p110γ catalytic subunit. Class I activation occurs downstream of receptor-ligand interaction on the cell surface via

phosphorylation of the regulatory subunit thus facilitating binding of PIP_2 by the catalytic subunit and its subsequent phosphorylation. In the case of Class IA and Class IB PI3Kinase, these are activated downstream of tyrosine kinase receptors and $G_{\alpha i}$ –containing GPCRs [256] respectively. However, recent evidence has demonstrated significant cross-talk in the activation both classes through the presence of a Ras binding domain (RBD) in the p110 subunit rendering both class IA and IB sensitive to the GTPase Ras [257]. Ras and Gβγ are now known to act synergistically to control PI3Kinase activation. The structure and function of the PI3Kinase family has been extensively reviewed in number of excellent review articles [255,258-261].

Table 1.5 PI3Kinase Isoforms in Mammalian Cells [255,258-260]

		Regulatory Subunit	Catalytic Subunit	Product	Activation	Cellular Processes	Localization
Class I	A	p85 or p55	p110 α β δ	PIP_3	Tyrosine Kinase Receptors (Integrins, FcγR); Ras-GTPase; Insulin Receptor A [262]	Neutrophil Directional Movement [263]	Cytoplasmic then recruited to the plasma membrane upon activation
	B	p101 or p84	p110 γ	PIP_3	Gβγ subunit of $G\alpha_i$-proteins; Ras-GTPase; Insulin Receptor-A [262]	Neutrophil Chemokinesis [264]	
Class II		None*	C2 α β γ	PI(3)P PIP_3 [265]	TC10-GTPase [266]; Clathrin [267]; Insulin-Receptor-B [265]; Calcium [268]; Chemokines; Integrin Engagement**	LPA-mediated migration [269] , endocytosis	Associated with membrane structures e.g. Clathrin coated vesicles [270]
Class III		p150	hVsp43p	PI(3)P	?	Intracellular trafficking e.g. autophagy [271] and phagosome formation [272]	Intracellular membranes e.g. Golgi & endosomes[273]

*Although Class II does not, at present, appear to have a regulatory subunit per se; its protein structure does contain several protein domains through which interaction with adaptor proteins is thought to regulate activation [274].
**Mechanisms for activation for Class II PI3Kinase remain poorly understood with some current mechanisms being the result of speculation based in the crystal structure of the protein [274]

Following activation, PI3Kinase catalyses the production of PIP_3 which accumulates at the leading edge of migrating cells [275] and further recruits PH – domain containing proteins such as 3-Phosphoinositide-Dependent Protein Kinase-1 (PDK1), GEFs such as P-Rex1, Bruton's tyrosine kinase (Btk) and PKB/Akt [276] which then activate effector proteins such as members of the Rho-GTPase family and the serine/threonine kinase Akt thus propagating the initial signal, eliciting a functional response.

The role of PI3Kinase in controlling migration has been somewhat controversial with some groups reporting PI3Kinase to be an absolute requirement for migratory processes [263,277], while others report only limited involvement of PI3K [264,278]. There is also evidence of migratory processes occurring independently of PI3Kinase [211]. These discrepancies are likely to arise from the experimental model used as well as the type and length of stimulation. By considering chemokinesis (speed) as a separate parameter to chemotaxis (direction), a subtle but discrete role for both PI3Kinase-δ and –γ as the major PI3Kinase isoforms involved in migratory processes is now widely accepted. This observation may explain the earlier discrepancies observed regarding the involvement of PI3Kinase in migratory processes. PI3Kinase-γ has been shown to be the dominant PIP_3 producing isoform at the leading edge during migration, as neutrophils from a PI3Kinase-γ deficient mouse produced negligible amounts of PIP_3 in response to chemoattractant stimulation [279,280]. In contrast, PI3Kinase-δ has been shown to facilitate neutrophil spreading and polarisation across fibronectin-coated surfaces [281]).

PI3Kinase signalling, and therefore PIP_3 production, is regulated through the action of the 3- and 5- phosphatases Phosphatase and Tensin Homolog (PTEN) and Phosphatidylinositol-3,4,5-triphosphate 5-phosphatase (SHIP1) respectively. These phosphatases are responsible for regulating PI3Kinase and confining PIP_3 to the leading edge through de-phosphoryation of PIP_3 producing a further lipid product phosphatidylinositol- 3,4 – bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol – 4,5 – bisphosphate ($PI(4,5)P_2$). In this manner PI3Kinase activity is antagonised maintaining cellular homeostasis.

1.4.2.2 Protein Kinase B (PKB)/Akt

Akt, also known as protein kinase B (PKB), is an ~60kDa serine/threonine kinase that was originally identified as a retroviral oncogene [282] and most well-known for its ability to prolong neutrophil survival in response to pro-inflammatory cytokines [283]. Akt is abundantly expressed in mammalian cells as three highly homologous isoforms Akt-1 (PKBα), Akt-2 (PKBβ) and Akt-3 (PKBγ). Akt1 and Akt2 are the most abundantly expressed isoforms with Akt-3 being solely expressed in the brain and testis where it has been shown to have a role in post-natal brain development [284,285]. Akt has been demonstrated as an essential component of a number of cellular processes including cellular proliferation [286], superoxide generation [287] and migration [288].

During migratory processes, Akt in known to translocate to the leading edge of migrating neutrophils from the cytosol by virtue of its PH domain recognising membrane localised PIP_3 produced by active PI3Kinase [289]. Current studies [290] suggest it is the Akt2 isoform that is recruited in this setting however, conclusive evidence of this is required before definitive conclusions can be drawn. Once at the membrane, Akt is phosphorylated by the action of two independent kinases, PDK1 and mTOR-complex 2 (mTORC2), these phosphorylate Threonine-308 (Thr308) and Serine-473 (Ser473) residues respectively. PDK1 is a kinase that has been shown to be dependent on PIP_3 but unaffected by wortmannin, a broad-spectrum PI3Kinase inhibitor, placing the action of PDK1 on Akt downstream of PI3Kinase [291]. For a long time the identity of the Ser473 kinase, now identified as mTORC2 (mTOR when in a rapamycin-insensitive complex with sin-1 and rictor) [292,293] was unclear and has previously been referred to as PDK2 or hydrophobic motif kinase (HM kinase).

When phosphorylated solely at Thr308, Akt shows partial activation and in this state it is capable of activating mTORC1 (mTOR when complexed with raptor) through phosphorylation of PRAS40, a dominant negative regulator of mTORC1 [294]). Activity in this manner allows Akt-mediated regulation of protein synthesis and cell growth through the action of mTORC1. Phosphorylation of both Ser473 and Thr308 results in full-activation of

Akt increasing the number of Akt phosphorylation targets. These targets include caspase-9 (phosphorylation of which prevents its protease activity [295]); Iκ-B Kinase (IκK) (affecting NF-κB gene transcription [296]); mTOR [297]; $p47^{phox}$ (mediating the respiratory burst [287]) and p21 Activated Kinase (PAK1) (influencing migration [298]).

In order to maintain homeostasis, Akt signalling is antagonised through de-phosphorylation of Thr308 by protein phosphatase 2A (PP2A) [299], and Ser473 by PH-domain leucine-rich-repeat-containing protein phosphatase 1 or 2 (PHLPP1/2) [300]. This is further controlled by de-phosphorylation of PIP_3 by PTEN/SHIP1 to form PIP_2.

1.4.2.3 Rho-GTPases

The Ras superfamily are a large (with approximately 150 members) conserved family of small (~21kDa) monomeric G-proteins consisting of 5 main groups (Ras, Rho, Rab, Ran and Arf) with Rho-family proteins forming the major branch. These GTPases act as molecular switches regulating a diverse range of cellular process by cycling between an active, Guanosine triphosphate (GTP) -bound and an inactive, guanosine-diphosphate (GDP) -bound state. This process is tightly regulated by both guanine nucleotide exchange factors (GEFs), which catalyse the exchange of GDP for GTP and thus activate the molecule, and GTPase activating proteins (GAPs), which inactivate the GTPase by exchanging GTP for GDP. Incorporation of prenyl groups (mainly geranylgeranyl pyrophosphates (GGP) and Farnesyl Pyrophosphate (FPP)) during biosynthesis allows the insertion of GTPases into the membrane. This supports activation through the exchange of GDP for GTP, a reaction catalysed by guanine nucleotide exchange factors already present in the membrane. In their GDP, inactive state, GTPases are sequestered in the cytoplasm where they are retained by GDP dissociation inhibitor (GDI) proteins by forming a high-affinity but biologically inert GDI-GTPase complex. GDI proteins provide another checkpoint in GTPase activation by binding to prenyl groups and preventing their interaction with the membrane. In addition, GDI proteins are known to stabilize the binding of Mg^{2+}, the dissociation of which is required for

nucleotide exchange. For full activation, GDP-bound GTPases must first dissociate from their GDI, a process that is catalysed by GDI dissociation factors (GDFs) whose identity still remains elusive however a single human GDF has been recently identified, PRA-1 [301]. Following dissociation, the GDP-bound GTPases must translocate to the membrane where, through interaction with GEFs, GDP is exchanged for GTP and thus activating the GTPase allowing interaction with effector molecules. It has been demonstrated that the interaction between the GDP-bound form of Rac-GTPase is modulated by inositol lipids, particularly PIP_2 [302]. This process has been elegantly reviewed elsewhere [303].

In their active conformation, Rho GTPases have been shown to be key regulators of cytoskeletal rearrangement, stress fibre formation and cell-cycle progression and have also been implicated in a number of cellular functions including adhesion, migration, phagocytosis and ROS production, most likely owing to their ability to dynamically control remodelling of the cytoskeleton. Three Rho GTPases are particularly prominent in the control of migration – Ras homolog gene family member A (RhoA), Cell Division Control Protein 42 (Cdc42) and Rac [304].

During migration, Rac and Cdc42 are localized to the leading edge where they are involved in the assembly of the filamentous actin (F-actin) network through recruitment of actin polymers and in maintenance of the leading edge [305]. Rac- and Cdc42-GTPase are activated by GEFs such as P-Rex-1 [306], DOCK1/2 [307] and β-Pix localized in the membrane through association with PIP_3. Once activated, these GTPases have been shown to modulate cytoskeletal rearrangement through activation of suppressor of cAR (SCAR)/ WASP family Verprolin-homologous protein (WAVE) family members and subsequently the actin nucleating complex actin-related protein 2/3 (Arp2/3) [308,309]. In addition, these proteins have also been shown to inhibit actin de-polymerization through phosphorylation and subsequent inhibition of Cofillin via LIM Kinase (LIMK) [310]. In comparison RhoA-GTPase is found localised to the rear of the cell in the uropod where it mediates tail retraction and dissociation of adhesive complexes [311].

1.4.2.4 Reactive Oxygen Species (ROS)

Production of ROS has been shown to occur at the leading edge of migrating neutrophils in an NADPH oxidase dependent manner [312]. The GTPase activating protein GIT2 links GPCR signalling at the surface with recruitment of NADPH oxidase [313] and subsequent ROS production at the leading edge. Chemoattractant-induced ROS production appears to function by amplifying the PIP_3 signal though localized oxidation of PTEN thus suppressing its phosphatase activity [314-316]. Phosphorylation of Akt, itself is reduced in the presence of N-acetyl-L-cysteine (NAC) [317] suggesting a role for redox-mediated feedback in the regulation of migration.

1.4.2.5 Actin Cytoskeleton

The actin cytoskeleton is a dense network of actin fibres present within the cytoplasm that acts as a cellular scaffold governing cell shape, intracellular transport, cell division and, in motile cells, membrane protrusion and tail retraction. Protrusion in this manner is driven by polymerization of actin filaments just under the plasma membrane forming one of two basic actin structures: branched filament networks leading to sheet-like protrusions (lamellipodia) or un-branched actin bundles leading to finger-like protrusions (filopodia and pseudopodia) characteristic of the leading edge in motile cells (see **Figure 1.4**). The dense actin network present in the lamellipodia provides the scaffold upon which motility machinery can localize within the leading edge (reviewed extensively in [318]).

Actin structures, once initiated, can continue extending owing to the abundance of monomeric actin within the cytoplasm and are only stopped through the action of capping protein concealing the barbed end of a growing filament [319]. However, for nucleation of new actin filaments, assistance is required from nucleating proteins such as the Arp2/3 complex and the Formin family of proteins including the diaphanous-related formins (DRFs also known as mDia1-3). Arp2/3 is responsible for the formation of branched networks by nucleating actin polymerization at a 70° angle from the sides of pre-existing filaments [320],

while formins act to nucleate un-branched actin filaments [321] creating linear bundles required for membrane spikes and pseudopodia. However, these proteins do not act in isolation, Wiskott-Aldrich syndrome proteins (WASP) family members, PIP_2 and Cdc42 are all required in addition to nucleating proteins to efficiently stimulate actin filament elongation [322].

In order to maintain motility and the supply of monomeric actin in the cytoplasm, actin disassembly must also take place. This occurs through the action of cofillin by dissociating Arp2/3 and its associated branches from actin filaments resulting in debranching of the actin network [323]. This forms a cycle beginning with actin nucleation and depleting cytoplasmic stores of monomeric actin and ending with replenishment of those stores through actin filament dissociation, this cycle is known as the dendritic nucleation model [324] ensuring a constant supply of monomeric actin for actin nucleation and driving the leading edge forward. See **Figure 1.5**

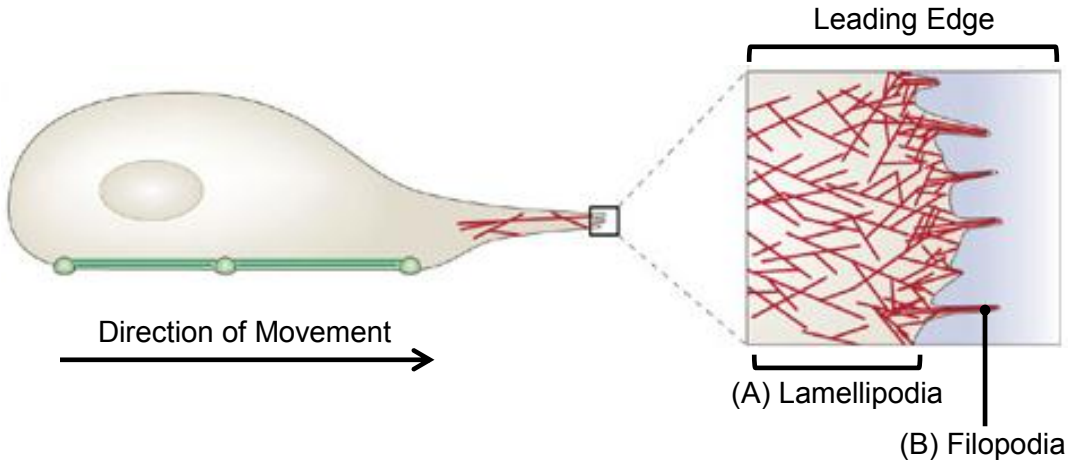


Figure 1.4 Actin Structures at the Leading Edge.

Growing actin filaments are found within the leading edge driving forward protrusion of the plasma membrane and generating motility. Actin filaments are found as two main conformations: (A) Lamellipodia, a dense actin network forming sheet-like protrusions and (B) Filopodia, bundles of parallel actin filaments creating finger-like protrusions. *Adapted from [325].*

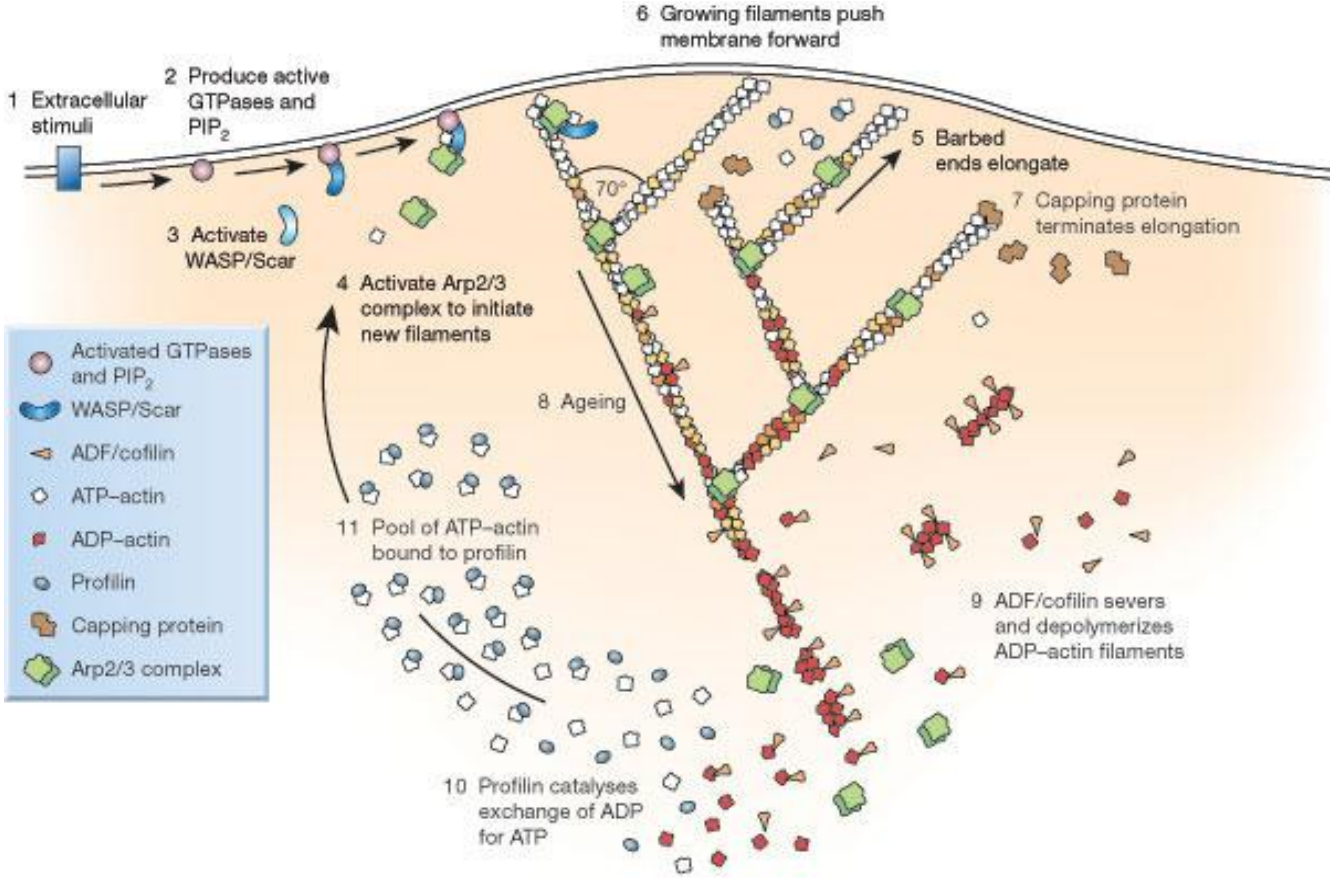


Figure 1.5 The Dendritic Nucleation Model.

This model describes the retrograde flow of monomeric actin ensuring an adequate supply for nucleation of new fibers and the subsequent protrusion of the plasma membrane at the leading edge driving cell motility. *Figure taken directly from [324]*

1.4.2.6 Other Molecules controlling migration

Although PI3Kinase and the Rho GTPases are widely accepted as mediators of accurate migration, a number of other signalling pathways have been implicated in the process. Mitogen activated protein kinase (MAPK) family members including ERK, p38 MAPK and Jun N-terminus kinase (JNK) have been shown to regulate migration through interaction with Paxillin, a docking protein that recruits certain proteins to focal adhesion complexes including serine/threonine kinases and GTPases [326] and Spir, a member of the WASP family of proteins, both of which results in reorganisation of the actin cytoskeleton (reviewed in [327]). Although activated downstream of PI3Kinase [328], inhibition of ERK had no effect on neutrophil migration in response to IL8 [329] implying that neutrophil migration is largely independent of MAPK activation.

Genetic silencing of Phospholipase-D_2 (PLD_2), an enzyme involved in phospholipid metabolism in the plasma membrane, has been shown to induce arrest of migration [330]. However mechanisms underlying this involvement remain largely unknown. Peng *et al* showed PLD_2 to contain two Cdc42/Rac Interactive binding (CRIB) domains within its structure allowing direct interaction with Rac-GTPase [331] highlighting a possible mechanism by which PLD_2 could be involved in migration. PLC has also been shown to be important in the regulation of migration through the translation of integrin-dependent signals catalysing PIP_2 to produce inositol-triphosphate (IP_3) and DAG, inducing the release of calcium from intracellular stores subsequently enhancing ROS production [332].

In addition, in the presence of PI3Kinase inhibitors murine neutrophils are still able to migrate, albeit with significantly reduced efficiency of migration. This phenomenon has been attributed to PKBR1, a membrane-bound isoform of Akt that is uniformly distributed throughout the cytoplasm but exhibits localised activation by mTORC2 upon detection of a chemotactic gradient [333]. Upon activation PKBR1 acts through as yet undetermined mechanisms to modulate both actin nucleation and disassembly (reviewed in [334]).

1.4.3 Pathway integration resulting in co-ordinated control of migration

We are still to fully understand the mechanisms involved in the control of directional migration; until recently directional migration was thought to be under the control of a single linear pathway governing both motility (chemokinesis) and directionality (chemotaxis) giving rise to a compass like model of migration. The role of the compass needle was attributed to PI3Kinase due to the essential localisation of PIP_3 at the leading edge [275]. However, inhibition of PI3K in murine neutrophils causes a reduction in the absolute number of cells responding but does not affect their speed or directionality [335]. This suggests there are additional control mechanisms acting independently of PI3K and that chemokinesis and chemotaxis are independently regulated.

The idea of a complex signalling network existing not only at the leading edge but also throughout the cell is now widely accepted [334]. Integration of concomitant signalling originating at both the leading edge and uropod determine polarity leading to the spatial and temporal orientation of signalling molecules and laying the foundation for a function that is both robust and generic whilst also exhibiting exquisite sensitivity. The extent to which individual elements of this signalling network contribute remains unclear. The process of directional migration can be divided into its three components parts: polarity, motility and directional movement in order to understand the pathways that govern migrational control.

1.4.3.1 Polarity

Polarisation refers to the morphological changes that occur as a direct consequence of chemoattractant-mediated cellular activation generating a polarised phenotype with a flattened ‘leading edge’ at the up-gradient edge of the cell and a tail, or uropod, at the down-gradient edge. Concomitantly, intracellular components exhibit their own, interior polarisation generating an internal gradient of signalling molecules causing the formation of distinct domains around the cell periphery.

The leading edge is characterised by the accumulation of F-actin [336] and the localisation of a number of proteins involved in cytoskeletal re-organisation and gradient sensing. These proteins include: PI3Kinase, particularly the p110γ isoform [337] and therefore it's lipid product PIP_3, the actin-nucleating protein Arp2/3 and the small GTPases Rac and Cdc42 which play fundamental roles in both the formation of the leading edge as well as its location and subsequent stability [305]. Addition of a cell-permeable ester of PIP_3, PIP_3/AM, to human neutrophils induces cell polarisation with 70% of cells exhibiting a flattened morphology with a contracted tail. In addition, cells treated with PIP_3/AM show a significant accumulation of F-actin at the leading edge and significantly increase their speed of migration [338]. Conversely, the uropod is devoid of these proteins and instead contains proteins such as PIP_2 and RhoA-GTPase (which is not detected at the leading edge). Simultaneous signalling originating from the leading edge and uropod have been shown to antagonize each other's activity [339,340] ensuring signalling networks are compartmentalised, generating and subsequently maintaining a self-organising polarity in response to a chemoattractant gradient. An excellent example of this is the lipid product PIP_3 which has been shown to promote the activity of Rac- and Cdc42-GTPase at the leading edge and RhoA-GTPase in the uropod [341]. In the case of PIP_3, polarity is further maintained through the presence of the phosphatases PTEN and SHIP1 at the sides of the cell and in the uropod which de-phosphorylate PIP_3 to produce $PI(4,5)P_2$ and $PI(3,4)P_2$ respectively. Using $SHIP1^{-/-}$ and $PTEN^{-/-}$ knock-out mice, PTEN appears to be involved in the maintenance of the anterior-posterior PIP_3 gradient and is the main antagonist of PI3Kinase activity whilst SHIP1 is involved in the regulation of PIP_3 production upon cell adhesion preventing the development of a top-down PIP_3 gradient [281]. Membrane composition and integrity have also been shown to be essential in the maintenance of polarity; depletion of cholesterol disturbs membrane organisation and inhibits cell polarisation and migration by preventing the sustained activation of signalling molecules necessary for maintenance of the migratory phenotype [342].

The importance of polarity, both morphologically and internally, in orchestrating migration has been demonstrated using neutrophils deficient in various proteins involved in the process. SHIP1 deficient neutrophils, which are not able to localise PIP_3 to the leading edge, exhibit an extremely flattened and weakly polarised morphology with significantly reduced speed [343], inhibition of RhoA or its downstream effector Rho-associated kinase (ROCK), does not appear to affect motility of the cell body (therefore the leading edge) but prevents detachment of the uropod thereby reducing the overall speed of migration [311]. However, in light of this, polarity does not appear to be a response to a chemotactic gradient *per se* as neutrophils exposed to a uniform stimulus i.e. one that is constant across the length of the cell, still display a polarized morphology [344].

Overall, polarity arises from the action of a localised activator and a global inhibitor, giving rise to the characteristic polarised phenotype and providing the foundation for locomotion and gradient sensing.

1.4.3.2 Motility (Chemokinesis)

In order for a cell to become motile it must first achieve a number of parameters: forward propulsion at the leading edge, retraction at the rear with release of adhesive contacts and maintenance of membrane tension and integrity preventing cell lysis.

Signals involved in the maintenance of polarity have been grouped into 'frontness' and 'backness' signals which are generated at the leading edge and uropod respectively [345]. 'Frontness' signalling refers to the protrusion of the leading edge driven by polymerized F-actin, which generates the power required to force protrusions of the plasma membrane commonly known as pseudopods (finger like projections) and/or lamellipodia (sheet-like projections). 'Backness' signalling, on the other hand, refers to retraction and 'de-adhesion' of the uropod facilitating forward motility. Both of these pathways occur downstream of chemoattractant stimulation but are independently regulated. Frontness signalling is under the control of G_i coupled GPCRs acting through PI3Kinase, the Rho family GTPases Cdc42

and Rac1 and Arp2/3, which stimulates actin polymerization [346,347], see **Figure 1.6**. 'Backness' signalling occurs downstream of $G_{12/13}$ coupled GPCRs, activating RhoA and its downstream kinase ROCK, which mediates detachment of the uropod and contraction of myosin fibres [339] generating a centripetal force against which actin filaments can push in order to force protrusions of the membrane at the leading edge, see **Figure 1.7**.

Actin polymerisation occurs at areas of dense PIP_3 accumulation and is driven by the recruitment and activation of Arp2/3 by members of the WASP family, particularly SCAR/WAVE, which is itself dephosphorylated for activity by the small GTPase Rac [348]. The production of PIP_3 in this context has been attributed to the action of the p110γ isoform of PI3Kinase and although this isoform appears to be dispensable for gradient sensing (see subsequent section), it is critical for the initiation of general motility or chemokinesis [264,349]. In addition, a role for Akt but not Cdc42 has also been demonstrated in generating motility [350].

In the uropod, RhoA-GTPase-/ROCK activation occurs following activation of p115Rho GEF by the $G\alpha_{12/13}$ subunit of heterotrimeric G-proteins and results in the activation of the calcium dependent enzyme myosin light chain kinase (MLCK). This enzyme is responsible for the light chain phosphorylation of Myosin II initiating filament contraction and therefore retraction of the uropod. Neutrophils treated with either a myosin inhibitor or a MLCK inhibitor exhibit a dose dependent reduction in chemokinesis due to impaired uropod retraction [351]. ROCK has also been shown to be able to act directly on Myosin II [352] whilst MLCK can also be activated downstream of Ras, Mitogen Activated Protein Kinase Kinase (MEK) and ERK [353]. Rho-GTPase stabilizes actin within actin-myosin bundles through its action on LIMK-2 and Cofillin [354] and enables detachment of adhesive contacts within the uropod [311].

The cell membrane also has a role to play in the regulation of motility. Apart from acting as a physical barrier, separating the interior and exterior of the cell and acting as a signalling platform, the membrane also generates an inward force mechanically restraining actin protrusions [355,356] and contributing to retractions of the uropod [357]. This allows both

‘frontness’ and ‘backness’ signalling to set the rate of motility: if one of these functions occurs at a slower rate than the other, it becomes the rate-limiting factor in speed of cell movement.

1.4.3.3 Directional Sensing (Chemotaxis)

Being able to sense the direction of a chemotactic gradient is the crux of chemotaxis, the process of generating polarity and chemokinesis are redundant without navigation. The exact mechanism of gradient sensing remains elusive, but is likely to involve integrated signalling from multiple pathways.

To date the most appropriate model to accurately describe gradient sensing is proposed by King and Insall in 2009 [254]. Here, the Bifurcation and Bias model suggests that *de novo* synthesis of pseudopods rarely occurs and instead the splitting of existing pseudopods creates ‘new’ pseudopods in a receptor occupancy dependent manner; the most accurate pseudopods (those that sense the highest chemokine concentration) are maintained while others are retracted. As receptor localisation appears to remain uniform across the plasma membrane during migration e.g. C5a-Receptor (C5aR) [289], (although in steep gradients, a higher concentration of chemoattractant at the leading edge will provide greater sensitivity) the internal gradient of signalling molecules appears to provide the sensitivity required to sense direction.

Production of ROS has been suggested as a mechanism by which cells collapse their less accurate pseudopods as inhibition of NADPH oxidase leads to the formation of multiple, sustained pseudopods within the leading edge [312]. Microtubule stability has also been shown to have a role in selecting and maintaining the accurate pseudopods allowing neutrophils to choose between protrusions to give direction [358]. In addition, murine neutrophils lacking the protein GIT2, the GEF that links GPCR signalling with NADPH oxidase recruitment, exhibit a loss in directional migration [313]. mTORC2 has also been shown to be involved in the generation of directional migration acting through PKBR1 [333].

An essential role for PI3Kinase-δ has been observed in the control of directional migration by selectively amplifying the PIP_3 signal in localised areas of the cell above that of the PI3Kinase-γ mediated PIP_3 signal. However, inhibition of PI3Kinase-δ does not block the synthesis of F-actin or prevent adhesion to the substratum [263]. Furthermore, signalling pathways involved in the molecular control of migration are not unidirectional and there are positive feedback loops existing within the leading edge involving PI3Kinase, PIP_3, multiple G-proteins including heterotrimeric G-proteins and small GTPases as well as NADPH oxidase/ROS. This governs directional migration, bringing together polarity, movement and direction. For example, PIP_3 has been shown to be able to activate PI3Kinase [338], while Rac- and Cdc42-GTPase have been shown to be able to act upstream of Akt in governing cell motility [350].

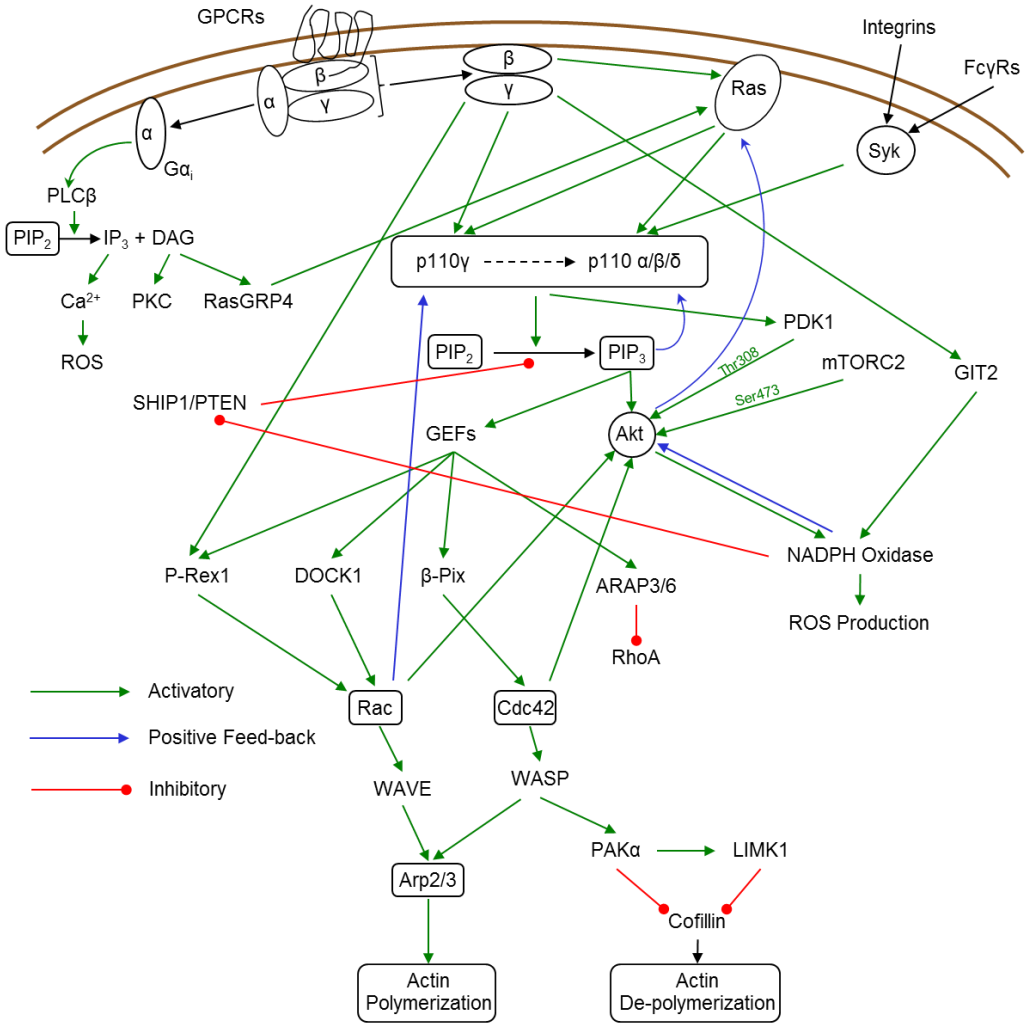


Figure 1.6 Signaling Networks at the Leading Edge of Migrating Neutrophils.

Neutrophil migration is under the control of a dense network of signaling pathways which interconnect to deliver a migratory phenotype that is both robust and sensitive. Signaling at the leading edge originates from activation of surface receptors and results in the polymerization of actin filaments causing protrusion of the leading edge and forward motility. GPCRs; G-protein coupled receptor; R, receptor; PLCβ, phospholipase C-β; PIP_2; phosphatidylinositol-2-phosphate; IP_3, inositol-triphosphate; DAG, Diacylglycerol; PKC, protein kinase-C; ROS, reactive oxygen species; PIP_3, phosphatidylinositol-(3,4,5)-phosphate; SHIP1, phosphatidylinositol-3,4,5-triphosphate 5 phosphatase; PTEN, phosphatase and tensin homolog; PDK1, 3-phosphoinositide dependent kinase-1; GEFs, guanine nucleotide exchange factors; mTORC1, mammalian target of rapamycin complex-2; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; WASP, Wiskott-Aldrich syndrome proteins; Arp2/3, actin-related protein-2/3; LIMK1, LIM-Kinase-1.

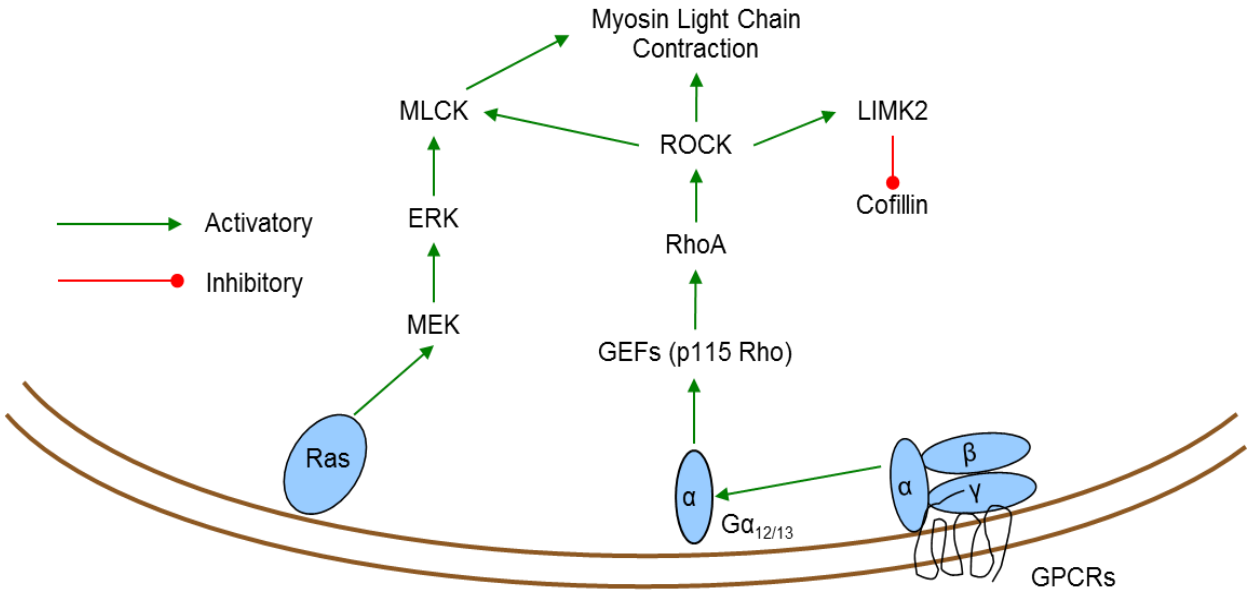


Figure 1.7 Signaling Networks within the Uropod of Migrating Neutrophils.

Retraction of the uropod occurs due to the contraction of myosin light chain mediated though the activation of RhoA and myosin light chain kinase (MLCK) filaments. Uropod signaling originates from interaction of G-protein coupled receptors (GPCRs) coupled to the $G\alpha_{12/13}$ subunit. GEFs, guanine nucleotide exchange factors; RhoA, RhoA-GTPase; ROCK, Rho-associated Kinase; LIMK2, LIM-Kinase-2; ERK, extracellular-signal-related protein kinase; MEK, mitogen-activated protein kinase kinase; Ras, Ras-GTPase

1.5 Plasma Membrane Structure

Biological membranes, consisting mainly of phospholipids, such as phosphatidylcholine (PC), glycolipids such as sphingomyelin (SM) and cholesterol, provide a physical barrier distinguishing the cell interior from the extracellular environment. Transport across the lipid membrane is a tightly controlled process made possible by the presence of proteins embedded within the fluid lipid structure.

1.5.1 Phospholipids

Phospholipids (PLs) are a major class of membrane lipid. The most common form is composed of a glycerol backbone attached to a phosphorylated alcohol and two esterified fatty acid chains, creating an amphipathic molecule with both a hydrophilic, polar head group and a hydrophobic tail. Once in the bi-layer conformation, PLs are capable of interaction with aqueous media found in the extracellular environment or cellular cytosol whilst simultaneously forming a hydrophobic barrier.

Cellular PL biosynthesis begins with the synthesis of phosphatidate which then reacts successively with DAG and an alcohol (one of which must be activated by cytidine triphosphate [CTP]) in a multi-step process forming the complete PL. Some of the more common PLs found in cell membranes include PC which accounts for 50% of phospholipids in eukaryotic membranes [359], Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylglycerol (PG), Phosphatidylserine (PS) and SM. See **Figure 1.8** for the chemical structure of these molecules.

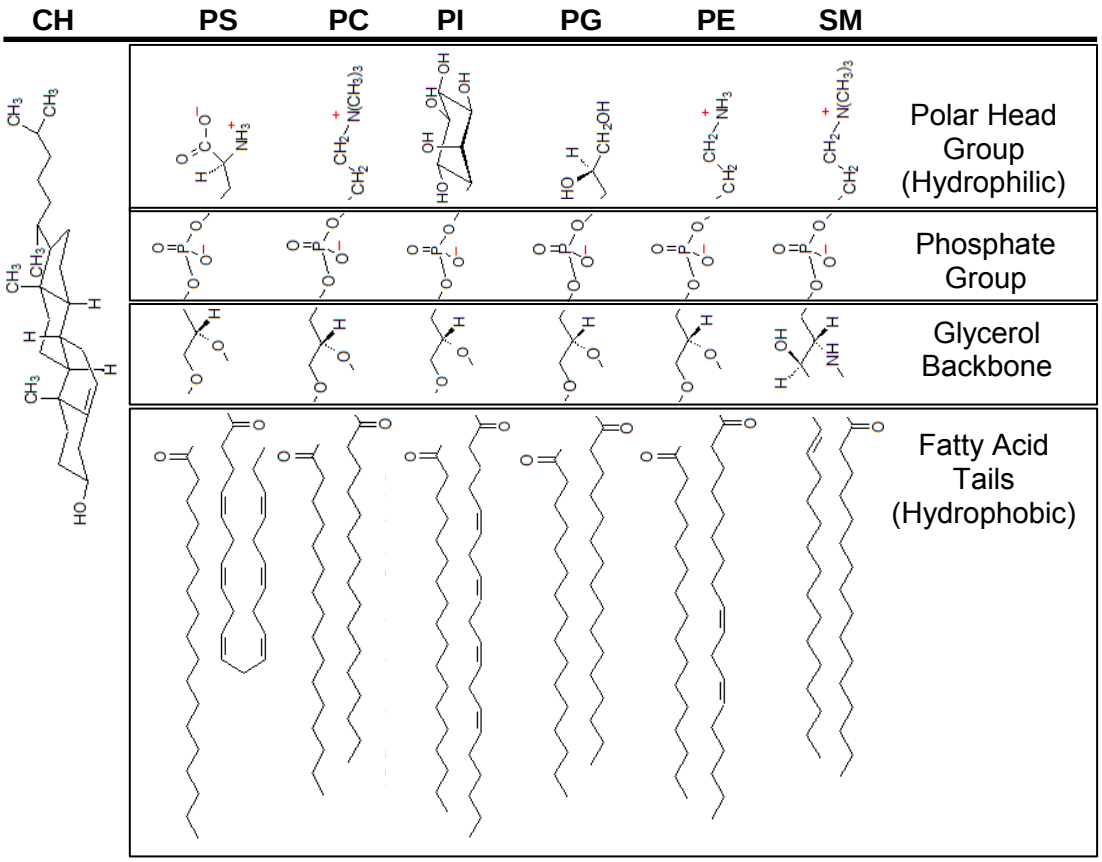


Figure 1.8 Structure of Common Membrane Lipids.

Chemical structure of lipid species commonly found in eukaryotic membranes. CH, Cholesterol; PS, Phosphatidylserine; PC, Phosphatidylcholine; PI, Phosphatidylinositol; PG, Phosphatidylglycerol; PE, Phosphatidylethanolamine; SM, Sphingomyelin.

The main site of phospholipid biosynthesis is the endoplasmic reticulum; however, synthesis also occurs within the golgi body and mitochondria [359], see **Figure 1.9**. Newly synthesized phospholipids are used to populate various cellular membranes including but not limited to, the plasma membrane and endosomes. As the cytosol is an aqueous solution, intracellular transport occurs via the endocytic pathway in the form of small membrane bound vesicles produced through budding of the endoplasmic reticulum and/or golgi body, or with the help of transport proteins [359,360]. Upon reaching the plasma membrane, PLs are distributed asymmetrically throughout the structure with SM and PC restricted to the exoplasmic face and PS and PE restricted to the cytosolic leaflet (the leaflet of the bi-layer that interacts with the cytoplasm) [361]. Asymmetry is achieved through the action of membrane-bound lipid translocases, which transport lipids across the lipid bi-layer in both an adenosine-triphosphate (ATP) - dependent and –independent manner. Enzymes responsible for this movement include the P4 subfamily of P-type ATPases such as aminophospholipid translocase, responsible for the restriction of PC and PS to the cytosolic leaflet and Scramblase, a less specific translocase that has been shown to be important in the exposure of PS on the exoplasmic face during apoptosis [362], see **Figure 1.10**.

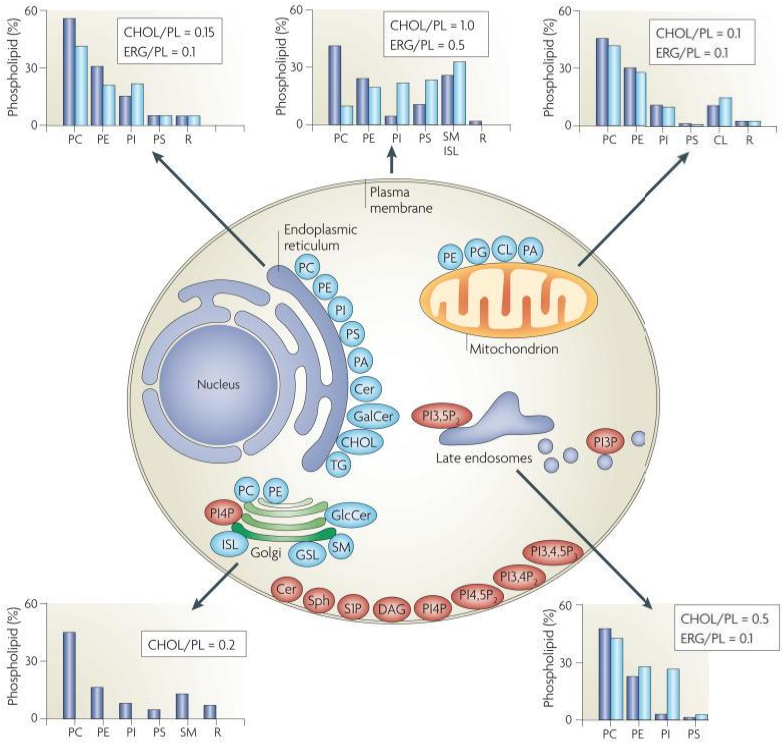


Figure 1.9 Differential Membrane Composition of Organelles in the Steady State. Membrane phospholipid (PL) composition is shown as a percentage of total PL in mammals (blue bars) and yeast (light blue bars) with sterol content, expressed as a molar ratio of cholesterol (CHO) or ergosterol (ERG) to PL, is also shown as a measure of membrane sterol content. Sites of synthesis are shown (central panel) for major phospholipids (blue) and organelle recognition and signalling pathways (red). Most lipid synthesis occurs in the endoplasmic reticulum (ER) and includes synthesis of Phosphatidylcholine (PC), phosphatidylethanolamine (PE), Phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), Ceramide (Cer) galactosylceramide (GalCer), cholesterol and ergosterol. The major exceptions are Sphingomyelin (SM) and complex glycosphingolipids (GSLs) which are synthesized in the golgi lumen. Some synthesis of PC also occurs within the golgi. Lipids populating the mitochondrial membrane are synthesized within the organelle. R, remaining lipids; TG, triacylglycerol; $PI(3,5)P_2$, CL, cardiolipin; BMP, bis(monoacylglycerol)phosphate); ISL, inositol sphingolipid; Sph, sphingosine; DAG, diacylglycerol; phosphatidylinositol-(3,5)-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol-(4,5)-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol-(3,4,5)-triphosphate; PI4P, phosphatidylinositol-4-phosphate; S1P, sphingosine-1-phosphate. *Taken directly from [359].*

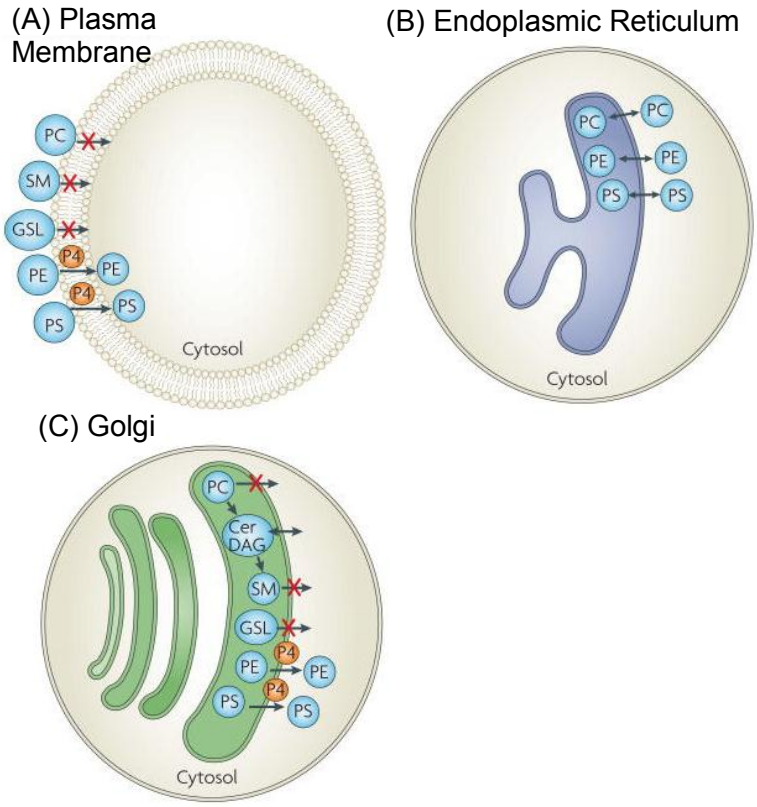


Figure 1.10 Membrane Asymmetry in Intracellular Compartments.

(A) Asymmetry within the plasma membrane occurs through P4-ATPase –dependent transport of Phosphatidylserine (PS) and Phosphatidylethanolamine (PE) to the cytosolic leaflet of the plasma bilayer whilst little transport of sphingomyelin (SM) and phosphatidylcholine (PC) exist confining these species to the outer (or exoplasmic) leaflet (B) Little asymmetry occurs within the endoplasmic reticulum which non-specific transport occurring resulting in near symmetrical distributions across the bilayer (C) Asymmetry across the golgi bilayer is the result of P4-ATPase transport of phosphatidylserine (PS) and phosphatidylethanolamine (PE) to the cytosolic face. SM is produced on the luminal leaflet and remains there due to the lack of transporters within the membrane structure. *Taken directly from [359].*

The composition of different phospholipid species within the plasma membrane of neutrophils is characteristic of neutrophils. This is due to the fatty acid structure located at positions sn1 and sn2 of phosphatidylcholine (PC). Characteristically, neutrophils have been shown to be depleted in arachidonoyl-containing PC species common in other cell types instead containing a greater amount of the 1-alkyl-2-acyl species with the fatty acid located at position sn2 to be joined to the head group via an ether- rather than an ester-bond.

The primary role of membrane phospholipids is their barrier function, but they also have a role in cellular metabolism, by releasing large quantities of ATP and are important signalling molecules involved in many signal transduction pathways. The phosholipase-A_2 (PLA_2) family of enzymes cleave the fatty acid at the sn2 position of many membrane-bound phospholipids producing arachidonic acid and lysophospholipids. Arachidonic acid metabolism leads to the production of pro-inflammatory mediators such leukotriene B_4 (LTB_4), which propagate the inflammatory response and attract more cells to the site of infection [254]. Other phospholipids such as PS have a regulatory role in ROS production [363] and are also essential in the activation of other enzymes such as phospholipase-C (PLC) [363] which is required for the release of calcium ions from the endoplasmic reticulum, an important second messenger in propagating the signalling cascade.

1.5.2 Glycolipids

Glycolipids, such as sphingomyelin, are a class of membrane lipids derived from sphingosine that consist of a sphingosine backbone linked to a single saturated fatty acid chain and 1 or more carbohydrate units such as glucose or galactose. It is the presence of the sugar units within the molecule that gives glycolipids their characteristic structure and their asymmetrical distribution across the membrane favouring the luminal leaflet. Orientated in this manner, the carbohydrate moiety functions as a recognition molecule and provides membrane stability. Within the membrane, glycolipids pack together much tighter than phospholipids due to the presence of a fully saturated fatty acid chain which are therefore devoid of the ‘kinks’

commonly seen in the fatty acid chains of phospholipids, caused by the presence of C=C double bonds. This reduces the mobility of the membrane in glycolipid rich areas and is thought to further concentrate signalling molecules. These areas are known as lipid rafts and will be discussed in subsequent sections.

1.5.3 Membrane Cholesterol

Cholesterol is the major sterol species found in vertebrate membranes. Although cholesterol falls within the lipid class of molecules, it has a very unique structure consisting of a fatty acid chain and a hydroxyl group separated by 4-linked hydrocarbon rings providing the bulk to the structure (see **Figure 1.8**). Cholesterol is an important precursor to a number of biologically important substances including bile acids, important for absorption of dietary lipids, and steroid hormones such as testosterone. Within the plasma membrane, cholesterol is known to have an important role in regulating both membrane permeability and fluidity through association with the acyl chains of other membrane lipids [364], particularly glycolipids. Cholesterol is also an integral part to other biological process such as caveolae- and clathrin-dependent endocytosis, the process by which external material in internalized utilizing membrane pits coated with calveolin or clathrin respectively which then bud into the cell from the plasma membrane forming specialized coated vesicles within the cytoplasm [365].

Cholesterol biosynthesis occurs mainly in the liver however, a limited number of other organs, such as the brain, are capable of producing cholesterol [366]. Cholesterol biosynthesis begins with the conversion of 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to Mevalonate by the enzyme HMG-CoA Reductase, the rate-limiting step in cholesterol biosynthesis. Mevalonate is then further metabolised in a multistep process resulting in the production of cholesterol and a number of prenyl groups used in the post-translational modification of small GTPases such as Ras and Rho family members [367]. In addition to hepatic biosynthesis, cholesterol can also be absorbed though the intestine from the diet, however, increased dietary uptake will attenuate *de novo* cholesterol biosynthesis in

the liver through cholesterol-mediated inhibition of HMG-CoA Reductase. This negative feedback loop ensures homeostasis is maintained regulating the availability of cholesterol for incorporation into membranes or for further metabolism.

Due to the amphipathic nature of the cholesterol molecule, cholesterol remains insoluble in the blood and must therefore be transported around the body by carrier proteins known as lipoproteins. Lipoproteins are classified according to the density of the protein: high-density lipoproteins (HDL), intermediate-density lipoproteins (IDL), low density lipoproteins (LDL) and very low-density lipoproteins (VLDL). These carrier proteins not only solubilise the lipid allowing transport but also contain cell-targeting moieties. Uptake of cholesterol occurs through expression of HDL and LDL receptors on the surface of target cells.

1.5.4 Membrane Fluidity and Lipid Rafts

Together, phospholipids, glycolipids and cholesterol are all important molecules in regulating membrane fluidity including the ability of proteins to move laterally within the bi-layer. Both the length of the fatty acid chain, their degree of saturation and cholesterol content contribute to membrane fluidity and subsequently the rate of lateral diffusion within the membrane. Long, saturated fatty acid chains with high level of interaction with cholesterol favours a more rigid membrane whereas short, unsaturated fatty acids with little interaction with cholesterol favours a more fluid state [368].

Overall, membrane fluidity is not thought to be continuous throughout the entire membrane structure. Instead, there exist small (26-70nm) lipid-ordered zones within the more fluid, liquid dis-ordered structure. These liquid-ordered zones, often termed rafts, are detergent insoluble glycolipid and cholesterol rich areas of the membrane that are thought to be present in the cell membranes at all times [369]. Upon stimulation, rafts are thought to aggregate, increasing in size, becoming visible by light microscopy [304] and forming ‘large’ areas of the membrane within an otherwise fluid structure. Aggregated rafts establish a

platform from which signal transduction can occur, bringing molecules into the correct juxtaposition.

Little work exists on the effects of age on neutrophil membrane composition and fluidity. Larbi *et al* found the cholesterol content of lipid rafts present in the plasma membrane of T cells was significantly increased with age and that membrane fluidity was decreased as a consequence [370] however, Alvarez *et al*, remain the only study to date who consider the effects of age on neutrophil plasma membrane composition. These authors found an age related decrease in the cholesterol: phospholipid ratio in neutrophils, the result of a decrease in free cholesterol and in increase in phospholipid content in neutrophils from rat peritoneum. This also correlated with an observed increase in membrane fluidity [371]. However, this work was carried out in neutrophils isolated from rat peritoneum and therefore further research is required before absolute conclusions can be drawn with regard to human cells.

1.6 Effects of Age on Neutrophil Chemotaxis

Age related changes in neutrophil migration have been reported since 1978, however there is still no consensus as to the influence of age on chemokinesis or chemotaxis. A number of groups have reported no effect for age [150,372-374] while an equal number report reduced migratory function with increasing age [162,375-379], see **Table 1.6**. Of the studies describing no effect for age on neutrophil migration, the average age of older adults was commonly in the late 70s to 80s. Alonzo-Fernandez *et al* demonstrated migratory dynamics in healthy centenarians that were comparable to those observed in healthy younger adults (aged 25 - 35 years) [378]. It is however conceivable that no age-related changes in migration were observed in these studies due to the successful ageing of participants included in the study. Phair *et al* used an old cohort aged 65 to 85 years [372], therefore including a more diverse older cohort with respect to ageing. However the young cohort used in this study ranged from 27 – 43 years and although no data currently exists on the effects of age on migratory dynamics across the lifespan, changes may not be observed when young and middle aged groups are combined. In addition, Biasi *et al* used the SENIEUR protocol to select their older adults [373], a protocol designed in 1984 by Ligthart *et al* [380] to standardize the health status on older adults being included in gerontological research by distinguishing *extremely* healthy older adults from those considered to be 'almost healthy' in order to eliminate any co-morbidities. However, this means that the older subjects included in this study were not representative of the general population and therefore results cannot be applied generally.

A number of studies presented found neutrophil migratory dynamics to be significantly reduced with increasing age. This collection of studies includes both *in vitro* and *in vivo* studies and includes the effect of ageing in humans and rodents. There are a greater number of studies finding reduced migration with increasing age compared to those observing no

differences while also including studies utilising the SENIEUR protocol as well as assessing migration in cohorts with an average age skewed towards older participants.

The majority of the studies observing both no change and significant reductions in migratory dynamics have done so using a modified Boyden chamber. This experimental set-up requires neutrophils placed in an upper chamber to migrate across a filter, which itself could affect migration, towards a chemotactic source placed in a lower chamber. Studies use filters of varying pore sizes (0.8μm-5μm) making conclusive arguments difficult to draw. In addition, the limitation of the Boyden chamber is principally that it assesses chemokinesis and not chemotaxis. Wenisch *et al* are the only group to have considered the effects of ageing on chemokinesis and chemotaxis as separate parameters [161]. In response to fMLP, chemokinesis appeared to be maintained while a trend toward reduced chemotaxis was observed in the elderly. However, this study utilised the under-agarose assay and can only provide rough estimations of cell movement and cannot comment on individual cell migratory behaviour.

As migration relies upon a complex signalling network in order to regulate a process that is both robust and extremely sensitive, it is conceivable that signal transduction itself may be altered as a consequence of increasing age thus compromising migration. Expression of triggering receptor expressed on myeloid cells-1 (TREM-1) and TLR2 and TLR4 remain unchanged with age [162]. However in the basal state TREM-1 has been shown to be present in the lipid rafts in the leading edge of neutrophils from elderly donors which cannot be further increased upon stimulation [381], implying dysregulated signal transduction as opposed to altered receptor expression as a mechanism for distorted migratory function in the elderly. This has been further explored by Lipschitz *et al* who demonstrate a significant reduction in the generation of a number of second messengers including IP_3, DAG and PIP_2 and Ca^{2+} in neutrophils from the elderly [163]. In addition, neutrophils from elderly donors exhibit reduced actin polymerisation in response to fMLP stimulation [382]. To date, alterations in signalling pathways have not been causally related to aberrant migratory

phenotypes of the healthy elderly. However, neutrophils isolated from patients with COPD, a chronic inflammatory disease common in the elderly population in which neutrophils exhibit aberrant migration has been attributed to aberrant PI3Kinase signalling [383], suggesting that altered neutrophil migration may indeed be a feature of ageing and may be driven by dysregulated signal transduction.

Table 1.6 Current Literature Examining the Effects of Increasing Chronological Age on Neutrophil Migration

	Author (Year)	Method	Chemotactic Factor	Primed	Young n (mean; range)	Old n (mean; range)
Unchanged	Phair *et al* (1978)	Modified Boyden Chamber	Insulin Generated Chemotactic Factor	No	20 (27 – 43)	70 (65-88)
	Biasi *et al* (1996)	Method not Reported	fMLP; Zymosan	No	* 25 (27.7 ± 2.59; 22 - 34)	* 25 (76.56 ± 7.36; 69 – 100)
	MacGregor *et al* (1990)	Modified Boyden Chamber with ^{51}Cr labelling of neutrophils; 5µm filters	Homologous C5a	No	10 (25.5)	10 (78.9)
	Wenisch *et al* (2000)	Under Agarose	fMLP	No	11 (27 ± 5; 21 - 36)	11 (71 ± 7.5; 62 – 83)
Reduced	Niwa *et al* (1989)	Under Agarose Boyden; filter size not reported	10^{-7}M fMLP; 10^{-7}M PAF; ZAP	No	30 (19 – 48)	
	Whyte *et al* (1993)	Boyden; 5µm and 0.8µm filter	fMLP; 10% ZAP	No	Isolated PMN aged 24 hours in culture	
	Izgüt-Uysal *et al* (2003) *(Rat Model)*	Boyden 3µm filter	ZAP	No	20 Rats aged between 2 and 24 months	
	Fulop *et al* (2004)	Boyden; filter size not reported	GM-CSF; LPS; fMLP	Yes (90min GM-CSF)	Cohort details not reported	
	Alonso-Fernández *et al* (2008)	Boyden; filter size not reported	10^{-8}M fMLP	No	* 30 (28; 25 – 35)	* 30 (69; 65 – 75)
	Brubaker *et al* (2003) *(BALB/c Mouse Model)*	*In vivo* recruitment to site of KC injection	1100-1000pg KC	*In vivo* inflammatory environment	12 (3-4 mo)	12 (18-20 mo)

fMLP, f-Met-Leu-Phe; C5a, Complement Protein 5a; KC, keratinocyte chemoattractant; ZAP, zymosan activated plasma, GM-CSF, granulocyte/macrophage-colony stimulating factor; LPS, lipopolysaccharide, PMN, polymorphonuclear neutrophils; mo, months old

* Utilized SENIEUR Protocol

Details of each study are presented as completely as possible based on information available in each publication

1.7 Potential Therapies

1.7.1 Anti - TNFα therapy

Potential interventions to improve aberrant neutrophil migration in the healthy elderly are severely lacking. Recent evidence has shown that Adalimumab, an anti-TNFα monoclonal antibody capable of neutralising TNFα currently used in the treatment of RA, is capable of restoring the migration of neutrophils isolated from the joints of RA patients to that comparable with healthy, disease-free patients [384]. Although this highlights the importance of TNFα in regulating neutrophil chemotaxis and points to a role for inflamm-ageing in driving defective neutrophil migration in older donors, TNFα is an essential cytokine involved in orchestrating the immune response and therefore neutralisation would likely increase the susceptibility of otherwise healthy older adults infectious diseases.

1.7.2 Statin Therapy

Statins are a class of drugs commonly prescribed to patients with hypercholesterolemia in order to lower serum cholesterol. This class of drugs includes 5 members, atorvastatin, fluvastatin, pravastatin, rosuvastatin and simvastatin, all of which act on the mevalonate pathway to competitively inhibit HMG-CoA reductase, preventing the conversion of HMG-CoA to Mevalonate, a precursor to cholesterol [385].This attenuates cholesterol biosynthesis and is associated with a reduction in total serum as well as low-density lipoprotein cholesterol. In 2011, simvastatin was the most commonly prescribed drug in the UK with a total of 41.2 million prescription dispensed throughout the year [386]. In recent years, the pleiotropic effects of statins have received significant attention with statins being able to reduce the risk of developing a number is diseases including dementia [387], lung cancer [388] and cardiovascular disease [389]. Of relevance to this thesis, a number of retrospective cohort studies and meta-analysis have also demonstrated the ability of statins to confer a survival advantage in older adults with pneumonia reducing rates of morbidity and mortality

[390-396]. These benefits are most apparent during the early stages in infection, which coupled with the knowledge that statins do not confer a survival advantage to patients with influenza [397], would suggest that in this situation statins may be acting on the innate immune response and may be able to modulate neutrophil anti-microbial function.

A number of studies have demonstrated the ability of statins to modulate neutrophil migration in both human and animal studies [398-401] by inhibiting the activity of RhoA-GTPase [398], post-translational modification of which occurs downstream of the mevalonate pathway [402]. Statins have also been shown to inhibit both MAPK activity and NF-κB-associated signalling leading to a reduction in the production of inflammatory mediators such as TNFα; IL-1β; IL-6 and IL-8 [402]. Levels of CRP have also been shown to be sensitive to modulation by statins [393,403].

Based on current evidence, statins present a likely candidate for therapeutic intervention to modulate migration in the healthy elderly having been shown to affect multiple pathways involved in the regulation of migration a concept that therefore needs further exploration.

CHAPTER 2

METHODS

2.1 Participants

Healthy volunteers were recruited from staff and students of the University of Birmingham and staff at Selly Oak and Queen Elizabeth Hospitals, alumni at the University of Birmingham, relatives of patients admitted into the Lung Investigation Unit, Queen Elizabeth Hospital and the Birmingham 1000 Elders Cohort, a research cohort of healthy older people aged over 60 years. To be considered "healthy" subjects were non-smokers, had no active symptoms or physical signs of acute or chronic disease (as assessed by attending physicians) and were medication free. Ethical approval was obtained and all volunteers gave informed consent prior to participation (REC 10/h1211/16) as per GCP guidelines.

Initially, participants were limited to younger (mean age 28 years; range 20-35years) or older (mean age 70 years; range 65-91 years) volunteers, but studies were then expanded to include subjects across a broad age range (from age 20 – 91).

2.2 Neutrophil Isolation

Human neutrophils were isolated from the peripheral blood of healthy donors and separated on a Percoll density gradient (pH 8.5-9.5; Sigma Aldrich) as described previously [138]. Briefly, whole blood was collected into sterile vacutainers coated with Lithium heparin (BD Biosciences) and mixed gently to prevent coagulation. Whole blood was then mixed with 2% Dextran (Amersham Bioscience) in a 6:1 ratio to sediment erythrocytes. The leukocyte fraction was then removed and layered on to a discontinuous Percoll gradient consisting of 56% Percoll overlaid onto 80% Percoll. Gradients were then centrifuged at 220 x g for 20 minutes at room temperature and granulocytes were removed from the interface between 56% and 80% Percoll. Isolated granulocytes were then washed in sterile phosphate buffered saline (PBS; Sigma Aldrich) and subsequently re-suspended at $2x10^6$/ml in sterile RPMI-1640 (with sodium bicarbonate and L-glutamine) (Sigma Aldrich). Sample purity was assessed using a commercial Giemsa statining kit (Reastain Quick-Diff Kit, Gentaur Europe)

and were routinely ≥ 95% neutrophils and neutrophil viability was above 98% as assessed by trypan blue exclusion.

2.3 Migratory Dynamics

Migratory parameters were assessed using an Insall Chamber [404] an improved chemotaxis chamber (Weber Scientific International Ltd, Teddington) as described previously [335]. Briefly, Bovine Albumin Fraction V (Sigma Aldrich) was added to a final concentration of 1.125% v/v to freshly isolated neutrophils re-suspended at $5x10^6$/ml. Neutrophils were then allowed to adhere to an albumin coated coverslip (22x22mm, Surgipath Medical Industries Inc. Europe) previously cleaned by acid washing (0.4M H_2SO_4) for 20 minutes at room temperature. Once neutrophils were adhered, the coverslip was inverted and placed onto an Insall chamber pre-filled with RPMI 1640. RPMI was then replaced with either fresh RPMI or a chemotactic stimulant (see chapter 3 for doses of stimulants used). Gradients were allowed to develop for up to 1 minute prior to assessment of migration.

For investigation of the role of PI3Kinase signalling, neutrophils were pre-incubated in the presence of either the non-selective PI3Kinase inhibitor LY294002 (Selleck, USA 1nM); PI3Kinase p110α inhibitor (PIK-75, [ChemieTek; CT-PIK75] 7.8nM), PI3Kinase p110β inhibitor (TGX-221 [Selleck Chemicals; S1169] 10nM); PI3Kinase p110γ inhibitor (AS-252424 [Selleck Chemicals; S2671] 33nM); PI3Kinase p110δ inhibitor (CAL-101 [Selleck Chemicals; S2226] 65nM). For investigation into the effects of statins on cell migration, cells were pre-treated Simvastatin (Sigma Aldrich; 1nM). All compounds were pre-incubated with neutrophils for 40 minutes prior to loading onto the Insall chamber. All incubation times included 20 minutes in which cells are allowed to adhere to an albumin-coated coverslip. Incubation times selected were based on pilot data collected by Dr. Elizabeth Sapey, prior to initiation of this project.

2.3.1 Chemo-attractant dose responses

To determine the optimum strength of chemotactic signal which would induce the most efficient migration over the 12-minute period, dose response experiments were conducted in the presence of RPMI-1640 alone (negative control) followed by increasing doses of each chemo-attractant (see **Table 2.1**)

Initial migration studies: stimulants selected for use in studies of migration and ageing were 100nM Interleukin 8 (IL8 [rhCXCL8/IL8] R&D Systems); 100nM Growth Related Oncogene α (GROα [rhCXCL1/GROα] R&D Systems); 10nM N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP, Sigma Aldrich); 10nM Leukotriene B_4 (LTB_4, Sigma Aldrich) and 1nM complement protein 5a (C5a, Sigma Aldrich).

2.3.2 Treatment with PI3Kinase Inhibitors

Concentrations of LY294002, a broad-spectrum PI3Kinase inhibitor, and all isoform specific PI3Kinase inhibitors (PIK75, TGX-221, AS252424 and CAL-101) used during migration studies corresponded to IC_{50} values (see **Table 2.2**).

2.3.3 Assessing migratory dynamics

Migratory dynamics were assessed using real-time video microscopy using a Zeiss Axiovert 200 microscope with HAL100 camera or a Leica DMI6000B with DFC360FX camera, capturing 36 frames over a 12 minute period with a 20 second delay between frames. Previous work has shown this duration of film provides similar migratory pathway data in comparison to longer filming times (30 minutes). A 20 second delay was optimal for studying migration between frames (personal communication, Dr. Elizabeth Sapey). Analysis was performed using vector analysis and ImageJ software (Wayne Rasband, NIH, Bethesda) on 10 randomly assigned cells per experiment. Cells were randomly selected by dividing image fields into equal segments and one cell tracked from each segment.

Neutrophil migration was assessed measuring 4 different parameters. Firstly, the average speed of movement (or chemokinesis) was measured from the distance travelled between frames in any given direction over time and subsequently expressed as μm/minute. All lengths and distances were initially recorded in units of pixels and converted to μm with reference to the resolution of the digital image itself, and the image magnification using a stage graticule (Electron Microscopy Sciences, Hatfield, USA).

Secondly, directional migration or chemotaxis, was measured as the average speed of migration in a consistent direction relative to the orientation of the chemotactic gradient, also expressed as μm/minute. Due to the orientation of the stage, the stable chemoattractant gradient was always formed in the y direction [404], therefore, only distance travelled in the y direction over time was included in calculations of chemotaxis.

Thirdly persistence, for each frame the orientation of the cell (as a line from tip to tail) was compared to the previous orientation and calculated by the cosine of the angle between cellular orientations in consecutive frames, expressed with reference to the y direction and expressed as a score between 0 to 1. Cells that tend to move in a straight line, or cells that execute slow changes in direction have a high persistence value (~1); whereas cells that move randomly and rapidly change direction do not (~0).

Finally chemotactic index - the accuracy of directional orientation of each cell relative to the chemotactic gradient throughout the duration of the film, calculated using the cosine of the angle between the cells direction and the orientation of the chemo-attractant gradient at each time from forming a vector analysis of movement. This parameter is expressed in a comparative scale ranging from -1 to 1 where 1 represents movement directly towards the chemo-attractant in all frames and -1 represents movement directly away from the chemo-attractant source in all frames.

Table 2.1 Chemoattractant Dose Responses

Chemoattractant	Dose Response (nM)
Interleukin-8 (IL8 [rhCXCL8/IL8] R&D Systems)	1; 10; 100; 250; 500
Growth Related Oncogene-α (GROα [rhCXCL1/GROα] R&D Systems)	1; 10; 100; 500
N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP; Sigma-Aldrich)	0.5; 1; 10; 100; 250
Leukotriene-B_4 (LTB_4; Sigma-Aldrich)	0.5; 1; 10; 100; 250
Complement Protein 5a (C5a; Sigma-Aldrich)	0.5; 1; 10; 100; 250

Doses used to determine the concentration of each chemoattractant capable of inducing the most efficient migration

Table 2.2 PI3Kinase Inhibitor Concentrations

Inhibitor	Concentation (nM)
LY294002 (Cell Signalling Technology)	1
PIK-75 - p110αinhibitor (ChemiTek)	7.8
TGX-221 - p110β inhibitor (Selleck Chemicals)	10
AS-252424 - p110γ inhibitor (Selleck Chemicals)	33
CAL-101 - p110δ inhibitor (Selleck Chemicals)	65

PI3Kinase Inhibitor concentrations used in migration studies

2.3.4 PI3Kinase Isoform selective Inhibitor cytotoxicity

The PI3Kδ inhibitor used in this study is currently in clinical trials to be used as a pharmacological intervention in patients with B cell malignancies due to its ability to induce apoptosis in these cells [405]. We therefore assessed neutrophil viability following incubation with all isoform selective inhibitors used in this study to determine the effects on neutrophil lifespan. Following isolation, neutrophils were incubated with isoform selective inhibitors for 40 minutes at room temperature and were then pelleted (250 x g, 4°, 5 minutes) and re-suspended in 1x binding buffer (0.1M HEPES [pH7.4], 1.4M NaCl, 25nM $CaCl_2$) before incubation with mouse anti-human Annexin-v (1:50 dilution, BD Biosciences), a marker of early apoptosis, for 10 minutes on ice. Sytox Blue (1:8000, Invitrogen, UK), a nuclear stain that is excluded from healthy cells, was then added to the cell suspension and cell viability measured by Flow Cytometry (CyAN, Beckman Coulter) counting 10,000 events to reduce sample variability. Cell viability was determined by the percentage of cells that remained both AnnexinV and Sytox Blue negative following 40-minute incubation with PI3K isoform selective inhibitors.

2.4 ELISA for neutrophil elastase activity

Neutrophil elastase (NE) activity was determined by quantifying the amounts of the elastase-specific fibrinogen breakdown product, $A\alpha Val^{360}$, in the plasma from both young and old volunteers. $A\alpha Val^{360}$ quantification was determined by competition ELISA as described previously [406]. Briefly, rabbit anti-sera raised against the free carboxyl group of $A\alpha Val^{360}$ was mixed with plasma samples at a 1:2500 dilution and incubated overnight at 4°C. This was then added into a 96-well tissue culture plate pre-coated with neutrophil elastase cleaved fibrinogen. Any free $A\alpha Val^{360}$ antibody remaining in the antisera binds the degraded fibrinogen and is detected and quantified using a europium-conjugated anti-rabbit IgG secondary antibody. Individual samples were assessed in triplicate with the average result quoted per person to reduce intra-sample variability.

2.5 Neutrophil Phagocytosis (pHrodo)

Neutrophil phagocytosis was measured using a commercially available assay and as per manufacturer's instructions. Briefly, neutrophils were isolated as described in **section 2.2** and re-suspended at $1x10^6$/ml in RPMI-1640 medium. 100ul aliquots were then incubated with either *Staphylococcus aureus* (*S.aureus*) or *Escherichia coli* (*E.coli*) bioparticles conjugated to the fluorescent dye pHrodo (Invitrogen Life Technologies, California, USA), which fluoresces at low pH i.e. once the particles, have been internalised into the phagosome. Un-opsonised particles were added at a final concentration of 0.33mg/ml for either 60, 45 or 30 minutes at 37°C. An equal volume of neutrophils were also stimulated with *S.aureus* or *E.coli* bioparticles and kept on ice for 60 minutes as a negative control. Following stimulation samples were washed (250 x g, 4°C, 5 minutes) in 2% cold PBS/BSA, transferred to cytometric tubes for analysis and quantified by flow cytometry ($CyAN_{ADP}$, Beckman Coulter). Data were expressed as a phagocytic index, as described previously [138]: Phagocytic Index = % of phagocytosing neutrophils X mean fluorescence intensity (MFI). Un-opsonised particles were specifically chosen to more closely replicate the pulmonary environment in which non-opsonized phagocytosis predominates as the airways are not a particularly serum-rich environment [407].

2.6 Reactive Oxygen Species Production

Neutrophil reactive oxygen species (ROS) production was measured in quiescent or stimulated neutrophils and quantified using a luminol-based assay which detects both extracellular and intracellular free radicals [408]. Freshly isolated neutrophils were re-suspended at $1x10^6$/ml in Hanks Balanced Salt Solution (HBSS: Gibco Invitrogen; 12.61mM $CaCl_2$, 4.93mM $MgCl_2$, 4.07mM $MgSO_4$ and Phenol Red Free). $1x10^5$ neutrophils were then plated out into 96-well white plates containing 25ul luminol (working concentration 100uM) and 75ul 1x HBSS. Neutrophil were then stimulated with 2.5μM fMLP or 1.25μM IL8 using 25nM PMA a positive control. ROS production was measured at 1 minute intervals for 60

minutes using a Berthold luminometer and ROS generation calculated as area under the curve.

2.7 Flow Cytometry

2.7.1 Antibody Titration

All antibodies used for flow cytometry were titrated to determine a minimum concentration that would saturate expression of all target molecules. **Table 2.3** shows concentrations used for each titration, the maximum concentration used for each antibody titration was that suggested in the manufacturer's instructions. Freshly isolated neutrophils or those in whole blood were stained for target molecules as described in subsequent sections (2.7.2 and 2.7.3). CD63 has been described as an activatory receptor on neutrophils [409], therefore expression was measured in whole blood to avoid artificially high expression levels due to cellular activation during the isolation process.

2.7.2 Neutrophil Degranulation

Surface and internal expression of primary granule marker CD63 was measured on neutrophils stained in whole blood following red cell lysis. 100µl whole blood collected in Lithium heparin vacutainers (BD Biosciences) was washed twice (250 x g, 4°C, 5 minutes) in 1%PBS/BSA to remove autologous plasma and re-suspended in 1% PBS/BSA. Samples were then incubated with relevant antibodies for 20 minutes in the dark on ice to allow relevant antibody binding. Post-incubation, samples were washed twice (250 x g, 4°C, 5 minutes) in cold 1% PBS/BSA and then red cells were lysed using FACS lysing solution (BD Biosciences) for 10 minutes at room temperature. Samples were then washed twice (250 x g, 4°C, 5 minutes) in cold 1% PBS/BSA and subsequently fixed and permeabilised using a commercially available kit according to the manufacturers instructions (Fix and Perm, Invitrogen-Life Technologies). During permeabilisation, relevant antibodies were added

simultaneously to facilitate the detection of internal epitopes and incubated for 30minutes in the dark on ice. Samples were then washed twice in cold 1%PBS/BSA and transferred to cytometric tubes for analysis. Neutrophil degranulation was assessed by flow cytometry (Accuri C6, BD Accuri).

Antibodies were sourced as follows: mouse anti-human CD63-PE 1:20 dilution (Invitrogen – Life Technologies, clone #: CLB-gran/12 (CLB-180). Isotype control antibodies were sourced as follows: IgG1-PE (BioLegend, clone #: MOPC-21).

Table 2.3 Antibody Titrations

Antibody	Titration	Selected Dilution
CXCR1	1:100; 1:50; 1:25; 1:17	1:25
CXCR2	1:100; 1:50; 1:25; 1:17; 1:10	1:17
FPR1	1:50; 1:25; 1:12.5; 1:8.3	1:8.3
C5aR	1:50; 1:25; 1:12.5; 1:8.3	1:12.5
BLT1	1:50; 1:25; 1:12.5; 1:8.3	1:8.3
CD63	1:200; 1:100; 1:50; 1:20	1:20

Dilution of all antibodies used to determine a minimum concentration that would saturate expression of all target molecules

2.7.3 Chemokine Receptor Expression

Surface expression of chemo-attractant receptors (CXCR1 [IL8 receptor], CXCR2 [IL8 and GROα receptor], FPR1 [fMLP receptor], C5aR [C5a Receptor] and BLT1 [LTB_4 receptor]) were measured on freshly isolated peripheral blood neutrophils. Neutrophils and all reagents were maintained on ice to reduce receptor down-regulation and/or shedding. $2x10^5$ neutrophils were re-suspended in 1% PBS/BSA (Sigma Aldrich) and incubated with relevant FITC-labelled antibodies for 20 minutes in the dark, on ice. Post incubation, samples were washed (250 x g, 4°C, 5 minutes) in 1% cold PBS/BSA and transferred to cytometric tubes for analysis. Receptor expression was determined by flow cytometry ($CyAN_{ADP}$, Beckman Coulter) counting 10,000 events to reduce sample variability.

All antibodies were sourced from R&D systems as follows: Mouse anti-human CXCR1 2µg/ml (clone #: 42075); mouse anti-human CXCR2 3µg/ml (clone #: 48311); mouse anti-human FPR1 3µg/ml (clone #: 350418); mouse anti-human BLT1 3µg/ml (clone #: 203/14F11); mouse anti-human C5aR1 2µg/ml (clone #: 347214). Isotype control antibodies were sourced from Dako as follows: mouse anti-human IgG2a 2µg/ml (clone #: DAK-GO5) and mouse anti-human IgG1 3µg/ml (clone #: DAK-GO1).

2.8 Mass Spectrometry

2.8.1 Sample Preparation

1ml aliquots of freshly isolated neutrophils were re-suspended at $5x10^6$/ml in RPMI-1640. Neutrophils from old donors were also pre-incubated ± 1nM Simvastatin for 40 minutes at room temperature. All samples were then centrifuged (6000rpm, 5 minutes [MSE, Micro Centaur]) and supernatants aspirated leaving a dry pellet. Pellets were then snap frozen in liquid nitrogen and stored at -80°C until use.

2.8.2 Lipid Extraction

Membrane lipids were extracted from whole cell lysates with acidified chloroform and methanol as described previously [410]. Dimyristoyl – phosphatidycholine (DMPC, 2nmoles), Low-substituted Hydroxypropyl Cellulose (LHPC, 0.2nmoles), Dimyristoyl-phosphatidic acid (DMPA, 0.24nmoles), Dimyristoyl-phosphatidylethanolamine (DMPE, 0.8nmoles), Dimyristoyl-phosphatidylglycerol (DMPG, 0.4nmoles) and Dimyristoyl-phosphatidylserine (DMPS, 0.4nmoles) were added as internal standards with values in brackets denoting amounts of each standard added. Briefly, cell pellets were re-suspended in 0.8ml Dulbecco's PBS (Ca^{2+} and Mg^{2+} free; Gibco) and briefly sonicated to ensure adequate dispersion of the pellet before addition of internal standards in the presence of butylated hydroxytoluene (BHT, 1g/50ml). Lipids were then extracted from cell lysates with the sequential addition of chloroform, methanol, chloroform (1:2:1) and allowed to separate overnight at -20ºC. The lipid containing, chloroform-rich fraction was removed and dried under oxygen free nitrogen at 37°C to remove chloroform and the dry samples were stored at -20°C until use.

2.8.3 Membrane Lipid Quantification

Membrane lipid content was determined by electrospray ionisation mass spectrometry (ESI-MS). Samples were reconstituted in 30ul of a solution containing 20% Butanol, 60% Methanol, 16% water and 4% concentrated aqueous ammonia ($NH_{3(aq)}$) and introduced by direct diffusion into a triple quadropole mass spectrometer (Quattro Ultima, Micromass, Manchester, UK) equipped with a nanoflow electrospray ionisation interface. Phospholipid species were selectively detected and quantified from a variety of precursor (P) and neutral loss (NL) scans. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were analysed in positive ionisation from tandem MS/MS precursor scans of the phosphatidylcholine fragment (P184) or neutral loss of the phosphoethanolamine fragment (NL141+) respectively. Phosphatidylinositol (PI) and phosphatidylserine (PS) were quantified directly from the negative ionisation spectrum. Data were processed using Mass Lynx

software (Waters) and analysed using a macro developed at the University of Southampton, as employed in previous studies [411]. The macro enabled spectra to be smoothed, background subtracted and converted into centroid format and exported into individual Excel sample files, which were subsequently imported into the analyser programme. Correction for the 13C isotope was performed prior to calculation of percentage composition and incorporation of labelled phospholipid head groups. Only species of PC, PE, PI and PS that made up >2% of the total molar percentage of the respective phospholipid class are reported for compositional analysis.

As free cholesterol does not ionise well using ESI-MS, it was first derivatised to cholesterol acetate by the action of acetyl chloride as described previously [412] using D6 cholesterol as an internal standard, Cholesterol species were quantified from neutral loss scans of the acetate fragment (NL77).

2.9 SDS-Page and Western blot

$1x10^6$ aliquots of isolated neutrophils were centrifuged at 1500xg for 2-minutes at 4°C, re-suspended in lysis buffer (20mM 3-[N-Morpholino] propanesulfonic acid (MOPS), 50mM NaF, 50nM β-glycerophosphate, 10mM Na_3VO_4, 1% Triton X-100 [Sigma-Aldrich, UK], 1mM 4-(2-Aminoethyl) benzensulfonyl fluoride hydrochloride (AEBSF, Sigma Aldrich), 1mM Dithiothreitol (DTT, Sigma Aldrich) and protease inhibitor cocktail [Sigma Aldrich]) and incubated on ice for 15 minutes, vortexing at 5-minute intervals. Lysates were then mixed 1:1 with 2x SDS-sample buffer (4% Sodium Dodecyl Sulfate (SDS), 0.1M Dithiothreitol, 20% Glycerol, 0.0625M Tris-HCl [pH 6.8], 0.004% bromophenol blue) and boiled for 10 minutes at 100°C. Cell lysates were then stored at -20°C until use. Prior to running the gel, defrosted samples were centrifuged at 3000rpm (MSE, Micro Centaur) for 2 minutes at room temperature.

Cell lysates were loaded onto a 5% stacking gel and 12% resolving gel and separated by SDS-polyacrylamide gel electrophoresis. Lysates and pre-stained molecular weight markers

(GeneFlow Ltd) were run at 140 volts and subsequently transferred onto a 0.45 micron polyvinylidene difluoride (PVDF) membrane (Geneflow Ltd) at 450mA for 90 minutes using a wet blotting system (BioRad). Membranes were incubated with 5% bovine serum albumin made up in TBS-Tween (0.1% Tween-20 in 1xTBS [20mM Tris; 150mM NaCl]) for 1 hour at room temperature to prevent non-specific binding of primary antibody. Membranes were then probed with either 1/1000 dilution of rabbit anti-human phospho-PI3K p85 (Tyr485)/p55 (Tyr199) (Cell Signalling Technology) or phospho-Akt (Ser473) XP (Cell Signalling Technology) both diluted in 5% BSA in 1xTBS-Tween overnight at 4°C with gentle agitation. Blots were subsequently washed 3 times in TBS-Tween for 15 minutes before incubation with anti-rabbit IgG secondary antibody conjugated to horse-radish peroxidase (HRP) (Dako Cytomation). Following incubation, blots were washed three times in 1xTBS-Tween as before and proteins were visualised using enhanced chemiluminescence (EZ-ECL; Geneflow Ltd) and autoradiography against X-ray film (Amersham Pharmacia). Equal protein loading was assessed by probing blots for β-actin using a mouse anti-human β-actin (Sigma Aldrich) primary antibody followed by anti-mouse HRP secondary antibody (Cell Signalling Technology) or rabbit anti-human Akt (pan) (Cell Signalling Technology) followed by anti-rabbit HRP secondary antibody (DakoCytomation) using the same protocol.

2.10 Retrospective study of hospital outcomes following admission with a diagnosis of community acquired pneumonia to assess whether statins impact upon outcomes.

This study was a retrospective analysis of data for patients admitted to the Queen Elizabeth Hospital Birmingham NHS Foundation Trust, UK with a clinical and radiological diagnosis of community acquired pneumonia occurring between November 2009 and October 2011.

Patients were included if they had an acute admission with a diagnosis of pneumonia in accordance with British Thoracic Society guidelines, namely symptoms and signs consistent

with an acute lower respiratory tract infection associated with new radiographic shadowing for which there is no other explanation, where the illness is the primary reason for hospital admission and is managed as pneumonia (see **Figure 2.1** for selection procedure). Patients with other acute respiratory presentations diagnosed from medical assessment or chest radiography were excluded from the study. See **Table 2.4** for diagnoses included and excluded from this study.

Patients were classified as being on statin therapy prior to admission if they were prescribed a statin within 24 hours of hospital admission.

2.10.1 Covariates

Using electronic medical records, a range of clinical parameters were also collated to determine potential confounding effects on pneumonia outcomes. In addition to diagnosis, statin therapy and patient outcome, data collected included age, gender, diabetic status, hospital length of stay, admittance onto the intensive care unit (ICU) and ICU length of stay (LoS). Levels of C-reactive protein (CRP) and numbers of circulating white blood cells (WBC) were also recorded to monitor the inflammatory state. Biochemical data collected included, Haemoglobin (Hb), clotting potential (expressed as International Normalized Ratio, INR). Sodium (Na), Potassium (K), Urea (Ur) and Creatinine (Cr) blood levels.

2.10.2 Pneumonia Outcomes and statistical analysis

The primary outcome for this study was defined as all-cause mortality during hospital stay. Patients who recovered and were subsequently discharged from hospital left the study without the occurrence of the primary outcome and therefore constitute censored data.

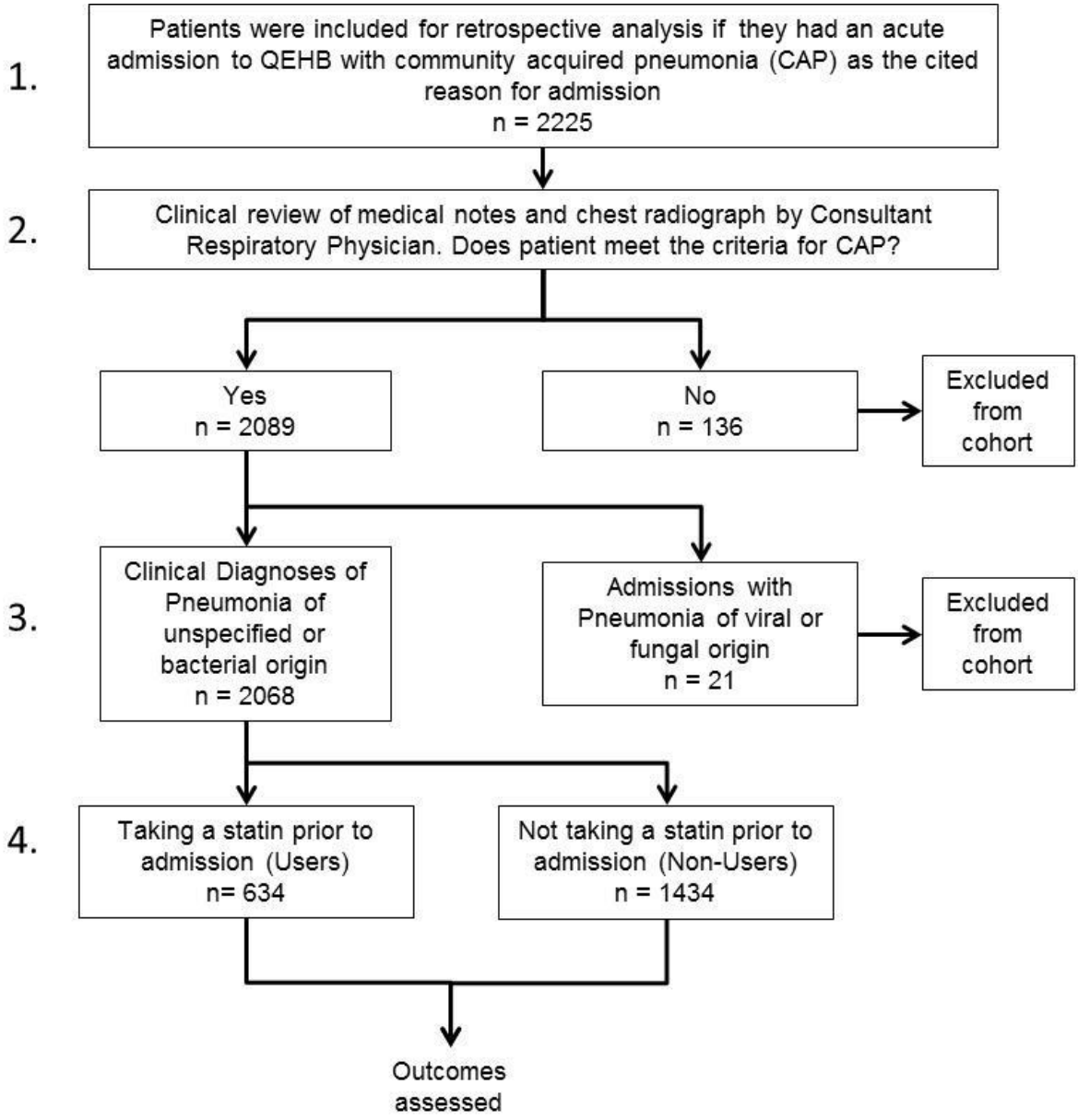


Figure 2.1 Selection Procedure for Retrospective Cohort Study.

Admission into the Queen Elizabeth Hospital, Birmingham UK between October 2009 and November 2011 were screened according to diagnosis to identify patients admitted with Pneumonia of unspecified of bacterial origin in their starting episode (stages 1-3). Qualifying patients were then split into users and non-users according to statin prescription prior to admission (stage 4)

Table 2.4 Inclusion and Exclusion Diagnoses for Retrospective Cohort Study

Inclusion	**Exclusion**
Pneumonia, unspecified	Pneumothorax
Bacterial Pneumonia, unspecified	Other spontaneous pneumothorax
Other Pneumonia, organism unspecified	Traumatic Pneumothorax
Lobar Pneumonia, unspecified	Traumatic Haemopneumothorax
Hypostatic Pneumonia, unspecified	Pneumocystosis
Bronchopneumonia, Unspecified	Pneumococcal meningitis
Pneumonitis	Influenza with Pneumonia
Pneumonitis due to food and vomit	Viral Pneumonia
Abscess of lung with Pneumonia	Cytomegalovirus Pneumonitis
Pneumonia due to *Haemophilus influenzae*	Septicemia due to *Streptococcus pneumoniae*
Pneumonia due to *Escherichia coli*	HIV disease resulting in Pneumocystis carinii pneumonia
Pneumonia due to *Klebsiella pneumoniae*	Abscess of lung without Pneumonia
Pneumonia due to *Mycoplasma pneumoniae*	Hypersensitivity pneumonitis due to unspecified organic
Pneumonia due to *Streptococcus pneumoniae*	Pneumoconiosis due to asbestos and other mineral fibres
Pneumonia due to other *Streptococcus sp.*	
Pneumonia due to *Staphylococcus sp.*	
Pneumonia due to *Pseudomonas sp.*	
Varicella pneumonia	

Inclusion and Exclusion diagnoses for to identify patients admitted into Queen Elizabeth Hospital with Pneumonia of unspecified or bacterial origin between November 2009 and October 2011.

The cohort used in this study was stratified into 2 groups - patients who were on statins upon admission into hospital and those who were not. The cohort was then summarised by comparing statin-users to non-users and continuous variables reported as means ± standard deviations when variables were normally distributed and as medians and interquartile ranges when variables were non-normally distributed. When considering categorical variables, data were reported as numbers with percentages in parenthesis. Normality was assessed using the Kologmorov-Smirnov test. Statin-users and non-users were compared using Mann-Whitney-U test when variables were continuous and χ^2-test for categorical variables.

Logistic regression was used to calculate a predicted probability of death and evaluate the effects of statin therapy on the predicted survival after pneumonia. This was done using an uncorrected model taking into account age and statin therapy in the first instance. This model was then updated to correct for gender, patient diabetic status and plasma levels of CRP. Significance was accepted at $p<0.05$.

2.12 Statistical Analysis

Statistical analyses were carried out using PASW v18.0 (Chicago, IL, USA). Data were tested for normality using the Kolmogorov-Smirnov or Shapiro-Wilk when samples sizes were small. Differences between two independent or paired samples were assessed using either a paired or un-paired T-test and a one-way, factorial or mixed analysis of variance (ANOVA) was used to compare more than two groups when data sets were normally distributed. When data sets did not follow a normal distribution, non–parametric Mann-Whitney-U tests were performed. Differences between categorical variables were assessed using χ^2 test. Post-hoc analysis was performed using Bonferroni correction for multiple comparisons where appropriate. The effects of chronological age on migratory parameters were assessed by linear regression to determine the relationship. Data were subsequently categorized by decade and assessed using a two-way ANOVA controlling for both age and

gender to determine the pattern of the relationship. In all instances, graphs are presented as mean ± standard error of the mean (SEM) and statistical significant was accepted at $p \leq 0.05$ unless otherwise stated. Unless otherwise stated all p-values are from a 2-tailed test.

CHAPTER 3

NEUTROPHIL FUNCTIONS IN THE HEALTHY ELDERLY

3.1 Introduction

Over the past 15 years, ageing research has uncovered numerous differences in immune cell function between young and old donors occurring as a consequence of natural ageing, termed immune-senescence. A significant proportion of this research has focussed on the adaptive arm of the immune response and the immune risk phenotype (IRP) which describes a set of parameters giving rise to an increased risk of mortality in the elderly population [44,413]. In comparison, our understanding of age-related alterations in innate immune function lags behind considerably. Ageing is associated with an increased susceptibility to bacterial infections [9,10,13,15] and reduced outcomes following infective events [20,21] which are suggestive of dysregulation of the innate immune compartment. Neutrophils are vital during the initial immune response to bacterial infection and therefore, these short-lived cells are ideal candidates for investigation into mechanisms driving immune decline associated with increasing age.

The existing literature on neutrophil migration with age is contradictory with reports of both unchanged [150,372-374] and reduced migratory function [375-379] with age. To date, only one study has assessed migration in terms of both speed of migration (chemokinesis) and direction of migration (chemotaxis) as independent parameters, describing maintained chemokinesis and a trend towards reduced chemotaxis in neutrophils isolated from the elderly [161]. However, this study utilised the under agarose assay which can only provide rough estimations of cell movement and cannot comment on individual cell migratory behaviour.

Altered neutrophil migration with age would clearly impact upon efficiency of pathogen removal, but could have consequences beyond this. There is evidence to support the hypothesis that neutrophils utilise proteinases to aid migration [228,239]. Neutrophil proteinases also facilitate cell adhesion and polarisation by interactions with integrins and cell signalling pathways [229]. Aberrant migration could be associated with increased

proteinase release, either as a consequence of cell activation by low grade systemic inflammation or could in fact contribute to increased systemic inflammation in older subjects as more proteinase would be released if the cells migratory pathways were not optimal [414]. If this were true, one would predict that neutrophils from older subjects may express more markers of primary granule degranulation on their surface, and systemically, there may be increased evidence of neutrophil proteinase activity, however, these data have not been collected in an older population.

Studies have suggested that other anti-microbial functions of neutrophils are altered in elderly adults [160,161,174], however, assessment of these functions have been performed by separate researchers, and there are no data to compare key neutrophil functions (migration, phagocytosis and ROS) in the same cohort of individuals. If all functions were suboptimal, this may have a significant impact on the ability of cells to reach and clear bacterial infection.

In this chapter it is hypothesised that neutrophils from elderly donors exhibit aberrant migration and that this would be associated with evidence of enhanced primary granule degranulation and neutrophil proteinase activity. Also where one neutrophil function was compromised with age, all others would be. This hypothesis was tested by the following aims.

1. To clarify the effects of increasing age on neutrophil migration by studying a cohort of healthy adults across the life-span;

2. To compare markers of azurophil granule degranulation and neutrophil elastase activity in *in vivo* in young and old adults;

3. To assess a broad range of neutrophil functions in a cohort of healthy young and old subjects.

3.2 Results

3.2.1 Neutrophils respond to chemotactic stimuli in a dose dependent manner

In order to determine the most appropriate concentration of chemokine at which to measure migration of neutrophils, migration of cells isolated from young donors was measured in response to increasing concentrations of Interleukin-8 (IL8); Growth Related Oncogene-α (GROα); Complement protein 5a (C5a); Leukotriene B_4 (LTB_4) and the bacterial peptide N-Formyl-Methionyl-Leucyl-Phenylalanine (fMLP). All concentrations were within the range noted in previous studies of biological secretions in man [415]. See **Table 3.1** for selected doses used in all subsequent migratory assays. Most chemokines tested showed a classic bell shaped response curve and the final concentrations for all subsequent studies were chosen based on the greatest chemotactic response (see **Figure 3.1**).

3.2.2 Neutrophils from older adults show decreased migratory accuracy

In order to assess the effects of increasing age on neutrophil migration, time-lapse video microscopy was used to track migration towards IL8, GROα, C5a, LTB_4 and fMLP at concentrations as described in **Table 3.1**. Neutrophils were isolated from healthy elderly (age > 65) and young donors (age < 35). Initial analysis of migratory tracks suggested a loss of migratory accuracy in the elderly in response to all chemoattractants tested. **Figure 3.2** A and B show representative images of cell tracks of neutrophils from a young and old subject towards IL8. **Figure 3.2** C and D show composite data for 5 healthy old and young subjects and demonstrate clear differences in migration patterns towards the chemoattractant source (top of figure). Cell tracks towards GROα, C5a, LTB_4 and fMLP showed similar characteristics (data not shown).

Table 3.1 Chemoattractant Dose Response

Chemoattractant	Dose Response (nM)	Optimum Dose (nM)
Interleukin-8 (IL8)	1; 10; 100; 250; 500	100
Growth Related Oncocgene-α (GROα)	1; 10; 100; 500	100
Complement Protein 5a (C5a)	0.5; 1; 10; 100; 250	1
Leukotriene B_4 (LTB_4)	0.5; 1; 10; 100; 250	10
f-Met-Leu-Phe (fMLP)	0.5; 1; 10; 100; 250	10

Neutrophil migratory parameters were measured in response to 0.5 – 500nM IL8, GROα, C5a, LTB_4 or fMLP. The optimum dose of each chemoattractant was selected based on the ability to efficiently stimulate neutrophil migration in comparison to all other doses and used in all subsequent migratory assays.

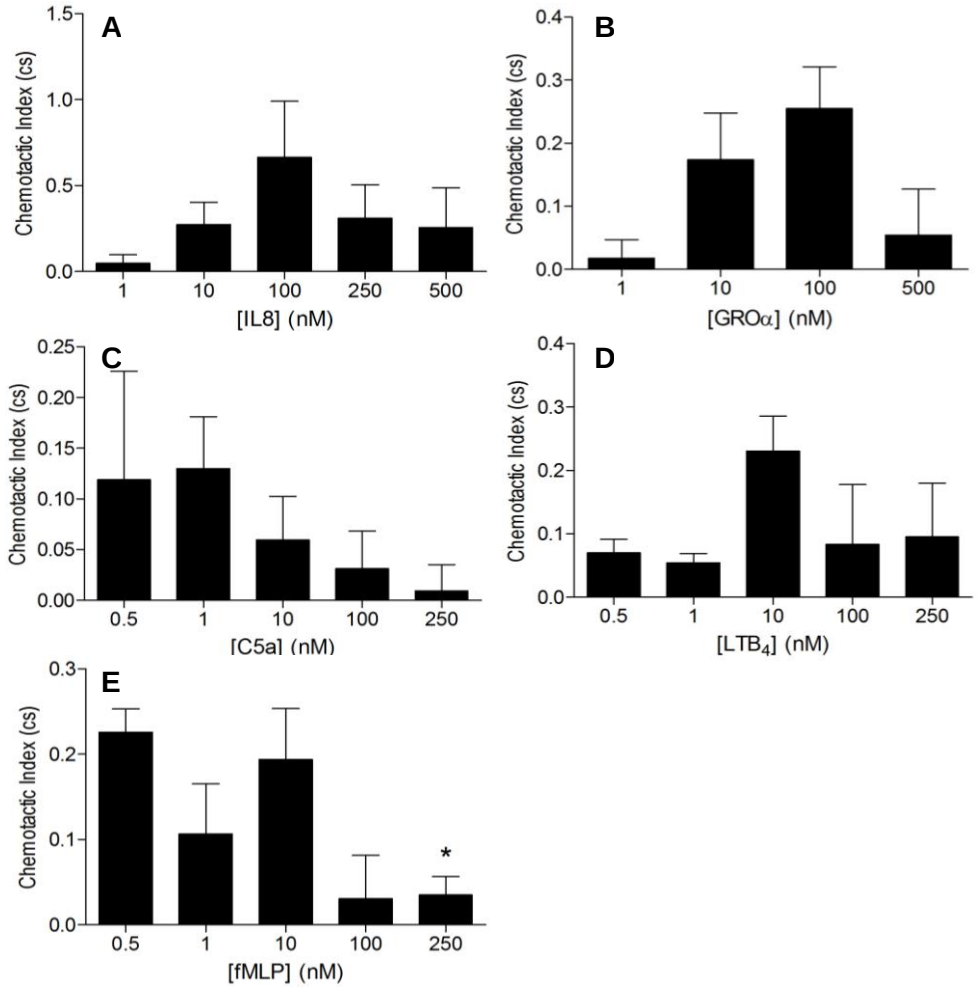


Figure 3.1 Neutrophil Chemotactic Index in Response to Increasing Concentrations of IL8 GROα C5a LTB_4 and fMLP. Neutrophil Chemotactic Index was measured in response to 0.5 – 500nM (A) IL8 (B) GROα (C) C5a (D) LTB_4 and (E) fMLP. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by repeated measures ANOVA.

* $p<0.05$ 0.5nM vs. 250nM. Data are mean ± SEM (n=3).

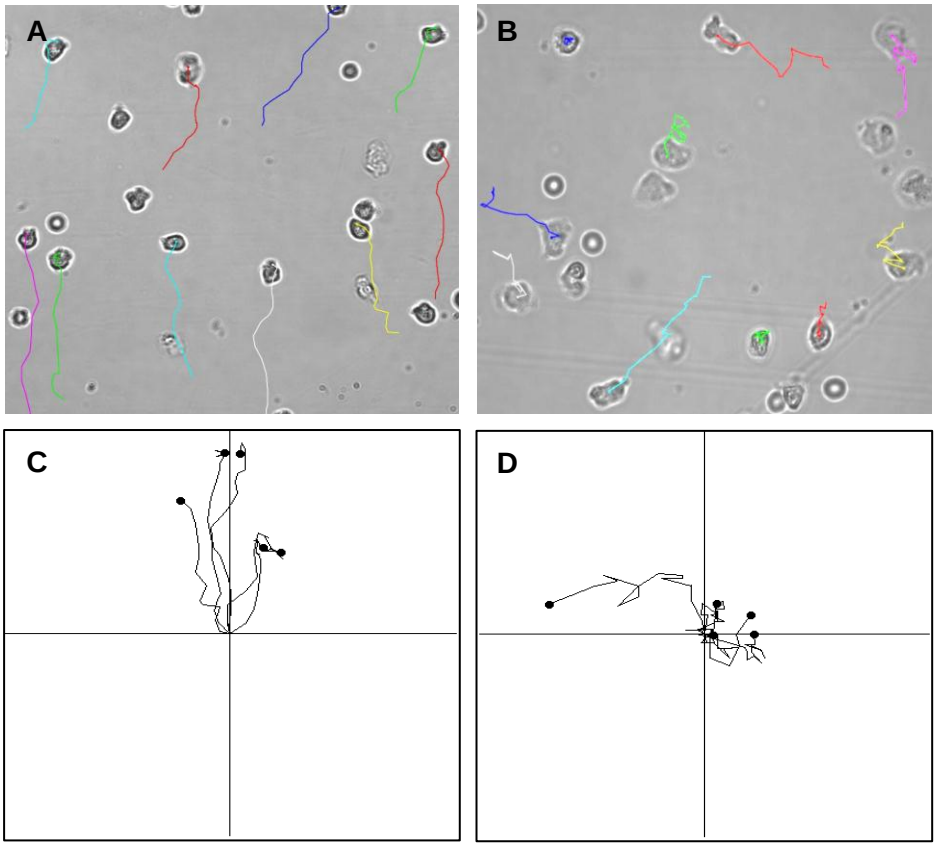


Figure 3.2 Migratory Tracks of Neutrophils isolated from Young and Elderly donors. Images show migratory tracks on a per cell basis following exposure to a chemotactic gradient of 100nM IL8 (greatest at the top of the image). Migratory trajectories of neutrophils from (A) young and (B) old donors were tracked using Image J. Each track begins at the starting position of each respective cell and ends at the final position of the cell following 12 minutes recording. Neutrophil migration was further studied by arranging 5 migratory tracks from (C) young and (D) old donors to show their origins at x = y = 0. The y axis of the tracks represents the direction of the chemotactic gradient and the x axis denotes deviation from a straight line. All images are representative plots of multiple experiments.

3.2.3 Chemokinesis is preserved but Chemotaxis is impaired in neutrophils from older adults

Neutrophil migration was then analysed using parameters of chemokinesis (non-directional speed of movement), chemotaxis (directional migration towards the chemoattractant source), directional persistence and chemotactic index. Neutrophils isolated from young and elderly donors demonstrated no difference in their chemokinesis (see **Figure 3.3**): Young vs. Old, mean ± SEM; IL8: 4.05 ± 0.19 vs. 4.06 ± 0.19, p=0.983; GROα: 3.73 ± 0.3 vs. 4.02 ± 0.16, p=0.381; C5a: 3.77 ± 0.15 vs. 3.58 ± 0.1, p=0.286; LTB_4: 3.93 ± 0.26 vs. 3.6 ± 0.12, p=0.191; fMLP: 3.58 ± 0.2 vs. 3.96 ± 0.21, p=0.327. However, chemotaxis was significantly reduced in the elderly when migrating in response to all chemoattractants studied: Young vs. old, mean ± SEM; IL8:1.41 ± 0.22 vs. 0.57 ± 0.20 p= 0.014; GROα: 0.78 ±0.19 vs. 0.09 ± 0.18 p=0.02; C5a: 1.24 ± 0.28 vs. 0.26 ± 0.16 p=0.01; LTB_4: 0.97 ± 0.21 vs. 0.10 ± 0.18 p=0.006; fMLP: 0.84 ± 0.11 vs. 0.29 ± 0.16 p<0.001 (see **Figure 3.3**).

Neutrophils from elderly donors also showed a significant reduction in migratory persistence, equating to a loss of cell orientation in respect to the chemotactic gradient, in response to GROα, C5a and fMLP: Young vs. old, mean ± SEM; GROα: 0.44 ± 0.03 vs. 0.24 ± 0.05 p=0.002; C5a: 0.42 ± 0.05 vs. 0.17 ± 0.06 p=0.007; fMLP: 0.49 ± 0.05 vs. 0.27 ± 0.05 p=0.007. Directional persistence was maintained with ageing towards IL8 and LTB_4: Young vs. Old, mean ± SEM: IL8: 0.37 ± 0.06 vs. 0.37 ± 0.04, p=0.878; LTB_4: 0.39 ± 0.06 vs. 0.27 ± 0.05, p=0.153 (see **Figure 3.4**).

Chemotactic index, an overall summary of migratory accuracy taking into account all parameters, was significantly reduced in neutrophils isolated from elderly donors compared to those isolated from young donors in response to all chemoattractants studied: Young vs. old, mean ± SEM; IL8: 0.27 ± 0.05 vs. 0.08 ± 0.04 p=0.011; GROα: 0.22 ± 0.05 vs. 0.04 ± 0.04 p<0.001; C5a: 0.26 ± 0.06 vs. 0.04 ± 0.04 p=0.008; LTB_4: 0.23 ± 0.05 vs. 0.01 ± 0.04 p=0.001; fMLP: 0.18 ± 0.04 vs. 0.04 ± 0.01 p=0.004 (**Figure 3.4**).

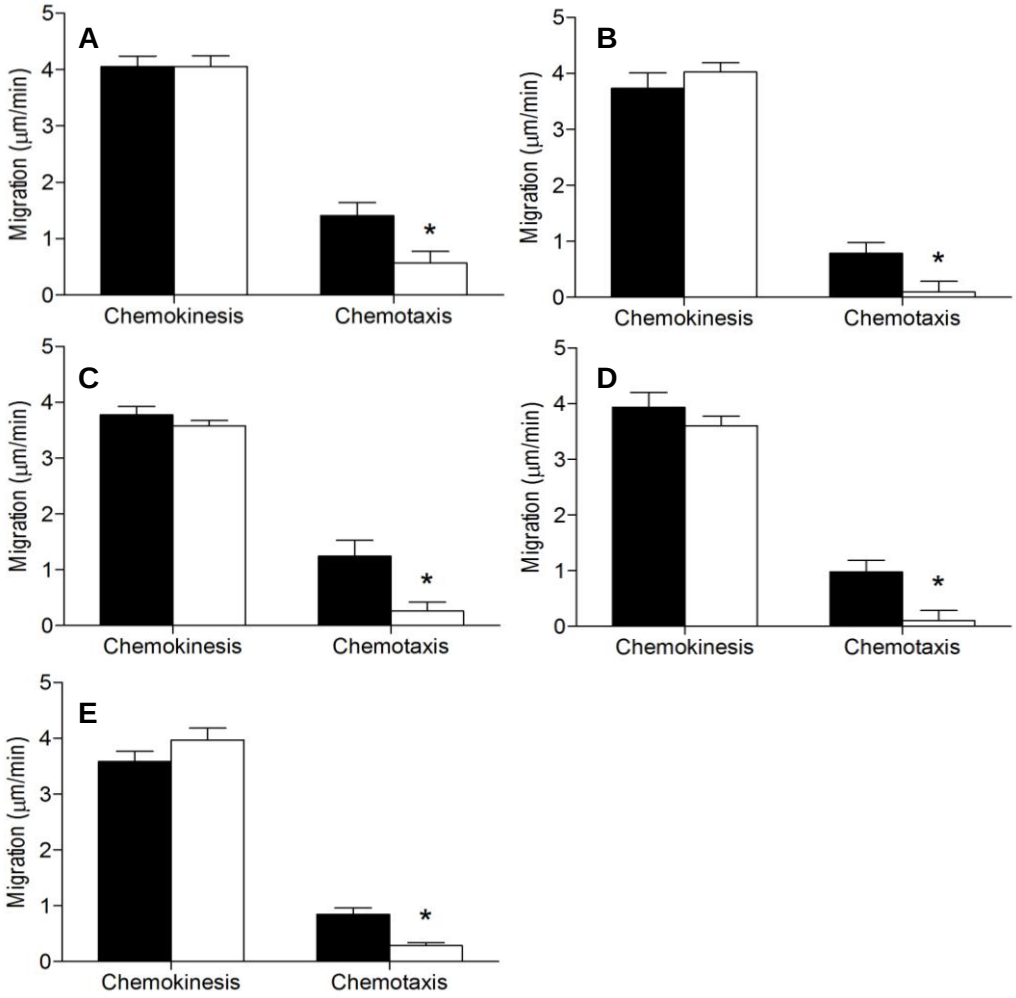


Figure 3.3 Neutrophil Chemokinesis and Chemotaxis in Response to IL8, GROα, C5a, LTB_4 and fMLP. Chemokinesis and Chemotaxis of neutrophils isolated from young (black bars) and old (white bars) in response to shallow gradients of (A) 100nM IL8; (B) 100nM GROα; (C) 1nM C5a; (D) 10nM LTB_4 and (E) 10nM fMLP. All data sets were normally distributed (Kolmogorov-Smirnov Test) and statistical significance measured using an independent samples T-Test. *$p<0.05$ young vs. old. Data are mean ± SEM (n=10).

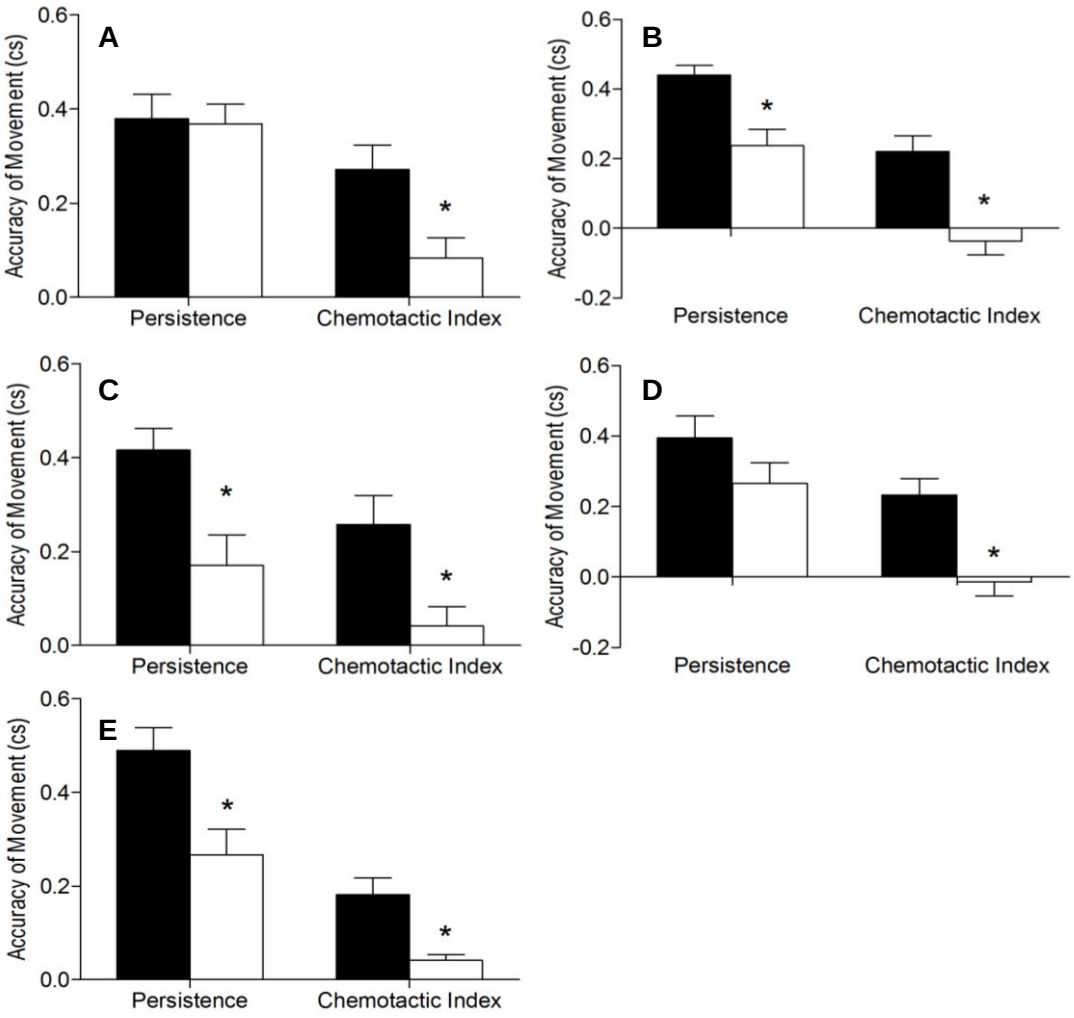


Figure 3.4 Neutrophil Persistence and Chemotactic Index in Response to IL8, GROα, C5a, LTB_4 and fMLP. Persistence (frequency of directional change) and Chemotactic Index (overall summary of migration) of neutrophils isolated from young (black bars) and old (white bars) in response to shallow gradients of (A) 100nM IL8; (B) 100nM GROα; (C) 1nM C5a; (D) 10nM LTB_4 and (E) 10nM fMLP. All data sets were normally distributed (Kolmogorov-Smirnov Test) and statistical significance measured using an independent samples T-Test. *$p<0.05$ young vs. old. Data are mean ± SEM (n=10).

3.2.4 Neutrophil migratory accuracy correlates with increasing age

The results above demonstrated reduced migratory accuracy in the healthy elderly (aged > 65) compared to those under the age of 35 years. It is therefore not clear whether a reduction in migratory accuracy is a late consequence of ageing, perhaps due to impending pathology, or occurs gradually during adulthood and is a biomarker of the ageing process. To assess migration across the life-course, migratory accuracy of neutrophils isolated from donors aged between 20 and 89 years old was determined. Donors were grouped according to decade with 10 participants in each group. See **Table 3.2** for subject demographics in this cohort.

Neutrophils isolated from 70 healthy donors (non-smokers with no clinical evidence of pathology and medication free) were migrated towards IL8 (100nM). When migratory parameters were plotted by age (see **Figures 3.5** and **3.6**) there was a significant relationship between increasing chronological age and chemotaxis ($F_{(1,68)}$= 28.32; β = -0.542; $p<0.001$) and chemotactic index ($F_{(1,68)}$= 46.42; β = -0.637; $p<0.001$). By applying a model of linear regression, these data show that ageing accounts for 28.4% (R^2=0.284) of the observed reduction in neutrophil chemotaxis and 39.7% (R^2=0.397) of the reduction in chemotactic index. There was no significant relationship between age and either chemokinesis or persistence, which was preserved across decennials.

Table 3.2 Cohort Demographics

	n	Age (years) mean ± SEM	Gender (male) n (%)
Total	70	55 ± 2.4	35 (50)
By Decade			
20-29	10	26.7 ± 0.4	5 (50)
30-39	10	34.0 ± 0.6	5 (50)
40-49	10	44.4 ± 0.8	5 (50)
50-59	10	55.0 ± 0.9	5 (50)
60-69	10	66.4 ± 0.5	5 (50)
70-79	10	75.9 ± 0.9	5 (50)
80-89	10	81.8 ± 0.6	5 (50)

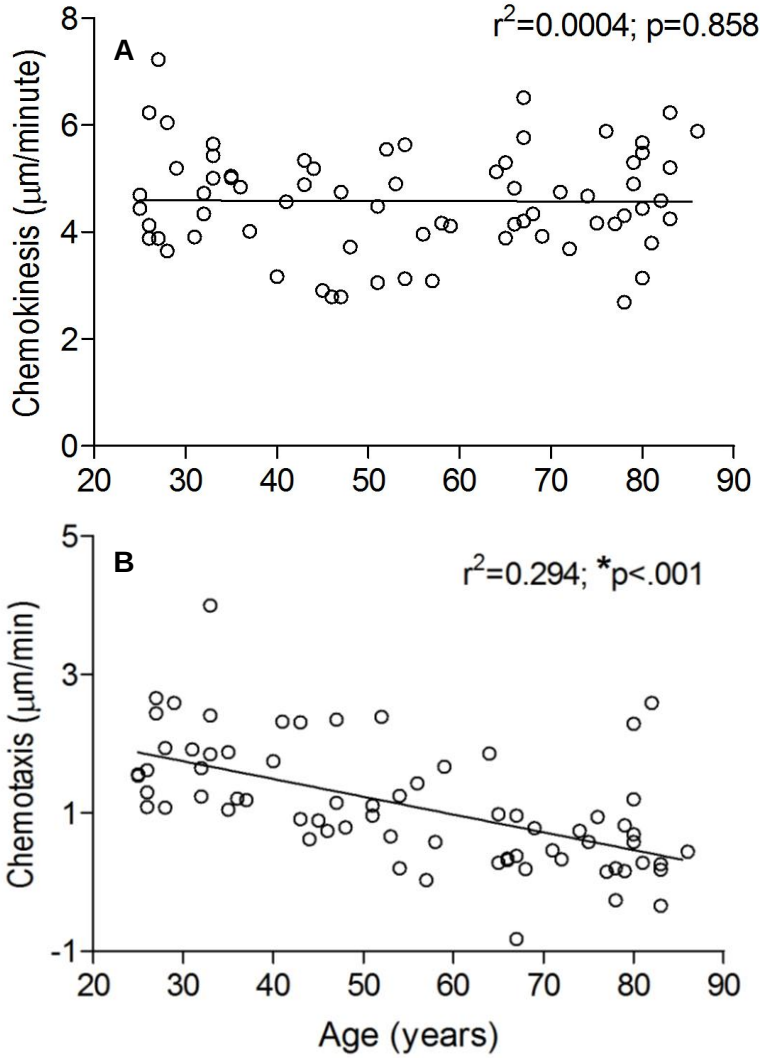


Figure 3.5 Relationship between Age and Neutrophil Chemokinesis and Chemotaxis. (A) Chemokinesis and (B) Chemotaxis of neutrophils isolated from donors aged between 20 and 89 years in response to shallow gradients of 100nM IL8. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by linear regression with significance accepted at * $p<0.05$.

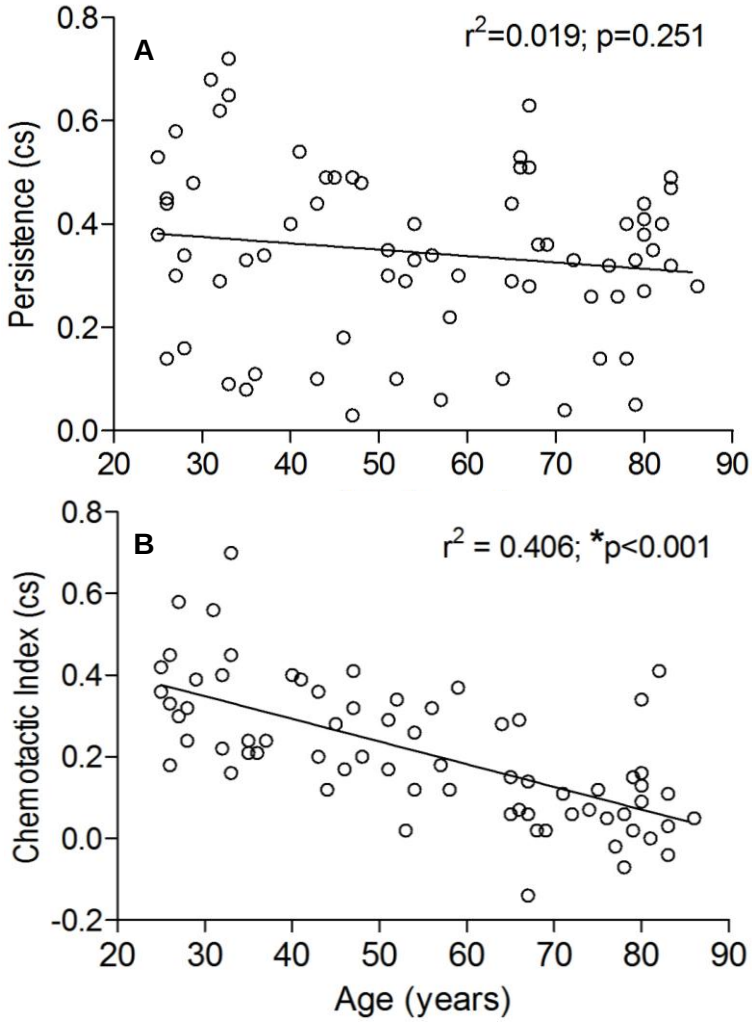


Figure 3.6 Relationship between Age and Neutrophil Persistence and Chemotactic Index. (A) Persistence and (B) Chemotactic Index of neutrophils isolated from donors aged between 20 and 89 years in response to shallow gradients of 100nM IL8. All data sets are normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by linear regression with significance accepted at * $p<0.05$.

3.2.5 Loss of migratory accuracy from 60 years of age

These data are a cross sectional sample of neutrophil function at one time point in each individual, and cannot be used to extrapolate what happens to an individuals neutrophil functions over time. However, they demonstrate a clear reduction in neutrophil migration with advancing age in a significant number of people. When migratory parameters were grouped according to decennial, there was a significant decline in chemotaxis and chemotactic index from the sixth decennial: Chemotaxis mean ± SEM; comparing 20-29 years to 60-60 years: 1.78 ± 0.19 vs. 0.53 ± 0.22 p=0.006; comparing 20-29 years to 70-79 years: 1.78 ± 0.19 vs. 0.41 ± 0.12 p=0.002; Chemotactic Index mean ± SEM; comparing 20-29 years to 60-69 years: 0.36 ± 0.04 vs. 0.09 ± 0.04 p=0.001; comparing 20-29 years to 70-79 years: 0.36 ± 0.04 vs. 0.05 ± 0.02 p<0.001; comparing 20-29 years to 80-89 years: 0.36 ± 0.04 vs. 0.13 ± 0.05 p=0.002 (see **Figure 3.7**).

3.2.6 Neutrophils from older adults exhibit increased expression of granule specific markers

To assess the extent to which neutrophils from older adults had mobilised their primary (Azurophil) granules, surface expression of CD63 (Granulophysin), a marker of Azurophil granule fusion with the plasma membrane was measured [147,416]. CD63 is also considered to be a marker of neutrophil activation [147] therefore, to minimise the extent to which neutrophils were artificially activated, expression of CD63 was measured in whole blood as described in chapter 2, **section 2.7.2**. Neutrophils from healthy older adults expressed significantly more CD63 on their surface compared to neutrophils isolated from young adults (mean ± SEM; 3218.56 ± 562.41 vs. 4364.61 ± 258.41 p= 0.044). There was no difference in the level of CD63 stored internally (see **Figure 3.8**).

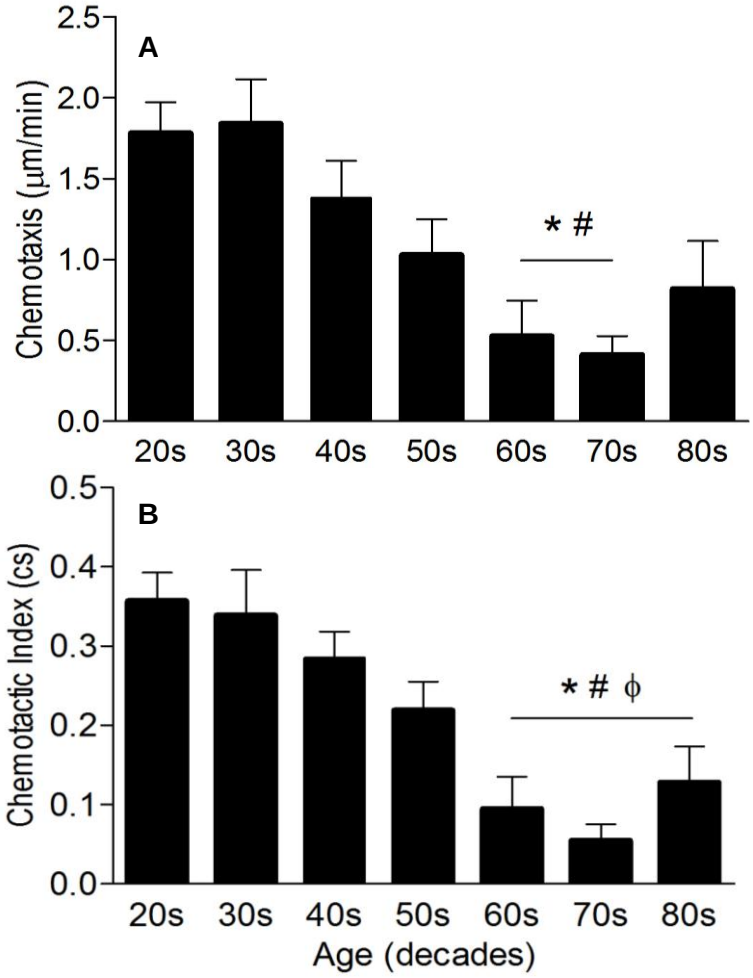


Figure 3.7 Neutrophil Migration across the Lifespan. (A) Chemotaxis and (B) Chemotactic Index of neutrophils isolated from donors aged between 20 and 89 years in response to shallow gradients of 100nM IL8. All data sets are normally distributed (Kolmogorov-Smirnov Test) and statistical significance measured by two-way ANOVA controlling for age and gender. There was no effect for gender on migratory parameters. *$p<0.05$ vs. 20yrs; # $p<0.05$ vs. 30yrs; ϕ $p<0.05$ vs. 40yrs. Data are mean ± SEM (n=10).

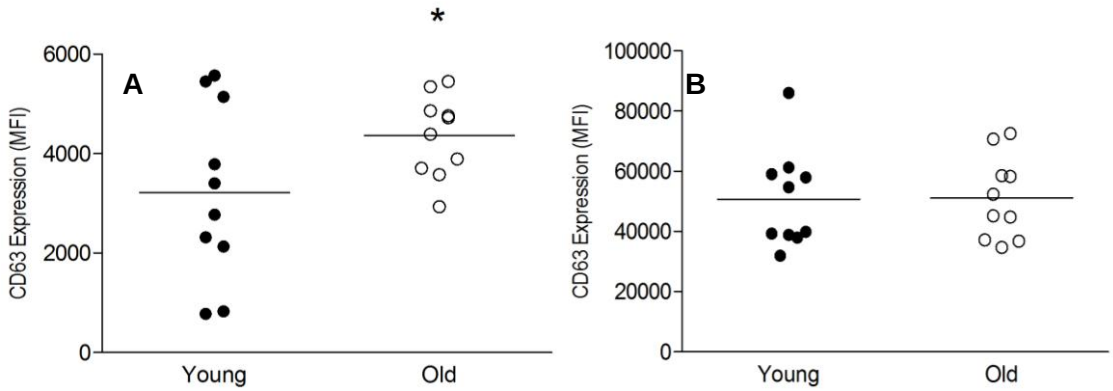


Figure 3.8 Neutrophil Degranulation in cells from the Healthy Elderly.

(A) Surface and (B) Internal expression (mean fluorescence intensity, MFI) of the primary granule marker CD63 on neutrophils isolated from young (filled circles) and old (open circles) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. * $p<0.05$ young vs. old. Bar denotes the mean for each group.

3.2.7 Increased Neutrophil Elastase activity with age

Neutrophil elastase cleaves fibrinogen leaving a stable by-product, termed Aα Val^{360}, which can be measured in plasma. Plasma levels of AαVal^{360} were measured as a footprint of neutrophil elastase activity in 10 healthy young and old donors. There was a significant increase in the concentration of AαVal^{360} in the plasma of old adults when compared with plasma from young adults (Young vs. old, mean ± SEM; 10.18 ± 0.87 vs. 25.92 ± 3.45 p=0.002) indicating increased neutrophil elastase release and activity in the healthy elderly (see **Figure 3.9**).

3.2.8 Neutrophil Phagocytosis of S.aureus but not E.coli is affected by increasing age

Data generated so far suggest that neutrophil migration is sub-optimal in older subjects. To assess whether all functions were equally compromised, migration, phagocytosis and ROS production were assessed in a cohort of healthy old people (aged > 65) where neutrophil migratory function was known to be reduced (n= 8) and a cohort of healthy young adults (age < 35) where migratory function was known to be preserved (n=8). The phagocytic capacity of neutrophils isolated from both young and old donors was measured in response to *Staphylococcus aureus* (*S.aureus*) or *Escherichia coli* (*E.coli*). Neutrophils were co-incubated for 30, 45 or 60 minutes and phagocytic capacity expressed as the phagocytic index (PI) calculated as the percentage of neutrophils capable of phagocytosing bacteria multiplied by the mean fluorescence intensity (MFI). When compared to neutrophils isolated from young donors, the PI of neutrophils isolated from old donors in response to *S.aureus* showed a significant reduction at all time points (young vs. old, mean ±SEM; 30 minutes: 73.03 ± 9.2 vs. 39.04 ± 11.8; p=0.019; 45 minutes: 100.6 ± 11.3 vs. 60.8 ± 15.8; p= 0.029; 60 minutes: 139.9 ± 13.8 vs. 86.7 ± 20.7, p= 0.025). There was no difference observed in the phagocytic index of neutrophils from young and old donors following co-incubation with *E.coli* (see **Figure 3.10**).

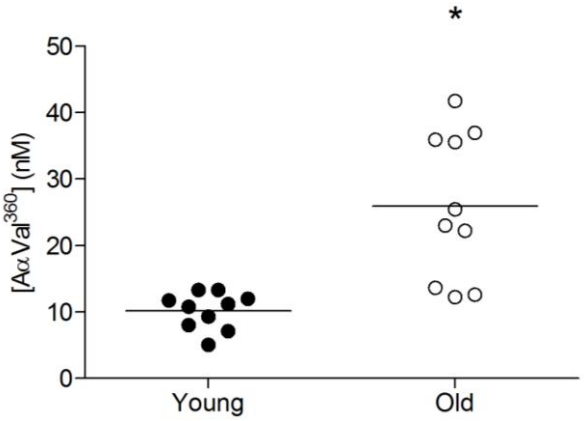


Figure 3.9 Neutrophil Elastase Activity in Neutrophils Isolated from Young and Elderly donors. Concentration of the neutrophil-elastase specific fibrinogen breakdown product $A\alpha Val^{360}$ in plasma collected from young (filled circles) and elderly (open circles) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. *$p<0.05$ young vs. old. Bar denotes the mean for each group (n=10).

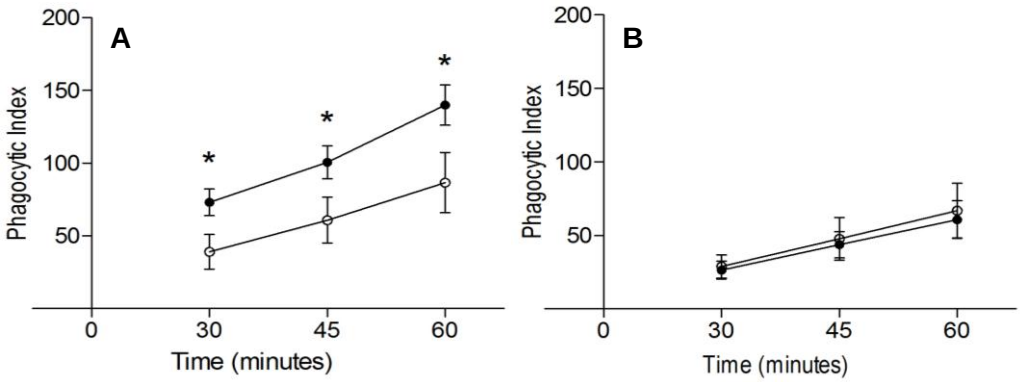


Figure 3.10 Neutrophil Phagocytosis of *S.aureus* and *E.coli*. Phagocytic Index of neutrophils isolated from young (filled circles) and old (open circles) in response to (A) *Staphylococcus aureus* or (B) *Escherichia coli* for 30, 45 or 60 minutes. All data sets are normally distributed (Kolmogorov-Smirnov Test) and statistical significance measured by repeated measures ANOVA. *p<0.05 young vs. old. Data are mean ± SEM (n=8).

3.2.9 Neutrophil Superoxide Production is not affected by increasing age

The production of reactive oxygen species (ROS) was measured in both quiescent and activated (fMLP or IL8 stimulated) neutrophils isolated from both young and old donors. PMA was used as a positive control. There was no significant difference in the amount of ROS produced either in the basal state or in response to 2.5μM fMLP, 1.25μM IL8 or 25nM PMA by neutrophils from either young or old donors (see **Table 3.3**).

In summary, aberrant migration in old subjects was associated with reduced phagocytosis to the gram positive pathogen *S.aureus*, but not to the gram negative pathogen *E.coli*, while reactive oxygen species was preserved with age.

Table 3.3 Neutrophil Reactive Oxygen Species Production.

	AUC (x1000)			
	Unstimuated	fMLP	IL8	PMA
Young	46.5 ± 9.7	169.7 ± 15.4	49.7 ± 10.2	312.7 ± 28.5
Old	45.8 ± 5.4	184.2 ± 38.3	62.8 ± 7.3	337.0 ± 40.6

Neutrophils isolated from young and elderly donors and either unstimulated or stimulated with 2.5μM fMLP, 1.25μM IL8 or 25nM PMA in the presence of 100μM luminol to measure the production of reactive oxygen species. There were no significant differences between groups. Data are mean ± SEM (n=8). AUC: Area under the curve.

3.3 Discussion

Neutrophil migration is the process by which neutrophils migrate through tissue towards the site of infection guided by sequential chemoattractant gradients consisting of cytokines, lipids, complement and bacterial proteins. Upon reaching the site of infection, neutrophils mediate bacterial clearance and degradation utilizing a variety of anti-microbial functions. Within the elderly population rates of both morbidity and mortality due to bacterial infections are significantly increased when compared to the younger population [165,417,418]. As neutrophils are the first cell to be recruited to the site of infection, the aim of this chapter was to accurately assess the effects of increasing age on neutrophil function with a particular focus on neutrophil migration. A number of studies have attempted to define the effects of increasing chronological age on neutrophil migration [150,373,376,379], however no consensus has been achieved. This study is therefore the first to provide a detailed analysis of migratory parameters of neutrophils isolated from healthy older adults when compared to those isolated from younger, gender matched controls and considered some of the physiological consequences of the changes observed.

Neutrophils isolated from healthy adults ≥ 60 years of age exhibited maintained chemokinesis but exhibit a significant reduction in chemotaxis and chemotactic index (overall accuracy) when migrating towards optimum concentrations of a number of physiologically relevant chemoattractants. Wenisch *et al*, the only report to date that has considered chemokinesis as an independent parameter to chemotaxis, reported maintained speed with a trend towards reduced directional migration in the elderly [161] supporting the findings presented here. However, due to the methodology used, direct comparisons cannot not be drawn. Discrepancies between work presented here and other published work reporting either no change or a reduction in neutrophil migration with age are also likely to be due to the methodology used. Most studies use a Boyden Chamber to assess migration, which does not allow for chemokinesis to be studied independently of chemotaxis and the results gained are influenced by the pore size of the filter which can vary between studies.

Data presented here are able to expand on the effect of age on neutrophil migration demonstrating a similar pattern of reduction in response to chemoattractants from different levels of the hierarchy e.g. intermediary chemoattractants (IL8 and LTB_4) and end-point chemoattractants (C5a and fMLP). Cross sectional analysis of migration across the life span demonstrated a significant correlation between age and both chemotaxis and chemotactic index with maximal velocity being achieved between 20 and 30 years of age and thereafter exhibiting a gradual decline in function, becoming significantly compromised from 60 years and onwards. It is noteworthy that this initial decline in migratory parameters coincides with the age at which hormone changes such as the adrenopause, a gradual decline in serum levels of the steroid hormone dehydroepiandrosterone–sulphate (DHEAS), become physiologically significant by 60-70 years of age resulting serum levels being reduced to 20-30% of those observed in younger cohorts [58]. However, DHEAS has been shown to have conflicting effects on neutrophil function; it enhances neutrophil superoxide generation *in vitro* through activation of Protein Kinase-Cβ (PKCβ) [419], but inhibits the migration of peripheral blood neutrophils although the mechanism of action remains unknown as this effect was shown to be independent of Akt, PKC, p38 and ERK, the classical regulators of migration [420]. This suggests that adrenopause is unlikely to be a driving factor behind reduced migratory accuracy with increasing age.

The analysis shown here suggests that increasing chronological age can only account for 28.4% of the reduction seen in chemotaxis and 39.7% of the reduction seen in chemotactic index. It is likely, therefore that other age-related changes must also influence migratory accuracy. Inflamm-ageing for example may affect cellular function by inducing a state in which neutrophils exhibit chronic low-level activation and are perhaps desensitized to chemokine stimulation as a result. Complement proteins present in the plasma of aged rats have also been shown to reduce migration of neutrophils isolated from young donors [421]. However, a link between these factors and an age-related reduction in neutrophil migration still remains to be proven and was not considered here.

Immune function has been shown to be an essential factor in increased longevity through the study of centenarian immune function. Alonso-Fernandez *et al* demonstrated neutrophil chemotaxis, phagocytosis and superoxide generation of neutrophils isolated from centenarians to be at a level comparable to that of a younger cohort (age 25 – 35 years) [378]. This suggests that those people who preserve their immune function are more likely to achieve extreme longevity. As shown in **Figure 3.7**, neutrophil chemotaxis progressively declines until the 8th decade after which the curve becomes more U-shaped. It may be that those people who have reached their 8th decade are those that exhibited a reduced level of immune-senescence in earlier life and are therefore the most likely to become centenarians. Only prospective longitudinal studies can determine how neutrophil functions decline with age and how this impacts on health. However, it may possible to compare an individual's 'immunological age' to that of a healthy cohort, in order to provide an indication as to how well their immune system is fairing against physiological changes that occur with increasing chronological age.

The natural ageing process has been causally related to a decline in organ function including the kidneys [422], liver [423] and lungs [424]. Specifically, decline in respiratory function has been shown to correlate with the systemic inflammatory burden in a dose-dependent manner [424] and it has been hypothesised that neutrophil infiltration may be a contributing factor. Certainly, neutrophils contain sufficient damaging proteins to cause tissue damage if released extracellularly, and neutrophil proteinases have been shown to cause significant cell and tissue damage *in vitro* and animal studies. Aberrant migration in the elderly may further exacerbate this situation due to the internal polarisation and exocytosis of serine proteases such as neutrophil elastase (NE) during migratory processes [223] leaving behind an area of obligate tissue damage [239]. Work presented here would support this hypothesis as surface expression of the azurophil granule specific marker, CD63 and plasma concentrations of the NE-specific fibrinogen breakdown product AαVal^{360} were raised in the elderly indicating an augmented release of neutrophil elastase and evidence of its activity.

Coupled to aberrant migration, increased neutrophil elastase release has the potential to severely compromise organ function, particularly in the lungs where function relies upon the elastic nature of elastin, the main substrate for NE. This is demonstrated in chronic obstructive pulmonary disease (COPD) where plasma concentrations of $AaVal^{360}$ correlate with disease progression and severity of emphysema [232].

Work presented here, although demonstrative of aberrant migration in the elderly with consequences for tissue integrity, included volunteers who were considered to be healthy i.e. medication free and having no inflammatory diseases or experiencing any infections. However *in vivo*, neutrophil migration occurs in response to infection or injury in the tissues with a concomitant increase in soluble inflammatory mediators favouring a pro-inflammatory micro-environment. It would be important to establish whether age-associated aberrant neutrophil migration was preserved or whether it was corrected during inflammatory insult, as this would have implications for the relevance of the described neutrophil phenotype *in vivo*. To investigate this, inflammation was compared during an acute episode of *Streptococcus pneumoniae* related community acquired pneumonia between young and old patients. Old patients (age > 65years) had significantly higher levels of inflammatory mediators and a greater sputum neutrophil count however, the bacterial load was also over 10 times greater than that observed in young patients (age < 35years) (see **Table 3.4**). This suggests that infiltrating neutrophils were either unable to efficiently phagocytose bacteria or exhibited delayed in arrival at the site of infection thus allowing a greater opportunity for substantial bacterial replication and infiltration. Neutrophil migration was also measured in these patients both during pneumonia infection and following recovery. Neutrophils isolated from young donors showed enhanced chemokinesis and chemotaxis during infection when compared to health, however this increase in function was absent in neutrophils isolated from old donors with chemotaxis exhibiting a significant reduction rather than an increase (**Figure 3.11**) (personal communication by Dr Elizabeth Sapey). These data suggest aberrant neutrophil functions are not corrected during heightened inflammation, are associated with worsening

inflammatory and infective profiles and therefore may be an important predictor of poor outcome and thus a target for therapeutic intervention.

Due to the number, age range, health status and array of stimuli used, we have demonstrated a robust, generic migratory phenotype that is a feature of natural immune-senescence and may be a contributing factor to increased rates of morbidity and mortality observed in the elderly population.

In addition to migration, both phagocytosis and the production of reactive oxygen species (ROS) have been reported to exhibit an age related functional decline in response to specific stimuli [161]. The data presented here suggest that if migration is impaired, phagocytosis of gram positive (but not gram negative) bacteria is also reduced, without an effect on ROS generation. The data for ROS generation were gained utilising pro-inflammatory mediators and not bacteria. Niwa *et al* also found no difference in ROS generation to the same chemoattractants [375] but interestingly, Wenisch *et al* demonstrated a significant reduction in ROS response to *S.aureus* stimulation but not *E.coli* [161], again suggesting a differential effect between gram positive and negative stimuli.

Table 3.4 The Inflammatory Response during Pneumonia Infection

	Age < 35	Age > 65
n	5	5
Streptococcus pneumoniae in Sputum	5	5
Bacterial Load (Sputum; cfu/ml); Median (IQR)	$6x10^8$ (3-8)	$8x10^9$ (5-10) *
Neutrophil Count/ml (Sputum); Median (IQR)	$15x10^6$ (11-25)	$23x10^6$ (14-30) *
IL8 (Sputum); nM (IQR)	33.9 (17-46)	60 (43-64) *
LTB_4 (Sputum); nM (IQR)	47.3 (14-72)	74.1 (18-84) *
TNFα (Sputum); pM (IQR)	15.6 (10-28)	40.3 (24-51) *
IL1β (Sputum); pM (IQR)	17.2 (9-22)	40.1 (22-63) *
CRP (Plasma); mg/dl (IQR)	67 (21-98)	102 (52-139) *
Neutrophil Count/ml (blood); Median (IQR)	$16.9x10^6$ (12-23)	$17.1x10^6$ (11-24)
$A\alpha Val^{360}$ (Plasma); nM ± SD	2.3 ± 0.3	4.1 ± 0.4 *

Inflammatory response of 5 young and 5 elderly patients admitted into QEHB with pneumonia infection. All data sets were normally distributed (Kolmogorov-Smirnov Test) and statistical significance measured using an independent samples T-test. *p<0.05 young vs. old.

IQR, Interquartile Range; IL8, Interleukin-8; LTB_4, Leukotriene-B_4; TNFα, Tumor Necrosis Factor-α; IL1β, Interleukin-1β; CRP, C-Reactive Protein; SD, Standard Deviation.

*Table provided by Dr. Elizabeth Sapey

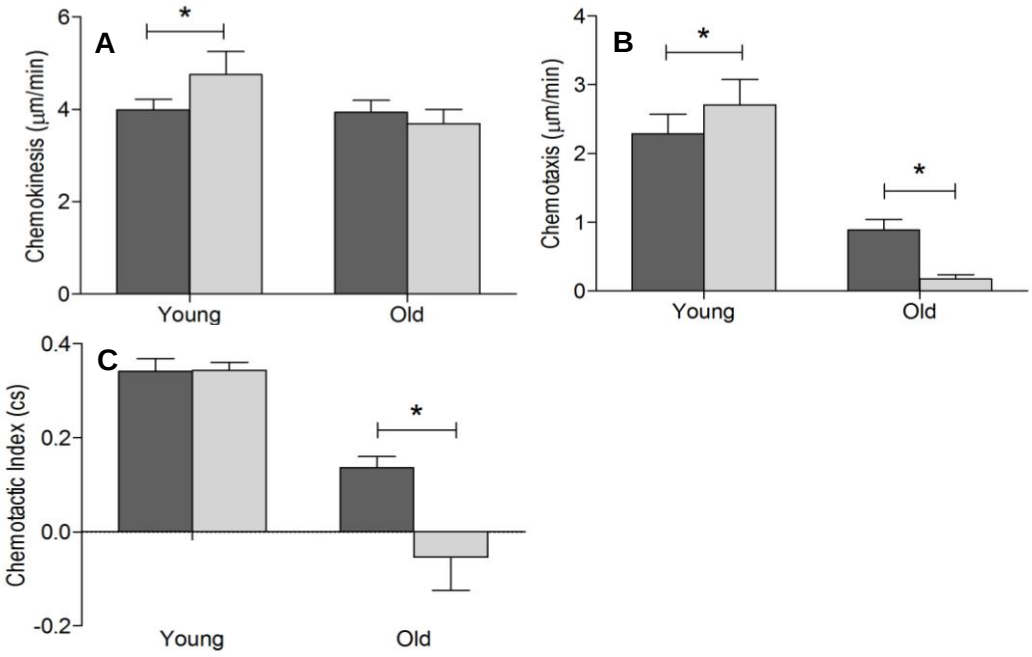


Figure 3.11 Neutrophil Migration in Response to IL8 during Pneumonia Infection.

(A) Chemokinesis (speed of migration), (B) Chemotaxis (direction of migration) and (C) Chemotactic Index (accuracy) of neutrophils isolated from both young and old donors during pneumonia infection (dark grey bars) and following recovery (light grey bars). Migration was measured in response to shallow gradients of 100nM IL8. All data sets were normally distributed (Kolmogorov-Smirnov Test) and statistical significance measured using a factorial mixed ANOVA. *$p<0.05$ health vs. disease. Data are mean ± SEM (n=5).

* Data provided by Dr. Elizabeth Sapey

This stimuli-specific response may reflect the nature of the response required when these stimuli are received *in vivo*. For example, exposure to IL8 would normally occur during the initial stages of migration where ROS production would be required to facilitate migration, whereas exposure to *S.aureus* would naturally occur at the site of infection where large-scale ROS production would be required to eliminate pathogenic material at the site of infection. In order to gain a more accurate picture of ageing and ROS production, stimuli should encompass a number of both particulate (e.g. *S.aureus*) and soluble (e.g. IL8, fMLP) stimuli, measured in both primed and un-primed neutrophils to more accurately represent the inflammatory environment and should use multiple chemiluminescent substrates in order to detect both intra- and extra-cellular ROS [425,426] and the different radical species produced. However, this is the first report where neutrophil functions have been studied in the same individuals where aberrant migration is coupled with poor phagocytosis of gram-positive bacteria. These findings represent significant impairments in vital innate host immunity which could contribute to the poorer responses seen to acute infection in the elderly, together describing the decline in the anti-bacterial capacity of from elderly donors neutrophils, both in travelling to and at the site of infection. This also provides a potential mechanism by which older adults exhibit increased susceptibility gram-positive infections such as pneumonia [21] and bacterial sepsis [427].

Here it is proposed that aberrant migration in the healthy elderly results in increased collateral damage and delayed arrival at the site of infection, potentially allowing invading bacteria a greater opportunity for host infiltration. This might contribute to the increased rates of morbidity and mortality observed in the elderly population. The cross sectional data suggest that the decline in migration might progress gradually over time and that loss of function is due to the accumulation of changes during the ageing process rather than a single catastrophic event.

Mechanisms driving this process remain unclear, however, in order to improve healthspan (the amount of time the average person can expect to spend in good health); a clearer

understanding of the mechanisms regulating neutrophil migration must be obtained. These data support the concept that the neutrophil is an immunotherapeutic target in both acute infections and chronic disease, however in order to ensure an adequate immune response remains, it would be important to normalise and not neutralise neutrophil function.

CHAPTER 4

MECHANISMS OF ALTERED NEUTROPHIL MIGRATION WITH AGEING

4.1 Introduction

Chapter 3 demonstrated a robust aberrant migratory phenotype in neutrophils isolated from old donors specifically, a reduced migratory accuracy with maintained speed of migration. This may result in delayed arrival at the site of infection and be a major contributing factor to the increased rates of morbidity and mortality associated with bacterial infections in the elderly population.

When a cell receives an external stimulus, it must first transmit this signal across the plasma membrane in order to evoke a cellular response, which in the case of cell migration would involve rearrangement of the actin cytoskeleton and modulation of adhesive contacts on the cell surface [428]. The first stage of this response is the binding of the stimulus to its complementary receptors on the cell surface e.g. IL8 binding to CXCR1 and/or CXCR2 inducing 'outside-in' with concomitant 'inside-out' signalling inducing increases in integrin avidity on the surface [429]. This then initiates an internal signalling cascade either through recruitment of cellular signalling molecules to the plasma membrane or through interaction with the cytoskeleton inducing rearrangement, all of which results in a functional cellular response. As previously mentioned, signalling pathways involved in neutrophil directional migration are only partially understood, however signalling molecules such as phosphatidylinositol-3-kinase (PI3Kinase) [430], Akt/PKB [431], mammalian target of rapamycin (mTOR) [432] and members of the Rho-GTPase family [305] have all been heavily implicated in the regulation of migration. In order to identify the mechanisms driving aberrant neutrophil migration in the elderly and potentially intervene to improve innate immunity in the aged, the effects of increasing age on signalling pathways must be better understood.

It was hypothesised that aberrant migration would not be caused by differential expression of chemoattractant receptors, as migration to a broad range of chemokines was affected by ageing, and would instead be driven by dysregulated cell signalling through PI3Kinase,

particularly the class 1 isoforms γ and δ. This hypothesis would be tested by the following aims:

1. To assess chemokine receptor expression on the surface of quiescent neutrophils isolated from young and old adults;

2. To measure the membrane phospholipid content of neutrophils isolated from young and old donors;

3. To compare PI3Kinase and Akt activity in neutrophils isolated from young and old donors;

4. To determine the effect of PI3Kinase inhibition on neutrophil migration using both broad-spectrum and isoform selective inhibitors.

4.2 Results

4.2.1 Effects of age on Surface Expression of Chemoattractant Receptors

To assess any age-related defects in the proximal step of signal transduction, surface expression of receptors for the chemoattractants used in chapter 3 were measured on quiescent neutrophils isolated from healthy young (n = 20) and healthy old (n = 20) donors. Surface expression of CXCR1 (Young vs. old MFI ± SEM 109.8 ± 8.2 vs. 66.9 ± 9.7; p=0.001) and CXCR2 (Young vs. old MFI ± SEM; 68.4 ± 5.7 vs. 49.01 ± 7.8; p=0.025) was found to be significantly reduced on quiescent neutrophils isolated from old donors compared to young donors. However, there was no significant difference in surface expression of C5aR, BLT1 or FPR1 in neutrophils isolated from old donors (see **Figure 4.1**).

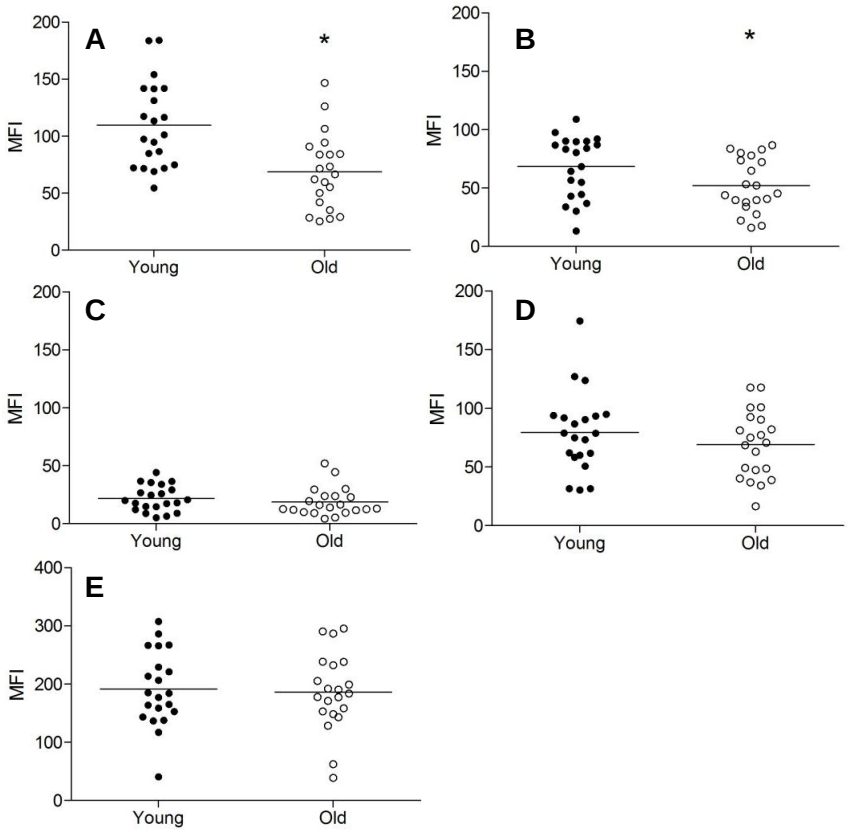


Figure 4.1 Surface Expression of Chemoattractant Receptors on Quiescent Neutrophils. Expression of (A) CXCR1, (B) CXCR2, (C) C5aR, (D) BLT1 and (E) FPR1 on the surface of quiescent peripheral blood neutrophils isolated from young (filled circles) and elderly (open circles) donors. Data sets were non-normally distributed (Kolmogorov-Smirnov test), statistical significance was assessed via Mann-Whitney U test. Bar denotes the mean for each group (n=21).

4.2.2 Ageing does not alter phospholipid composition of the plasma membrane

Alterations in the composition of phospholipids within the plasma membrane, particularly those with long fatty-acid chains, has been suggested as a possible mechanism by which age-related alterations in signal transduction may occur [162]. This may be due to reductions in membrane fluidity and therefore the ability of receptors to co-localize. This chapter therefore assessed the proportions of phosphatidylcholine (PC); phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) present in the membrane of quiescent neutrophils isolated from young and old donors. This work was done in collaboration with Professor Anthony D. Postle, Southampton Centre for Biomedical Research, NIHR Respiratory Biomedical Research Unit, University Hospitals Southampton, UK.

Neutrophils isolated from old donors showed a significant reduction in the proportion of total PS species present in the membrane (young vs. old, mean ± SEM; 12.03 ± 0.6 vs. 9.73 ± 0.6, p=0.009) while total proportions of total PC, PE and PI species remained unchanged with age (see **Figure 4.2**).

Individual phosphatidylcholine (PC) species showed minor alterations with age: PC16:0/22:6 and PC18:0/22:6 were significantly reduced (young vs. old, mean ± SEM; PC16:0/22:6: 0.9 ± 0.2 vs. 0.5 ± 0.06, p=0.037; PC18:0/22:6: 0.8 ± 0.2 vs. 0.4 ± 0.05, p=0.05). There were no age related alterations in the proportions of individual PE, PS and PI species (**Figures 4.3** - **4.6**).

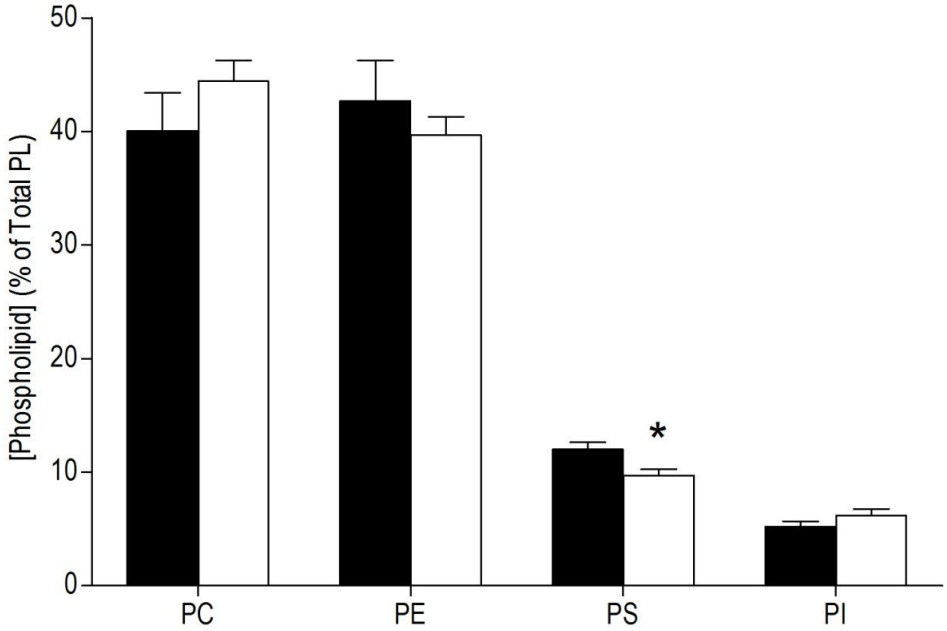


Figure 4.2 Plasma Membrane Phospholipid Composition

Percentage of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS) and Phosphatidylinositol (PI) present in the plasma membrane of human neutrophils isolated from young (black bars) and old (clear bars) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance testing by independent samples T-test. Data are mean ± SEM (n=10). *$p < 0.05$ young vs. old.

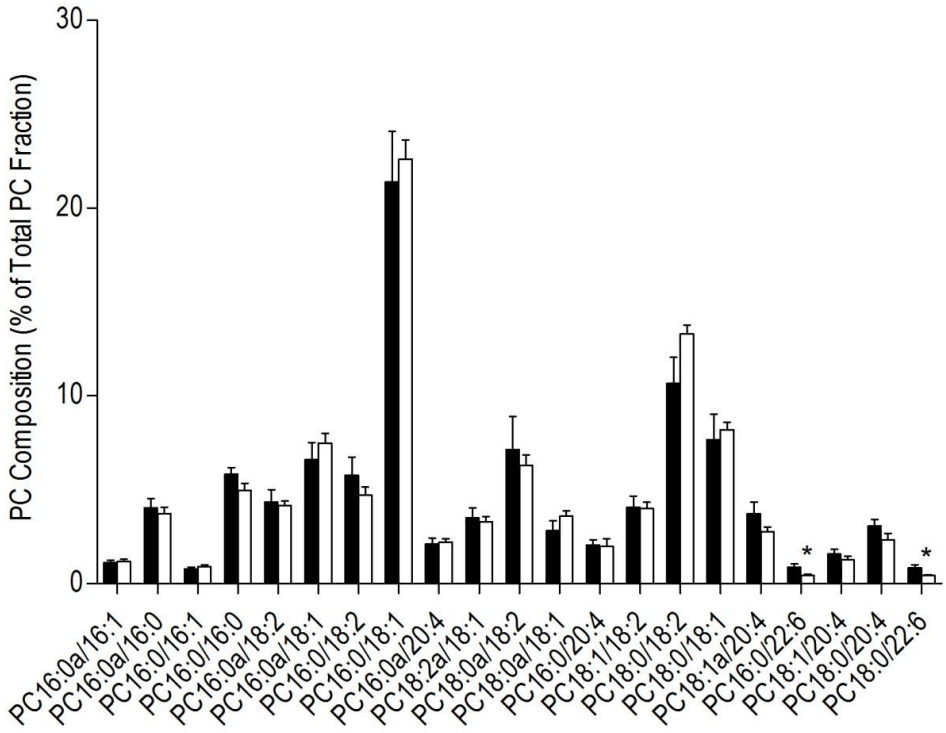


Figure 4.3 Composition of Phosphatidylcholine species in the Plasma Membrane

Percentage of Phosphatidylcholine (PC) species present in the plasma membrane of human neutrophils isolated from young (black bars) and old (clear bars) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance was assessed by independent samples T-test. Data are mean ± SEM (n=10). *$p < 0.05$ young vs. old.

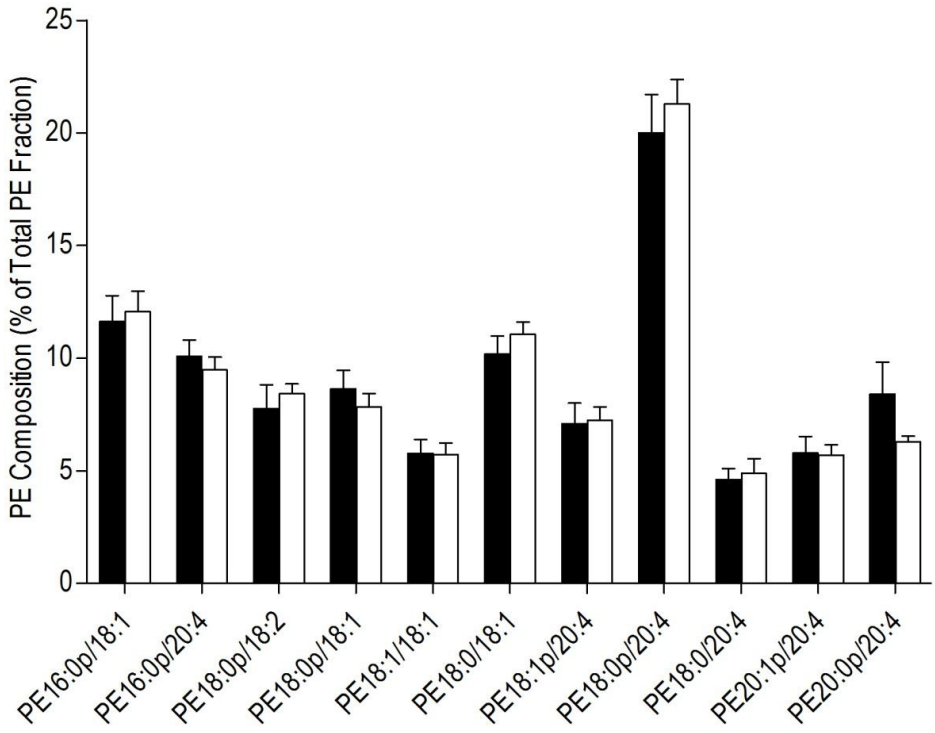


Figure 4.4 Composition of Phosphatidylethanolamine species in the Plasma Membrane

Percentage of Phosphatidylethanolamine (PE) species present in the plasma membrane of human neutrophils isolated from young (black bars) and old (clear bars) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. Data are mean ± SEM (n=10).

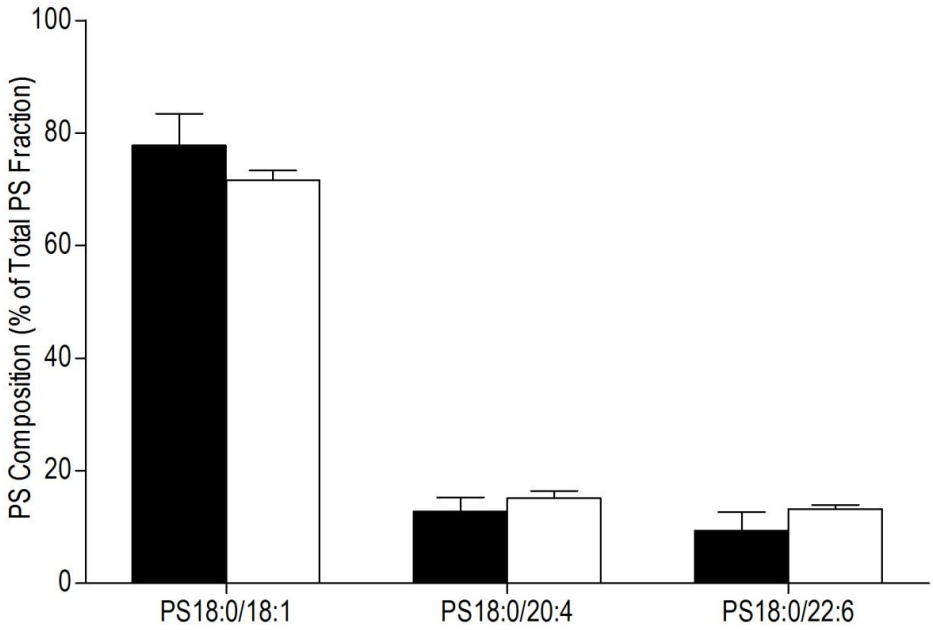


Figure 4.5 Composition of Phosphatidylserine species in the Plasma Membrane

Percentage of Phosphatidylserine (PS) species present in the plasma membrane of human neutrophils isolated from young (black bars) and old (clear bars) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance testing by independent samples T-test. Data are mean ± SEM (n=10).

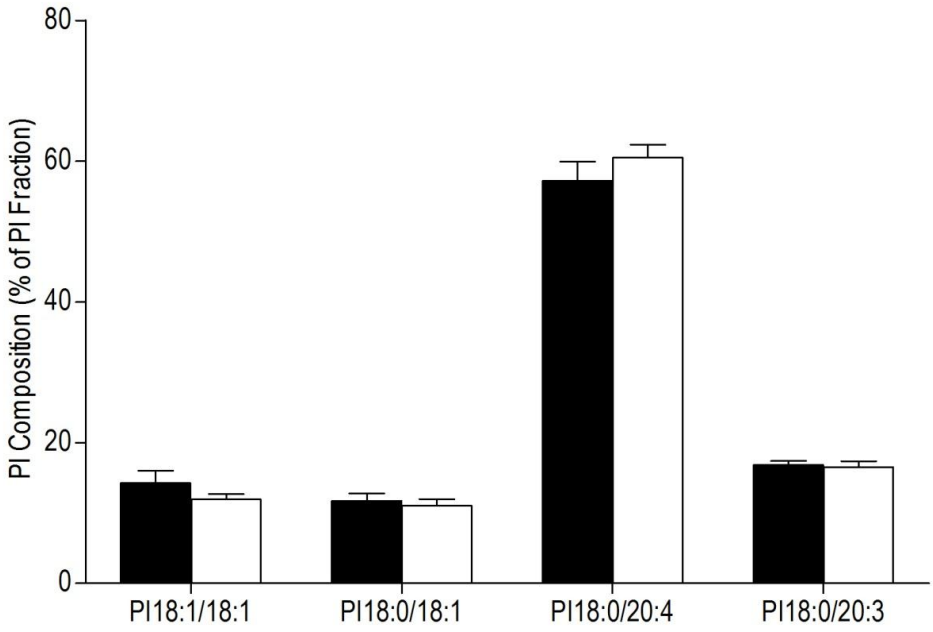


Figure 4.6 Composition of Phosphatidylinositol species in the Plasma Membrane. Percentage of Phosphatidylinositol (PI) species present in the plasma membrane of human neutrophils isolated from young (black bars) and old (clear bars) donors. All data sets follow normal distribution (Kolmogorov-Smirnov test) and statistical significance testing by independent samples T-test. Data are mean ± SEM (n=10).

4.2.3 PI3Kinase is constitutively active in Neutrophils from older adults

The effects of increasing chronological age were also assessed on signal transduction pathways known to be involved in migration. PI3Kinase is thought to be a major pathway involved in the control of directional migration generating an internal gradient of the inositol lipid PIP_3, concentrated at the leading edge of migrating cells [275]. In order to assess the effects of age on PI3Kinase activity, neutrophil lysates from young and old donors were probed for the phosphorylated form of the Class IA regulatory subunit p85 in the presence or absence of IL8. Phosphorylation was determined via western blot using β-actin as a loading control and subsequently quantified by densitometry.

Neutrophils from young adults demonstrated low-level activation in the basal state followed by a transient increase in p85 phosphorylation (peaking at 1 minute) in response to IL8. In contrast, neutrophils from older adults demonstrated constitutive, basal activation of PI3Kinase that was not increased further by stimulation with IL8 (see **Figure 4.7**).

4.2.4 Dysregulated PI3Kinase activation has differential effects on Akt activity

To determine the extent to which dysregulated PI3Kinase activity extended downstream, Akt/PKB activity was measured by phosphorylation of Serine473 (Ser473) in neutrophil lysates from both young (n = 3) and older (n = 3) adults. Akt plays a central role in multiple cellular responses in addition to the control of migration and can therefore be activated by numerous cellular signalling pathways. Neutrophils isolated from young and old donors showed little or no phosphorylation at Ser473 in the basal state but exhibited differential activation when stimulated with IL8 for 1 minute and 2 minutes which was lost by 5 minutes (see **Figure 4.8**).

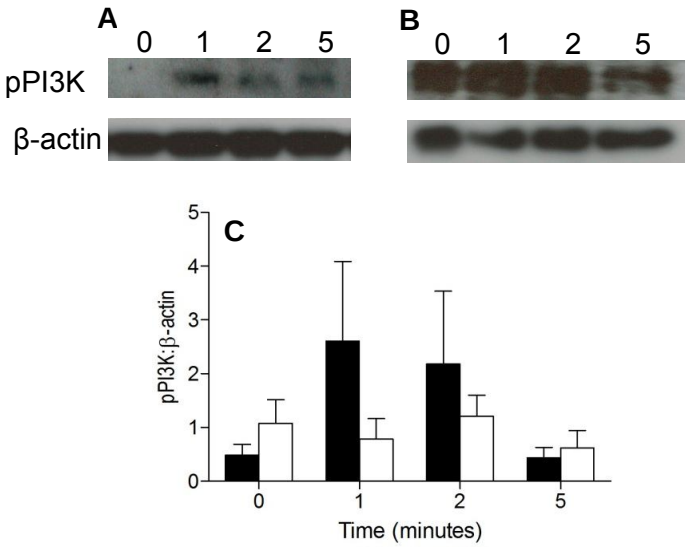


Figure 4.7 PI3Kinase Activity with IL8 Stimulation in Neutrophils from Young and Old donors. Peripheral blood neutrophils isolated from (A) young and (B) old donors were stimulated with 100nM IL8 for the times indicated and PI3Kinase activation measured by the phosphorylation of the regulatory subunit p85. β-actin was used as a loading control. (C) Levels of phosphoPI3Kinase were quantified by densitometry and expressed as a ratio of phosphorylated PI3Kinase (pPI3K):β-actin in neutrophils from both young (black bars) and old (white bars) donors. Images in (A) and (B) are representative of multiple independent experiments (3 young and 4 old) which are presented in (C) as mean ± SEM.

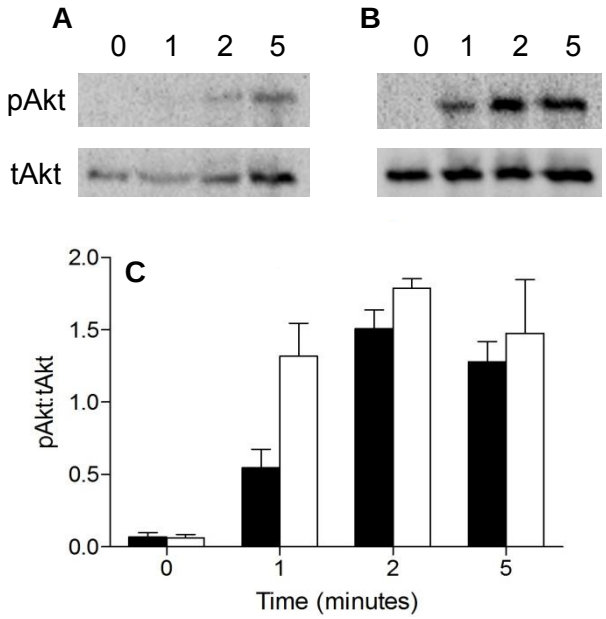


Figure 4.8 Akt Activity in response to IL8 in Neutrophils from Young and Old donors. Peripheral blood neutrophils isolated from (A) young and (B) old donors were stimulated with 100nM IL8 for the times indicated and Akt activation measured by the phosphorylation of Ser473. Total Akt was used as a loading control. (C) Phosphorylation of Ser473 was quantified by densitometry and expressed as a ratio of phosphorylated Akt (pAkt):total Akt (tAkt) in neutrophils from both young (black bars) and old (white bars) donors. Images in (A) and (B) are representative of 3 independent experiments which are presented in (C) as mean ± SEM. Statistical significance was measured by independent t-test.

4.2.5 Dysregulated PI3Kinase activity contributes to aberrant migration

To confirm a role for constitutive PI3Kinase activity in the manifestation of aberrant migration in neutrophils from elderly donors, neutrophils from both young and old donors were pre-incubated with 1μM LY294002 a broad-spectrum PI3Kinase inhibitor prior to measuring migratory parameters in response to IL8.

Pre-treatment of neutrophils from young donors with LY294002 significantly reduced chemokinesis (untreated vs. treated, mean ± SEM; 4.9 ± 0.3 vs. 3.3 ± 0.5 μm/min; p=0.002), chemotaxis (untreated vs. treated, mean ± SEM; 2.0 ± 0.2 vs. 0.8 ± 0.3 μm/min; p=0.001) and chemotactic index (untreated vs. treated, mean ± SEM; 0.4 ± 0.03 vs. 0.1 ± 0.03 μm/min; p=0.008). However, when neutrophils isolated from old donors were used LY294002 had no effect on chemokinesis but significantly improved both chemotaxis (untreated vs. treated, mean ± SEM; 1.1 ± 0.2 vs. 1.7 ± 0.1 μm/min; p=0.01) and chemotactic index (untreated vs. treated, mean ± SEM; 0.2 ± 0.02 vs. 0.4 ± 0.02 μm/min; p-0.03) to levels observed in young cells (see **Figure 4.9**). These data support the proposal that constitutive PI3Kinase signalling in neutrophils from old donors is involved in the generation of an ‘old migratory’ phenotype.

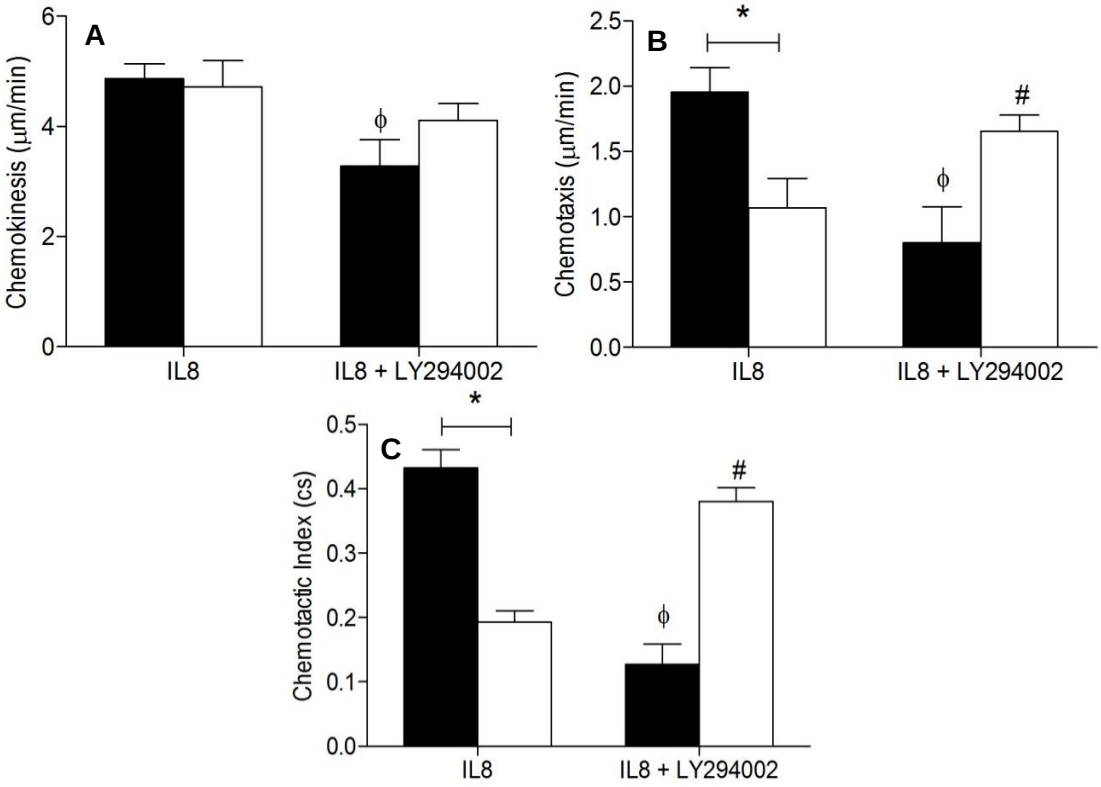


Figure 4.9 The effect of LY294002 on Neutrophil Migration.

Peripheral neutrophils isolated from young (black bars) and old (white bars) donors were incubated in the absence or presence of 1µM LY294002 and (A) chemokinesis, (B) chemotaxis and (C) chemotactic index were measured in response to 100nM IL8. All data sets were normally distributed and statistical significance measured by repeated measures ANOVA. * $p<0.05$ young vs. old; Φ $p<0.05$ young untreated vs. treated; # $p<0.05$ old untreated vs. treated. Data are mean ± SEM (n=10).

4.2.6 Inhibition of PI3Kinase p110 γ and δ isoforms restores migratory accuracy in neutrophils from older adults

Class I PI3Kinase consists of 4 different isoforms of the p110 catalytic subunit, (α, β, γ and δ) all of which are expressed in human neutrophils and involved in migratory processes [255]. Therefore, to further elucidate the role of specific PI3Kinase isoforms in aberrant neutrophil migration, migratory studies were repeated in the presence or absence of inhibitors selective for each isoform: p110α PIK-75 (7.8nM); p110β TGX-221 (10nM); p110γ AS-252424 (33nM) and p110δ CAL-101 (65nM); all concentration used correspond to IC_{50} values to ensure inhibitor selectivity.

Inhibition of p110α or p110β in neutrophils isolated from young donors resulted in a significant reduction in all migratory parameters: untreated vs. treated, mean ± SEM; p110α inhibitor: chemokinesis: 5.4 ± 0.3 vs. 4.1 ± 0.4 p=0.002; chemotaxis: 3.3 ± 0.3 vs. 1.5 ± 0.4 p<0.001; persistence: 0.5 ± 0.06 vs. 0.35 ± 0.05 p=0.012; and chemotactic index: 0.5 ± 0.06 vs. 0.3 ± 0.05 p=0.011; p110β inhibitor: chemokinesis: 5.4 ± 0.3 vs. 3.4 ± 0.3 p<0.001; chemotaxis: 3.3 ± 0.3 vs. 0.7 ± 0.2 p<0.001; persistence: 0.5 ± 0.06 vs. 0.2 ± 0.06 p=0.001; chemotactic index: 0.5 ± 0.06 vs. 0.1 ± 0.04 p=0.001 (see **Figure 4.10**). A significant reduction in chemokinesis was also seen in cells isolated from old donors: untreated vs. treated, mean ± SEM; p110α inhibitor: 4.6 ± 0.4 vs. 3.6 ± 0.5 p= 0.035; p110β inhibitor: 4.6 ± 0.4 vs. 3.6 ± 0.4 p=0.044. However, chemotaxis, persistence and chemotactic index were unaffected by inhibition of either isoform (see **Figure 4.10**).

In contrast, inhibition of p110γ had no effect on chemokinesis but significantly reduced: chemotaxis: untreated vs. treated, mean ± SEM; 2.9 ± 0.3 vs. 1.1 ± 0.2 p=0.002; persistence: untreated vs. treated, mean ± SEM; 0.5 ± 0.05 vs. 0.3 ± 0.05 p=0.001; and the chemotactic index: untreated vs. treated, mean ± SEM; 0.5 ± 0.05 vs. 0.2 ± 0.04 p=0.001 of cells isolated from young adults (see **Figure 4.11**). However in cells isolated from older adults, inhibition p110γ was able to partially restore migratory accuracy by improving chemotactic index: untreated vs. treated, mean ± SEM; 0.2 ± 0.04 vs. 0.4 ± 0.05 p=0.01 whilst preserving

chemokinesis, chemotaxis and persistence (see **Figure 4.11**). A similar effect was seen upon inhibition of p110δ which restored both chemotaxis: untreated vs. treated, mean ± SEM; 1.2 ± 0.2 vs. 2.7 ± 0.3 p=0.001, and the chemotactic index: untreated vs. treated, mean ± SEM; 0.2 ± 0.04 vs. 0.6 ± 0.04 p<0.001 (see **Figure 4.11**) of cells isolated from older adults to levels comparable with those isolated from their young counterparts. In young cells, inhibition of p110δ resulted in a significant reduction in all migratory parameters (see **Figure 4.11**).

Due to the widespread reductions seen in migratory parameters following inhibition of p110 isoforms, especially in cells isolated from young donors, the percentage of viable neutrophils remaining following inhibition with PI3Kinase isoform selective inhibitors (as determined by the percentage of cells remaining annexinV and Sytox negative following incubation) were measured to ensure the inhibitors used during migratory studies were not toxic.

Following a 40-minute incubation with isoform selective PI3Kinase inhibitors, the number of viable cells was unchanged when compared to untreated cells (see **Figure 4.12**).

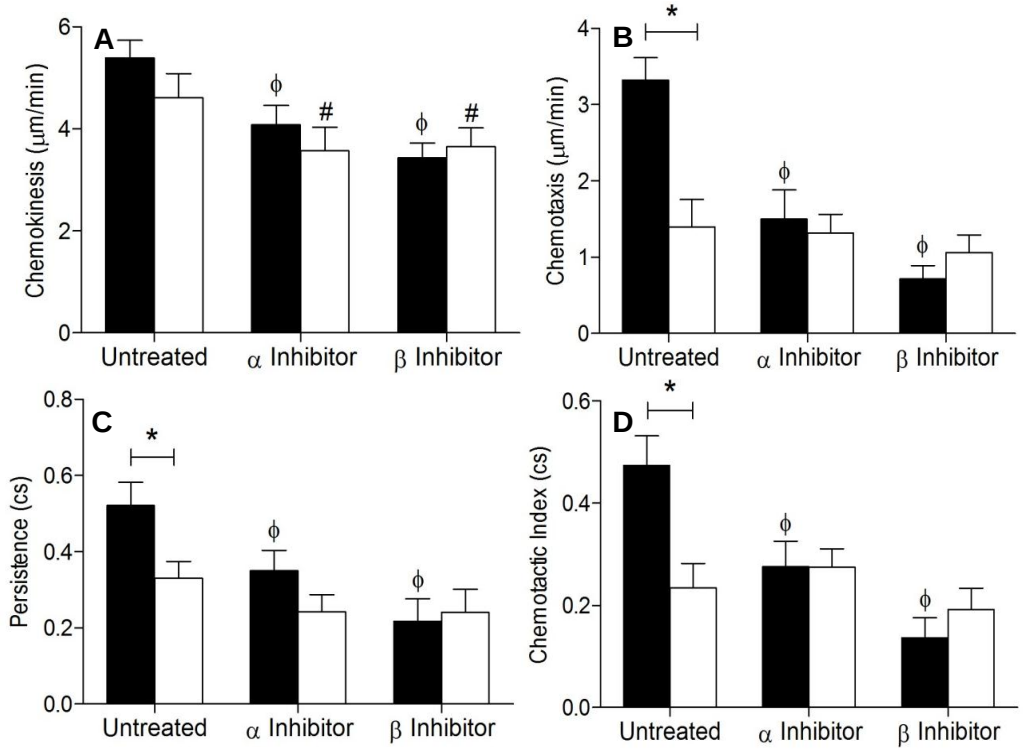


Figure 4.10 Effect of PI3Kinase p110 α and β selective inhibitors on Neutrophil Migration. Peripheral neutrophils isolated from young (black bars) and old (white bars) donors were pre-incubated in the absence or presence of PI3Kinase inhibitors selective for the p110α (PIK-75, 7.8nM) or p110β (TGX-221, 10nM) catalytic subunit of class I PI3Kinase and (A) chemokinesis, (B) chemotaxis, (C) persistence and (D) chemotactic index measured in response to 100nM IL8. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by factorial repeated measures ANOVA. * $p<0.05$ young vs. old; ϕ $p<0.05$ young untreated vs. treated; # $p<0.05$ old untreated vs. treated. Data are mean ± SEM (n=10).

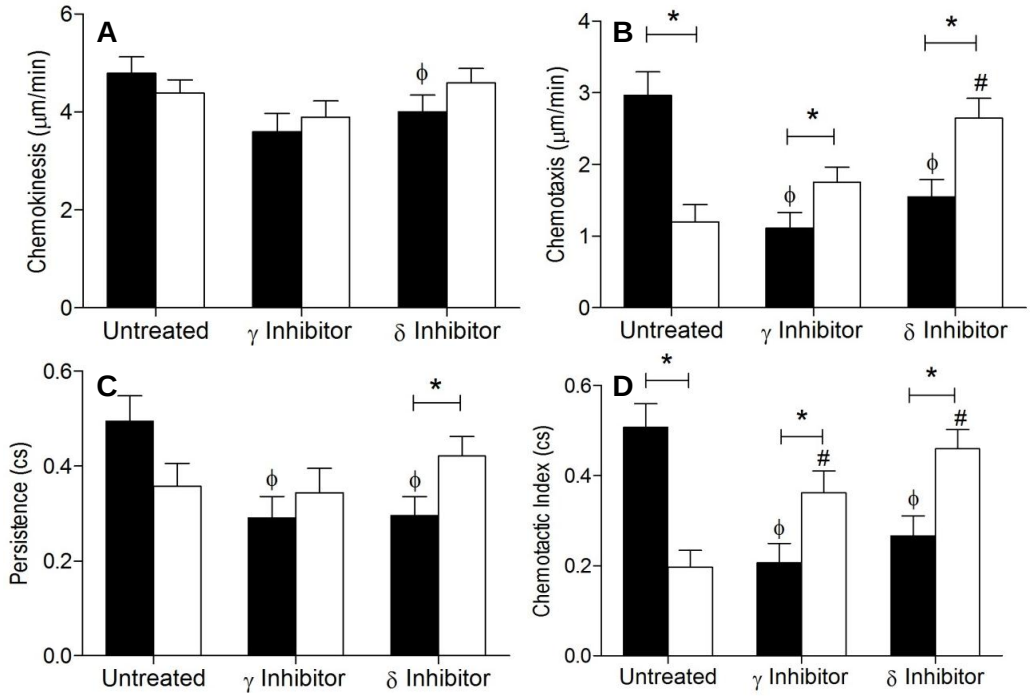


Figure 4.11 Effect of PI3Kinase p110 γ and δ selective inhibitors on Neutrophil Migration. Peripheral neutrophils isolated from young (black bars) and old (white bars) donors were pre-incubated ± PI3Kinase inhibitors selective for the p110γ (AS-252424, 33nM) or p110δ (CAL-101, 65nM) catalytic subunit of class I PI3Kinase and (A) chemokinesis, (B) chemotaxis, (C) persistence and (D) chemotactic index measured in response to 100nM IL8. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by factorial repeated measures ANOVA. * $p<0.05$ young vs. old; ϕ $p<0.05$ young untreated vs. treated; # $p<0.05$ old untreated vs. treated. Data are mean ± SEM (n=10).

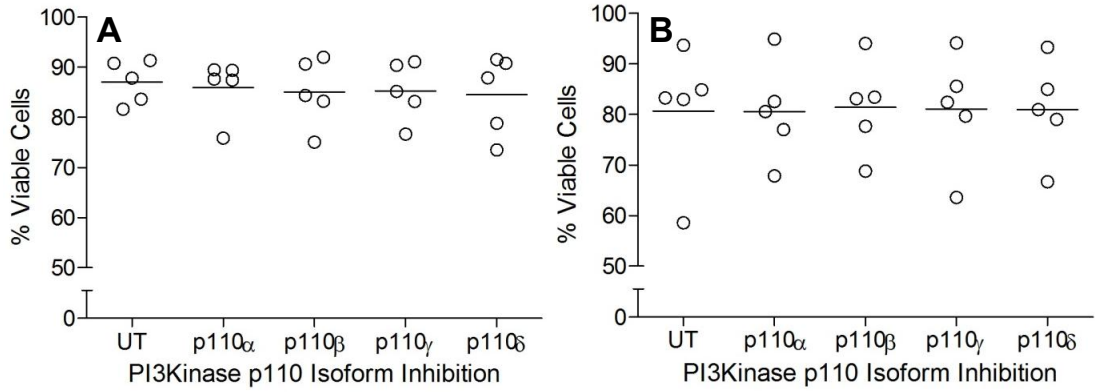


Fig. 4.12 Percentage Viable Cells in the Presence of PI3Kinase Isoform Selective Inhibitors. Percentage of viable neutrophils isolated from (A) young and (B) old donors following 45 minute incubation with isoform selective PI3Kinase inhibitors. Cell viability was determined as those that remain both Annexin V and Sytox negative following incubation ± PI3Kinase inhibitors. Bar denotes the mean for each group (n=5).

4.3 Discussion

Mechanisms driving neutrophil migration are no longer thought to exist downstream of a single linear pathway and instead occur through the complex interaction of multiple pathways when in the correct spatial and temporal orientation [254]. The aim of this chapter was to elucidate any age-related alterations in receptor expression, membrane composition and/or in the PI3Kinase/Akt signalling pathway that may be involved in the generation of the 'old-migratory phenotype' observed in chapter 3. The data show constitutive basal activation of Class I PI3Kinase, which is insensitive to further stimulation in the neutrophils of elderly donors but did not confirm data reported in rat neutrophils of a change in membrane fluidity with ageing [371]. Furthermore inhibition on PI3Kinase-γ and PI3Kinase-δ was able to correct the defects in migratory behaviour in the neutrophils from old donors.

Detection of a chemotactic gradient through GPCRs is the first stage in responding to a chemotactic gradient. Surface expression of CXCR1 (IL8 Receptor A) and CXCR2 (IL8 Receptor B which also ligates GROα, neutrophil activating peptide-2 (NAP2) and epithelial neutrophil activating protein-78 (ENA-78)) showed a significant reduction with increasing age. Neutralisation of CXCR1 has been shown to severely impair neutrophil migration [433] while inhibition of CXCR2 abrogates the age-associated increase in pulmonary inflammation following burn injury [434]. IL8 is considered to be an intermediary chemokine [211] therefore reduced expression of both IL8 receptors may impair migration during the early stages of recruitment. However, this discrepancy is unlikely to be the underlying cause of the aberrant migration observed here as the 'old-migratory phenotype' was also present when migrating towards C5a, fMLP and LTB_4 which signal independently of CXCR1 and CXCR2. This suggests a generic dysregulation of signal transduction downstream of receptor-ligand interactions as opposed to altered receptor specific signalling or expression.

Within the literature, few reports exist on the effects of age on neutrophil membrane composition and fluidity. Alvarez *et al* reported an age-related increase in the phospholipid

content of rat peritoneal neutrophils [371] while Ponnappan *et al* showed that increasing age did not affect the fatty acid composition of membrane phospholipids in human lymphocytes [435].To date these reports remain the only published work investigating the effects of ageing on membrane lipid composition. Here small but statistically significant changes in both the proportion of membrane phospholipid species and in individual phospholipid species were detected in neutrophils from old donors. The discrepancies with published work are likely due to the intrinsic differences between rat and human neutrophils or could also arise from the fact that here peripheral blood neutrophils were assessed, whereas Alvarez *et al* used peritoneal cells which would be activated. However, the changes observed in phospholipid species are small in magnitude and are therefore unlikely to underlie any changes in membrane fluidity and subsequently aberrant migration with ageing. The significant reductions in phosphatidylserine (PS), a phospholipid essential in the recognition apoptotic neutrophils, may impact on the resolution of inflammation through reduced clearance of apoptotic neutrophils, though this has not been tested here.

All chemoattractants utilized in this study signal through GPCRs with a common downstream event being the activation of class I PI3Kinase isoforms [436]. Work presented here demonstrated a constitutive activation of PI3Kinse in the basal state of neutrophils isolated from healthy elderly donors which could not be further activated following stimulation with IL8. PI3Kinase signalling is a significant contributor to the regulation of migratory processes acting as both an initiator and amplifier of the PIP_3 response during inflammation. Inflamm-ageing may be a contributing factor to the sustained PI3Kinase activity observed with ageing, with heightened inflammatory mediators, such as IL8 itself [437], leading to basal activation and desensitisation of immune cells. Indeed exposure to chemokines can result in internalisation of their receptor [211], which could explain the reduced CXCR1 and 2 seen in neutrophils from old donors.

Interestingly, inhibition of PI3Kinase-γ reduces mortality in a murine model of bacterial sepsis, partly by reducing the systemic inflammatory response syndrome (SIRS) [438]. This

thesis is the first study to demonstrate dysregulated PI3Kinase during natural ageing [439]. Although not directly related, mutations in the gene encoding PI3Kinase are a common feature in a number of cancers including glioblastomas (27% of cases), gastric (25%), breast (8%) and lung (4%) cancers which are causally related to aberrant migration, a requirement for invasive migration and metastasis [440].

Increased basal PI3Kinase activity did not directly translate into a downstream signal, at least not through Akt phosphorylation which was not raised in the basal state. At early time points after stimulation with IL8 Akt phosphorylation was significantly higher in the elderly than in the young however; this was not sustained over time. There are a number of possible reasons for this, firstly, phosphorylation of the p85 subunit may not represent a concomitant increase in the levels of membrane localised PIP_3, the p85 regulatory subunit controls the activity of class IA isoforms (α, β and δ) while PI3Kinase-γ, which is under the control of a p101 regulatory subunit, constitutes the dominant PIP_3 producing isoform at the leading edge [279,280]. However the ability of inhibitors of both PI3Kinase γ and δ to correct the aged migratory phenotype do not support this proposal.

Secondly, sustained, dysregulated PI3Kinase activity may not be sufficient to induce sustained up regulation of Akt activity. Additional levels of regulation exist for both PIP_3 and Akt independent of PI3Kinase in the form of PTEN and SHIP1 for PIP_3 [281] and PP2A [299] and PHLPP1/2 [300] for Akt. This possibility was not investigated here but the differential activity of either PIP_3 or Akt regulators in neutrophils from old vs. young donors should be included in future research. In the literature, there are a number of animal models demonstrating the importance of PI3Kinase and PIP_3 phosphatases in the control of directional migration. Dictyostelium discoideum lacking PTEN demonstrate preserved chemokinesis but reduced chemotaxis [441]; knockout of PTEN in Zebrafish results in reduced accuracy of migration, a phenotype that is amenable to correction by LY294002, a broad-spectrum PI3Kinase inhibitor [442] and finally, by using a green fluorescent protein

(GFP) construct specific for the PH domain of Akt, in the absence of SHIP1, Akt exhibits limited translocation to the leading edge of migrating murine neutrophils [343]. These models suggest that the balance between PI3Kinase and PTEN/SHIP1 activity in the regulation of PIP_3 production and localisation is essential in the co-ordination of chemotaxis and could be altered with ageing. This is supported by data presented here demonstrating a causal relationship between PI3Kinase activity and the generation of the 'old migratory' phenotype through inhibition of PI3Kinase using LY294002.

Thirdly, it is possible that the constitutive activation of PI3Kinase presented here does not result in irregular PIP_3 production *per se* (potentially due to compensation by PI3Kinase-γ and/or PTEN/SHIP1), and instead this activity results in inappropriate localisation of PIP_3.

Here directional migration was partly restored through inhibition of PI3Kinase-γ while inhibition of PI3Kinase-δ was able to recover migratory parameters in old donor neutrophils to levels comparable with the young. It is well documented within the literature the importance of these isoforms in the regulation of chemokinesis and chemotaxis [256,264,278]. Here it is proposed that 50% inhibition of these isoforms (the concentration used was in the IC_{50} range) during migratory processes is sufficient to normalize PIP_3 production reducing the background 'noise', allowing greater amplification of the PIP_3 signal upon activation of PI3Kinase-δ and better orientating the cell along the chemotactic gradient. This is supported by work carried out by Boulven *et al* who reported a biphasic PIP_3 response when stimulated with fMLP and attributed the early peak to the action of PI3Kinase-γ and the later peak to an unidentified Class IA PI3Kinase isoform [278]. The data shown here would support the notion that the other Class IA isoform involved is in fact PI3Kinase-δ.

Restoring neutrophil migration through inhibition of PI3Kinase-γ and –δ isoforms will reduce both collateral damage caused by aberrant migration and pathological inflammation thus improving clinical outcomes during infection. Aberrant signalling as a consequence of increasing age may be a function of inflamm-ageing but restoring these pathways may

prevent the concurrent amplification of inflammation thus restoring immune homeostasis and rebuilding neutrophil functions.

CHAPTER 5

SIMVASTATIN AND NEUTROPHIL MIGRATION

5.1 Introduction

So far the data in this thesis have established an 'old-migratory phenotype' exhibiting reduced migratory accuracy with maintained speed of migration in neutrophils from old healthy donors. This phenotype is causally related to constitutive PI3Kinase signalling and can be corrected by inhibition of either PI3Kinase-γ or –δ isoforms highlighting a mechanism by which therapeutic intervention may be possible.

Development of new drugs, from conception to clinical use, can take up to 20 years however in this study, it was decided to look for treatments which had the potential to impact upon immune-senescence in the short term. The influence of statins, specifically simvastatin, on neutrophil migration in the healthy elderly was selected as this drug is widely prescribed to treat high serum cholesterol. Importantly, a number of studies have also shown multiple members of the statin family to have pleiotropic effects independent of their cholesterol lowering abilities, particularly in conferring a survival advantage in older adults with pneumonia [390-396]. In addition, the inhibition of HMG-CoA Reductase by statins also leads to reduced prenylation of small GTPases such as Rho and Rac, inhibiting their function [398,443,444]. As Rac lies downstream of PI3Kinase it was possible that inhibiting this pathway with statins would have the same effect as a PI3Kinase inhibitor.

Considering these data, it was hypothesised that statins would be able to correct the migratory deficit in older adults and thus increase innate immunity. This hypothesis was tested by the following aims:

1. To evaluate the effects of statins on pneumonia outcomes in a retrospective cohort study;
2. To assess the effects of simvastatin *in vitro* on the migration of neutrophils isolated from young and old donors;
3. To identify a mechanism by which statins may act to influence neutrophil migratory dynamics in the healthy elderly.

5.2 Results

5.2.1 Statin Therapy prior to admission to hospital improves Pneumonia outcomes

To confirm published work on the effects of statin therapy on pneumonia outcomes, patients admitted into the Queen Elizabeth Hospital between November 2009 and October 2011 with pneumonia of a bacterial or unspecified origin as their starting episode, were grouped according to statin therapy and outcomes assessed based on their age, gender, inflammatory- and diabetic - status with the clinical endpoint being death.

Of the 2068 patients admitted into QEHB with pneumonia, 634 were taking a statin upon admission and 1434 were not. Clinical data relating to white blood cell (WBC) count, Haemoglobin, C-reactive Protein (CRP), Haemoglobin (Hb), Creatinine (Cr) and Diabetic status was also collated from the hospital database and stratified according to statin therapy.

Patients taking statins prior to admission into hospital had a significantly longer hospital stay than non-users (statin user vs. statin free, mean ± SD; 13.5 ± 15.2 vs. 8.7 ± 12.7 days, $p<0.001$), but were also more likely to survive (statin user vs. statin free, %; 83.1 vs. 75.5, $p<0.001$). Patients taking statins prior to admission were also significantly older (statin user vs. statin free, mean ± SD, 75.66 ± 11.7 vs. 69.2 ± 20.1, $p<0.001$), more likely to be admitted onto ITU during their hospital stay (statin user vs. statin free, %; 10.6 vs. 7.1, $p=0.008$) and more likely to be diabetic (statin user vs. statin free, %; 36.4 vs. 12.1, $p<0.001$). In addition, those on statin therapy prior to admission had higher levels of circulating potassium, urea and creatinine but lower levels of haemoglobin and sodium than those not on a statin. Although differences between these two groups are statistically significant they are not considered to be clinically significant and are therefore unlikely to impact on patient outcome. There was no difference in CRP or numbers of circulating WBC between the two groups (see **Table 5.1**).

Table 5.1 Cohort characteristics split according to Statin treatment

	Statin User	Statin Free	n (%)	p value
n (%)	634 (30.7)	1434 (69.3)	2068	
Age, mean ± SD (years)	75.66 ± 11.7	69.2 ± 20.1		<0.001
Gender, n (%)				
Male	356 (56.2)	691 (48.2)	1047 (50.6)	0.001
Female	278 (43.8)	743 (51.8)	1021 (49.3)	
Patient Outcome, n (%)				
Survived	527 (83.1)	1083 (75.5)	1610 (77.9)	<0.001
Died	107 (16.9)	351 (24.5)	458 (22.2)	
Diabetic, n (%)				
Yes	231 (36.4)	173 (12.1)	404 (19.6)	<0.001
No	403 (63.6)	1259 (87.9)	1662 (80.4)	
Hospital Length of Stay, mean ± SD (days)	13.5 ± 15.2	8.7 ± 12.7		<0.001
Admittance onto ICU, n (%)				
Yes	67 (10.6)	102 (7.1)	169 (8.1)	0.008
No	567 (89.4)	1332 (92.9)	1899 (91.8)	
ICU Length of Stay, mean ± SD (days)	8.6 ± 10.3	7.7 ± 8.86		0.483
CRP, median (IQR)	93 (34 - 211)	102 (43-215.25)	1801	0.188
Haemoglobin, median (IQR)	11.9 (10.7-13.3)	12.6 (11.1-14)	1572	<0.001
WBC, median (IQR)	12.8 (9.3-16.5)	12.6 (8.9-16.9)	1573	0.709
INR, median (IQR)	1.1 (1-1.3)	1.1 (1-1.3)	1270	0.907
Sodium, median (IQR)	138 (134-141)	139 (136-142)	1505	<0.001
Potassium, median (IQR)	4.2 (3.8-4.7)	4.1 (3.7-4.6)	1510	0.005
Urea, median (IQR)	8.5 (6.1-12.3)	7.3 (4.9-11.3)	1683	<0.001
Creatinine, median (IQR)	102 (70-176)	83 (61-134)	715	<0.001

Characteristics of patients admitted into the Queen Elizabeth hospital with pneumonia of unspecified or bacterial origin in their starting episode. ICU, Intensive Care Unit; IQR, Interquartile Range; CRP, C-reactive Protein; WBC, White Blood Cell Count; INR, International Normalized Ratio. Mann-Whitney U-test was conducted for continuous variables and χ^2-test for categorical variables

Utilizing logistic regression, it was possible to assess the predicted probability of death (PPoD) from pneumonia, controlling for age and gender, demonstrating a significant positive relationship between age and PPoD (see **Figure 5.1**). The unadjusted Odds Ratio (OR) for death from pneumonia was 1.045, meaning for every 1 year increment in age, the probability of death increases by 4.5% ($p<0.001$). However when on a statin prior to admission, the OR is reduced to 0.525 ($p<0.001$) (model 1). There was also a significant effect for gender on this model while diabetic status had no effect on PPoD ($p=0.946$) (model 2; see **Table 5.2**). This model was then further expanded to take into account the inflammatory burden, represented by plasma CRP levels (model 3). Taking CRP into account (in addition to age, statin therapy and gender), the OR increased by 0.7 to 1.744 ($p<0.001$). In this model, gender no longer had a significant effect on the PPoD. This is represented graphically in **Figure 5.2** where PPoD is shown as a function of plasma CRP levels in patients aged 79 years, an age bracket chosen to give the greatest spread of data between the groups.

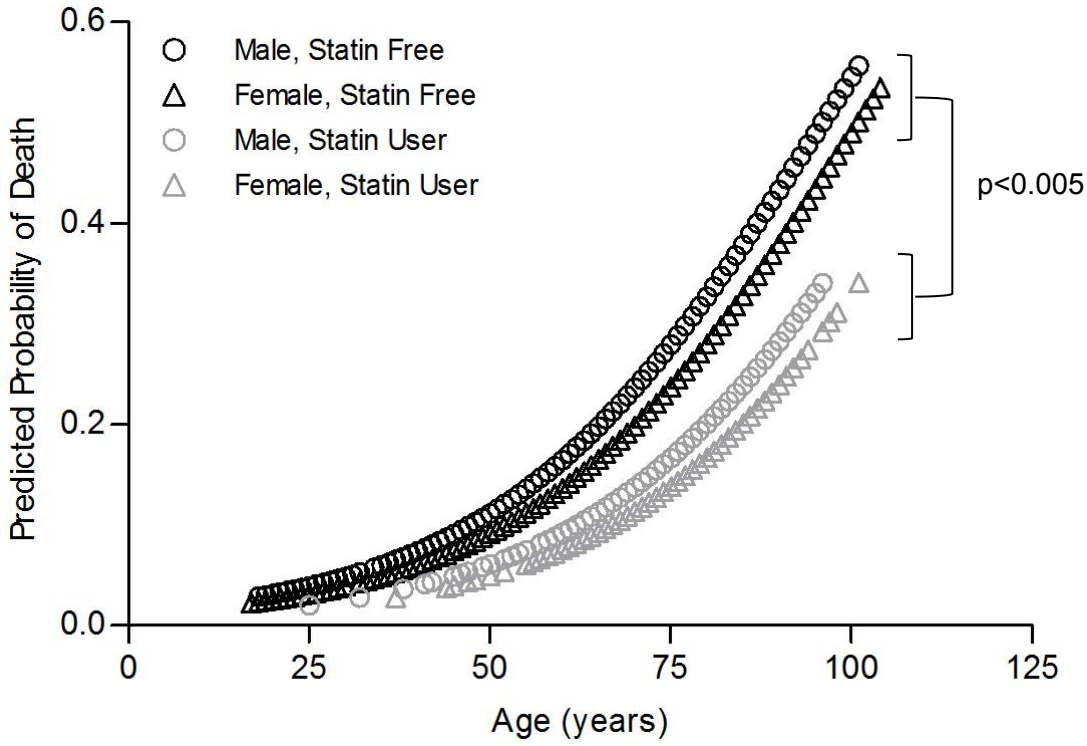


Figure 5.1 Relationship between Predicted Probability of death from Pneumonia and Increasing Chronological Age. The relationship between predicted probability of death from Pneumonia and increasing chronological age stratified according to statin usage prior to admission (not taking a statin, black lines; taking a statin, grey lines) and gender (male, circles; female, triangles). Relationships were assessed by linear regression controlling for age, statin therapy, gender and diabetic status.

Table 5.2 Relationship between Predicted Probability of Death from Pneumonia and Increasing Chronological Age

		β	S.E	p	OR	95% CI for OR	
						Lower	Upper
Model 1	Age	.044	.004	<0.001	1.045	1.037	1.053
	Statin User	-.645	.126	<0.001	.525	.410	.671
Model 2	Age	.045	.004	<0.001	1.046	1.038	1.054
	Statin User	-.652	.130	<0.001	.521	.404	.672
	Gender	.222	.112	.047	1.248	1.003	1.554
	Diabetes	-.055	.146	.707	.946	.710	1.261
Model 3	Age	.052	.005	<0.001	1.053	1.044	1.063
	Statin User	-.679	.137	<0.001	.507	.388	.644
	Gender	.145	.123	.240	1.156	.908	1.472
	CRP	.556	.126	<0.001	1.744	1.450	2.233

Values were obtained from logistic regression models. Model 1 was adjusted for Statin Therapy; Model 2 was adjusted for Statin Therapy, Gender and Diabetic status; Model 3 was adjusted for Statin Therapy, Gender and C-reactive Protein (CRP), diabetes was removed from this model as it has no significant effect in model 2.

S.E, Standard Error; OR, Odds Ratio

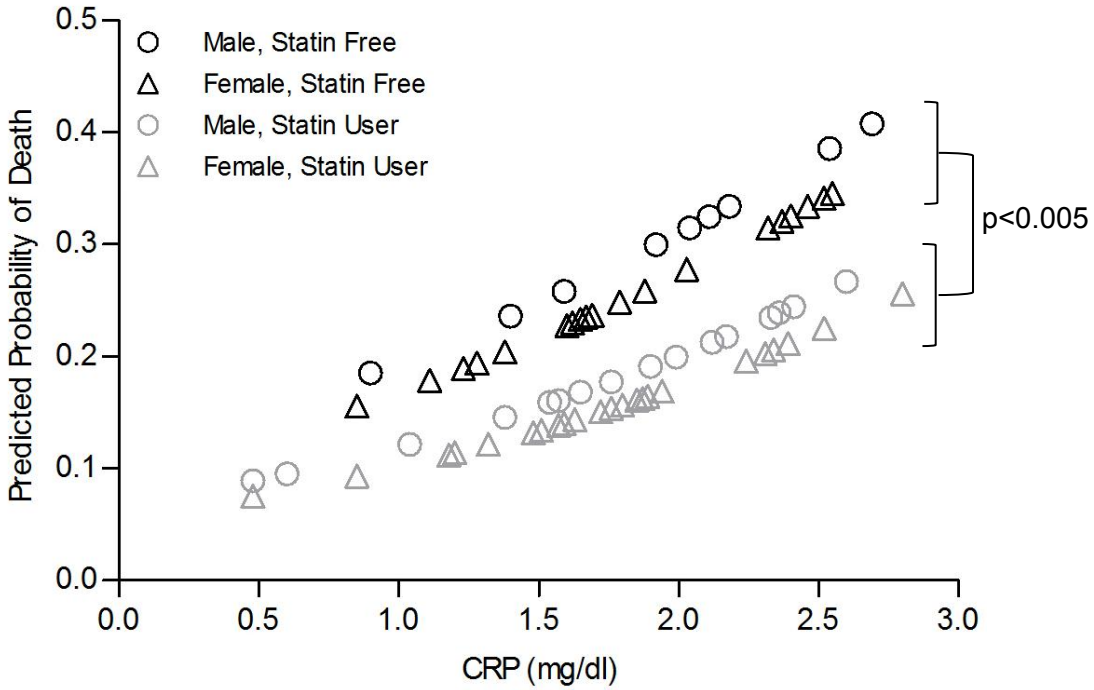


Figure 5.2 Relationship between Predicted Probability of death from Pneumonia and C-reactive Protein at 79 years old. The relationship between predicted probability of death from Pneumonia and increasing levels of C-reactive protein (CRP) at 79 years old stratified according to statin therapy prior to admission (not taking a statin, black lines; taking a statin, grey lines) and gender (male, circles; female, triangles). Relationships were assessed by linear regression controlling for CRP, age, statin therapy and gender. *p<0.05 statin free vs. statin given

5.2.2 Simvastatin restores directional migration of neutrophils from older adults

To begin establishing a causative relationship between statin therapy and pneumonia outcomes, migratory parameters were measured on freshly isolated peripheral blood neutrophils from both young and old donors in the presence or absence of 1nM simvastatin, a plasma concentration equivalent to clinical clinically prescribed doses. Migratory studies were repeated using the Insall chamber, imaged using real-time video microscopy and cell tracked using ImageJ in response to 100nM IL8 and 10nM fMLP.

Neutrophils isolated from, young (n=10) and elderly (n=10) donors demonstrated maintained chemokinesis and persistence in response to IL8 and fMLP as reported in chapter 3. Both chemotaxis and chemotactic index were significantly reduced in neutrophils isolated from elderly donors when compared to those isolated from young donors (young vs. old, mean ± SEM; IL8: chemotaxis 1.7 ± 0.2 vs. 0.4 ± 0.2 $p<0.001$; chemotactic index 0.4 ± 0.04 vs. 0.1 ± 0.04 $p<0.001$; fMLP: chemotaxis 1.7 ± 0.2 vs. 0.9 ± 0.2 $p=0.008$; chemotactic index: 0.4 ± 0.04 vs. 0.1 ± 0.03 $p<0.001$). Pre-incubation with 1nM simvastatin had no effect on migratory parameters of neutrophils isolated from young donors but significantly improved both the chemotaxis and chemotactic index of neutrophils isolated from old donors in response to both IL8 and fMLP (old untreated vs. treated, mean ± SEM; IL8: chemotaxis 0.4 ± 0.2 vs. 2.2 ± 0.1 $p<0.001$; chemotactic index: 0.09 ± 0.03 vs. 0.5 ± 0.04 $p<0.001$; fMLP: chemotaxis 0.9 ± 0.2 vs. 1.8 ± 0.2 $p=0.005$; chemotactic index 0.1 ± 0.03 vs. 0.4 ± 0.03 $p=0.001$) restoring these parameters to levels comparable to neutrophils isolated from young donors (see **Figures 5.3** and **5.4**).

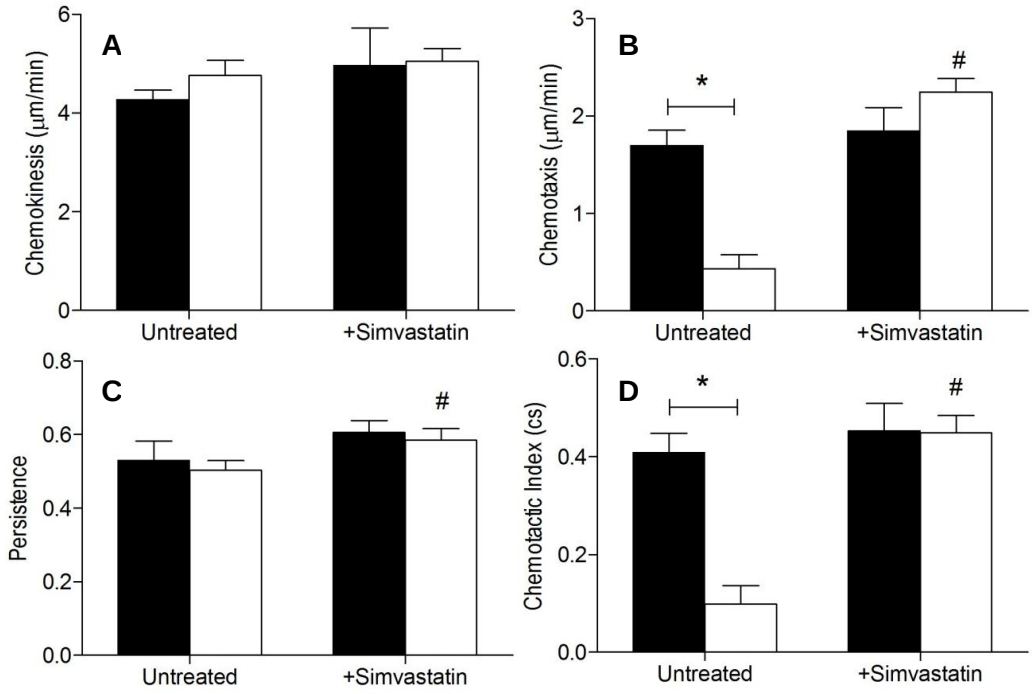


Figure 5.3 Effect of Simvastatin on Neutrophil Migration toward IL8.

Peripheral neutrophils isolated from young (black bars) and old (white bars) were pre-incubated for 40 minutes with 1nM Simvastatin and (A) chemokinesis (B) chemotaxis (C) persistence and (D) chemotactic index measured in response to 100nM IL8. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by factorial repeated measures ANOVA. *$p<0.05$ young vs. old; # $p<0.05$ old untreated vs. treated. Data are mean ± SEM (n=10).

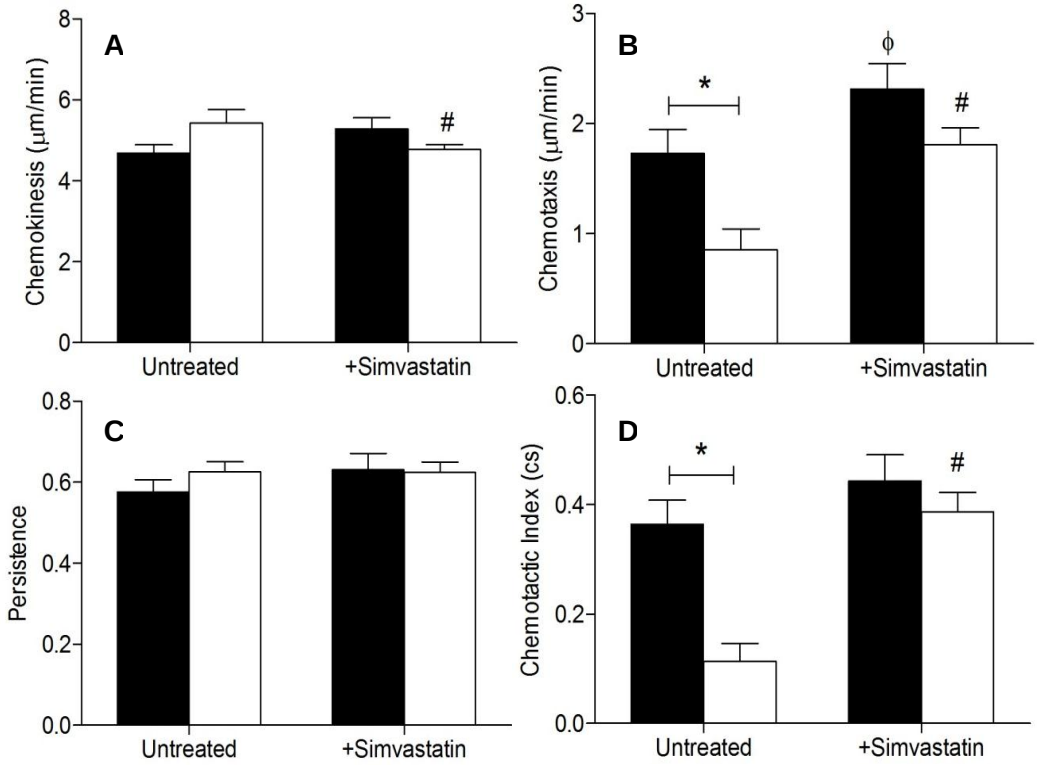


Figure 5.4 Effect of Simvastatin on Neutrophil Migration toward fMLP.

Peripheral neutrophils isolated from young (black bars) and old (white bars) were pre-incubated for 40 minutes with 1nM Simvastatin and (A) chemokinesis (B) chemotaxis (C) persistence and (D) chemotactic index measured in response to 10nM fMLP. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance measured by factorial repeated measures ANOVA. *$p<0.05$ young vs. old; ϕ $p<0.05$ young untreated vs. treated; # $p<0.05$ old untreated vs. treated. Data are mean ± SEM (n=10).

5.2.3 Simvastatin does not alter phospholipid composition of the plasma membrane

In order to ascertain a potential mechanism by which simvastatin was able to modulate neutrophil migration, the membrane phospholipid composition of neutrophils isolated from old donors pre-incubated for 40 minutes with 1nM simvastatin prior to lipid extraction was assessed. Compared to neutrophil isolated from older adults not pre-treated with simvastatin, there was no significant difference in the proportion of total PC, PE, PS and PI species in the plasma membrane (see **Figure 5.5**). Comparing individual phospholipid species, there were also no significant differences in the composition of PC, PS or PI species however, the proportion of PE 18:0p/18:2 in the membrane of neutrophils from older adults pre-treated with simvastatin was significantly reduced (old untreated vs. old treated, mean ± SEM; 8.43 ± 0.43 vs. 7.36 ± 0.68, p=0.05) (see **Figures 5.6 to 5.9**). This work was done in collaboration with Professor Anthony D. Postle, Southampton Centre for Biomedical Research.

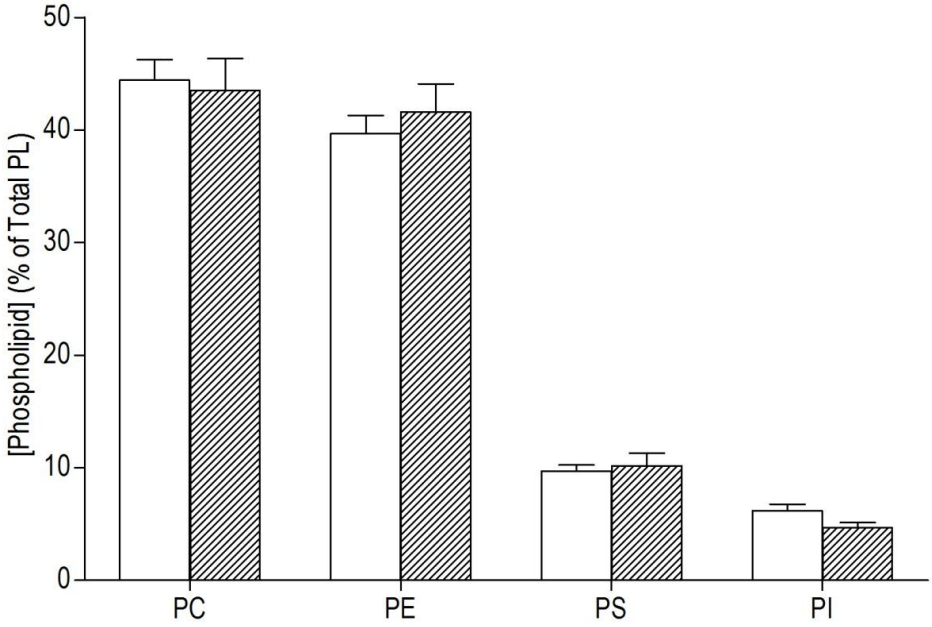


Figure 5.5 Effect of Simvastatin on Plasma Membrane Phospholipid Composition.

Percentage of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS) and Phosphatidylinositol (PI) present in the plasma membrane of human neutrophils isolated from old donors and pre-treated with (hashed bars) and without (clear bars) 1nM Simvastatin for 40 minutes prior to measurement. All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. Data are mean ± SEM (n=10).

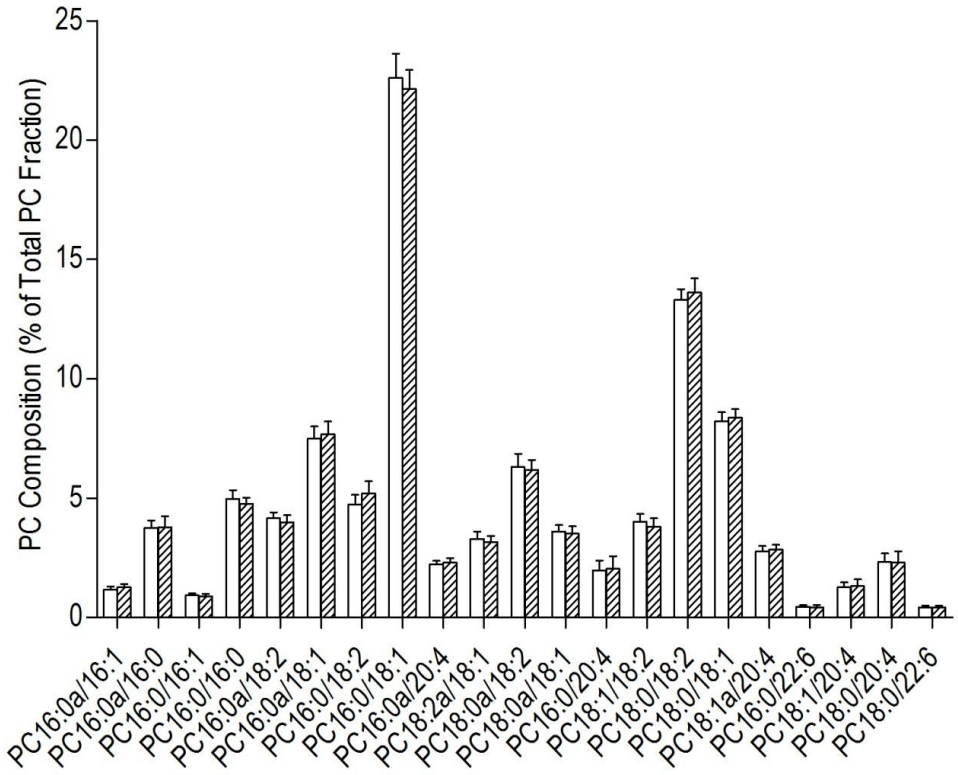


Figure 5.6 Effect of Simvastatin on Composition of Phosphatidylcholine Species

Percentage of Phosphatidylcholine (PC) species present in the plasma membrane of were measured in human neutrophils isolated from old donors (clear bars) a proportion of which were treated with 1nM Simvastatin for 40 minutes prior to measurement (hashed bars). All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. Data are mean ± SEM (n=10).

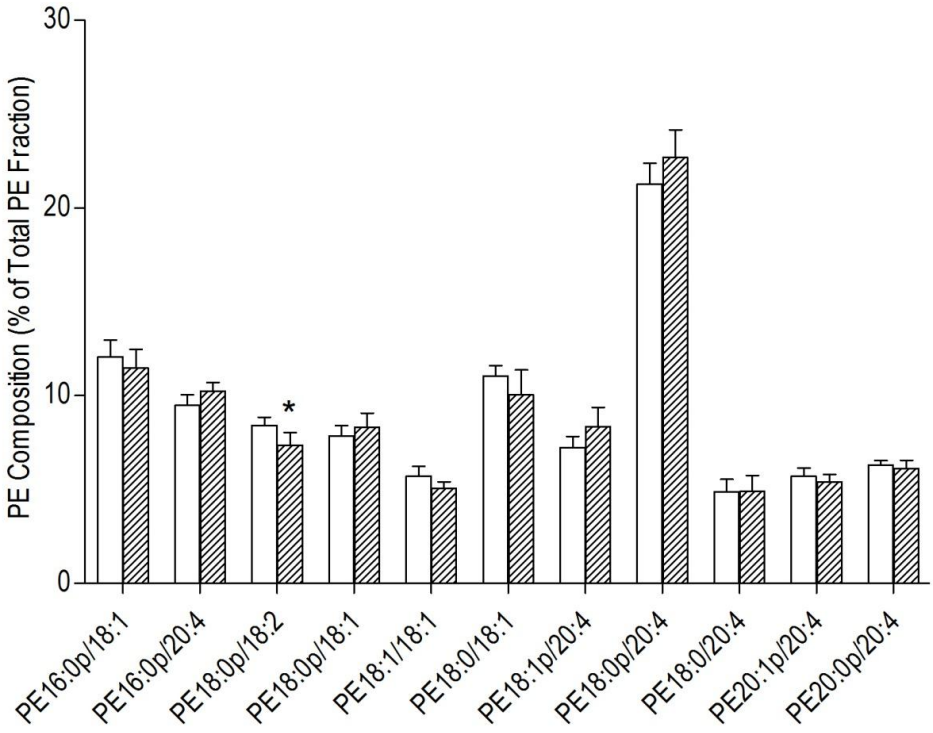


Figure 5.7 Effect of Simvastatin on Composition of Phosphatidylethanolamine Species.

Percentage of Phosphatidylethanolamine (PE) species present in the plasma membrane of were measured in human neutrophils isolated from old donors (clear bars) a proportion of which were treated with 1nM Simvastatin for 40 minutes prior to measurement (hashed bars). All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. Data are mean ± SEM (n=10). *$p<0.05$ untreated vs. treated.

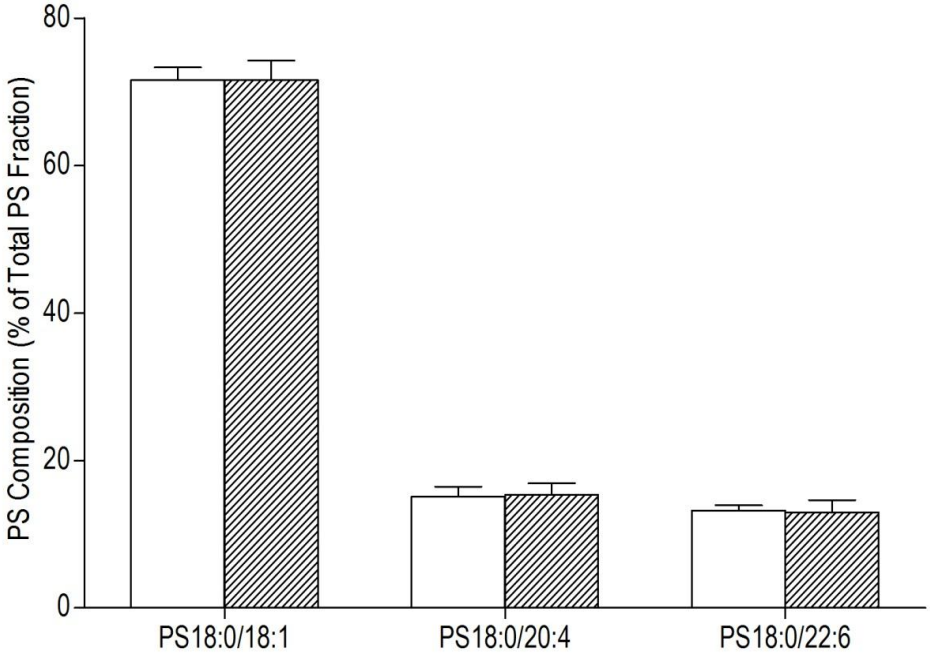


Figure 5.8 Effect of Simvastatin on Composition of Phosphatidylserine Species.

Percentage of Phosphatidylserine (PS) species present in the plasma membrane of were measured in human neutrophils isolated from old donors (clear bars) a proportion of which were treated with 1nM Simvastatin for 40 minutes prior to measurement (hashed bars). All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. Data are mean ± SEM (n=10).

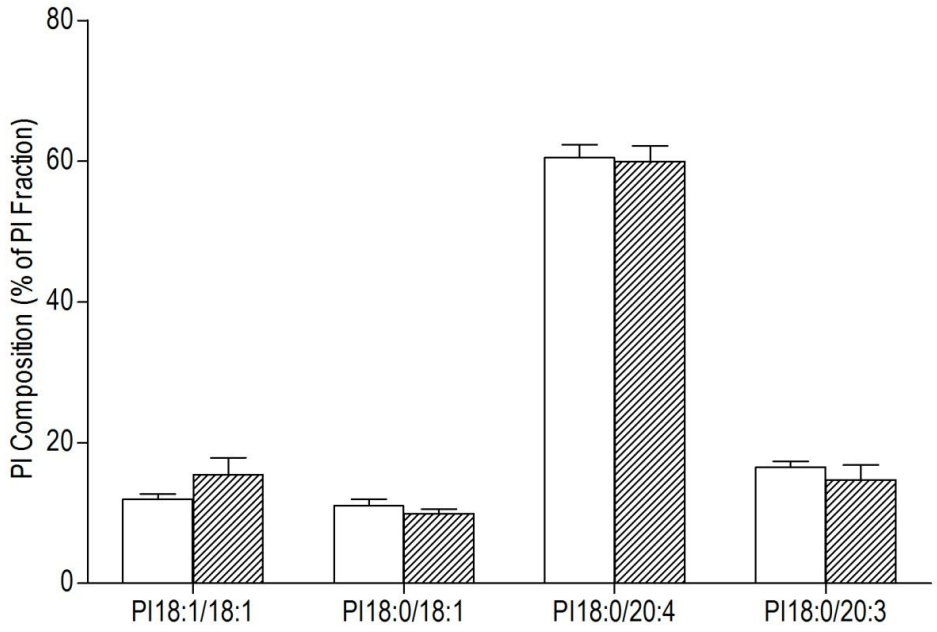


Figure 5.9 Effect of Simvastatin on Composition of Phosphatidylinositol Species.

Percentage of Phosphatidylinositol (PI) species present in the plasma membrane of were measured in human neutrophils isolated from old donors (clear bars) a proportion of which were treated with 1nM Simvastatin for 40 minutes prior to measurement (hashed bars). All data sets were normally distributed (Kolmogorov-Smirnov test) and statistical significance assessed by independent samples T-test. Data are mean ± SEM (n=10).

5.2.4 Membrane Cholesterol:PC Ratio is not altered by increasing age or Simvastatin treatment

Cholesterol is the major sterol species found within membranes and is known to play a significant role in influencing membrane fluidity through interactions with the fatty acid tails of membrane phospholipids thereby preventing lateral movement and inducing a more rigid membrane structure [368]. Serum cholesterol is known to increase with age however, little is known about the effects of age on membrane cholesterol content. Although it was felt unlikely that pre-incubation with 1nM simvastatin for 40 minutes would significantly alter membrane composition, statin therapy has been shown to modulate cholesterol levels of synaptosomal plasma membranes within the brains of patients with Alzheimer's disease ultimately altering membrane micro-domains and affecting membrane fluidity [445]. This possibility was therefore examined.

Neutrophils isolated from old donors showed a significant increase in membrane content of both cholesterol and PC when compared to young donors (young vs. old, mean ± SEM; cholesterol: 1.1 ± 0.2 vs. 1.8 ± 0.2 p=0.019; PC: 5.2 ± 0.5 vs. 7.8 ± 0.9, p=0.012) resulting in an unchanged cholesterol: PC ratio. Pre-incubation of neutrophils from old donors with 1nM simvastatin had no effect on either membrane cholesterol or PC content nor on the ratio of cholesterol:PC (see **Figure 5.10**).

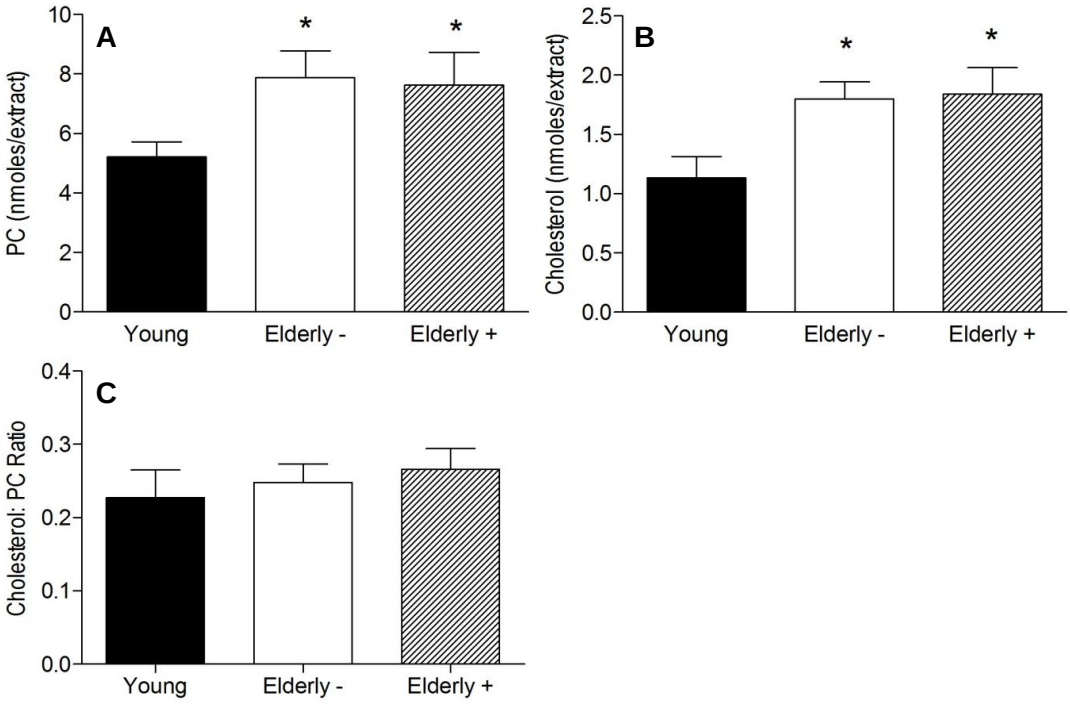


Figure 5.10 Composition of free Cholesterol and Phosphatidylcholine species in the Plasma Membrane. Proportion of (A) Cholesterol and (B) Phosphatidylcholine (PC) and (C) ratio of cholesterol: PC species present in the plasma membrane of human neutrophils isolated from young (black bars) and old (clear bars) donors. Neutrophils isolated from old donors were also pre-incubated with 1nM Simvastatin for 40 minutes (hashed bars) prior to measurement. All data sets follow normal distribution (Kolmogorov-Smirnov test). Data are mean ± SEM (n=10). *$p < 0.05$ old vs. young.

5.3 Discussion

Statins have been shown to have pleiotropic effects beyond their ability to lower serum cholesterol conferring a survival advantage to patients with pneumonia [390-396]. However their specific mechanisms of action remain elusive although effects on the innate immune system have been indirectly implicated. The aim of this chapter was to investigate the effects of simvastatin on the migratory dynamics of neutrophils isolated from young and old donors and identify any potential mechanisms by which this may occur. The data confirm previously published work documenting the ability of statins to confer a survival advantage during infection [390-396] and demonstrate for the first time how migration in the elderly but not the young is sensitive to modulation by simvastatin.

Statins have been shown to modulate the function of small GTPases known to be essential in mediating a number of neutrophil anti-microbial functions including phagocytosis, ROS production and migration due to their capacity to dynamically re-model the cytoskeleton [446]. In the case of migration, addition of C3 exo-enzyme, a specific inhibitor for Rho proteins, significantly impairs the ability of THP-1 monocytes to migrate towards monocyte chemoattractant protein-1 (MCP1) [401]. Statins are able to mediate this effect on small GTPases by preventing their isoprenylation [401,447] through inhibition of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in the mevalonate pathway. In the absence of a prenyl group (either farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGP)), small GTPases cannot be inserted into the membrane thereby preventing interaction with activatory molecules and inducing functional inactivity [447].

In recent years, the pleiotropic effect of statins have received significant attention with statins being suggested to reduce the risk of a number of diseases including dementia [387], lung cancer [388] and cardiovascular disease [389]. Stains have also been recently recognized as having anti-inflammatory properties through modulation of cytokine secretion [448] and prevention of endothelial dysfunction [449]. Although the observation is well documented, the

mechanism by which statins confer a survival advantage during pneumonia infection remains unclear. It was hypothesised that this may be due to the improvement of neutrophil function, strengthening defences previously weakened by natural ageing.

A number of previous studies have demonstrated the ability of statins to reduce neutrophil migratory efficiency in both human and animal studies [398-400], however these authors used statin concentrations 1,000 – 10,000 times that which would be considered therapeutically relevant. Data presented here indicate the ability of simvastatin, when used at therapeutic concentrations, to act directly on neutrophils in such a way as to reverse the age-associated decline in neutrophil migratory accuracy.

Statins are most well known for their ability to lower serum cholesterol which itself is known to increase with during ageing [450], however little is known on the effects of statins on membrane composition and therefore their ability to alter membrane fluidity. To date, no reports exist on the ability of statins to modulate neutrophil membrane phospholipid composition. Here the data show no change in the proportion of phospholipids in the membrane following pre-incubation of neutrophils from older adults with 1nM simvastatin with the exception of PE 18:0p/18:2. However the observed reduction on this single species is unlikely to constitute the mechanism by which statins modulate improvements in neutrophil migration especially as this phospholipid would not be considered to have a particularly long fatty acid chain and therefore its ability to alter membrane fluidity would be severely limited. The data also show a significant increase in the amount of cholesterol present in lipid extracts from neutrophils isolated from older adults. However the amount of phosphatidylcholine (PC), the most abundant phospholipid in eukaryote membranes [359], also showed a significant increase with age resulting in a cholesterol:PC ratio that was not affected by ageing. Pre-incubation of neutrophils from older adults with 1nM simvastatin prior to lipid extraction did not appear to have any effect on membrane composition and is therefore not a contributing factor to the ability of simvastatin to modulate neutrophil migration in the elderly.

It is proposed that restoring neutrophil migration through the action of simvastatin may have the potential to impact upon immune senescence in the short term reducing both pathological inflammation and the rates of morbidity and mortality from bacterial infections observed in the elderly population.

CHAPTER 6

CAN SIMVASTATIN IMPROVE NEUTROPHIL FUNCTIONS IN THE HEALTHY ELDERLY?

6.1 Introduction

The data so far have demonstrated a robust aberrant migratory phenotype in the healthy elderly which was restored in *in vitro* studies through inhibition of class I PI3Kinase isoforms, specifically PI3Kinase γ and δ, or through inhibition of the mevalonate pathway by treatment with simvastatin. Inhibition of either of these pathways may be particularly effective at improving innate immunity and thus reducing the rates of morbidity and mortality associated with infection. PI3Kinase-δ isoform inhibitors have been used in clinical trials of patients chronic lymphoid leukaemia for lymphoid malignancies with some suggestion of efficacy [451], however these trials have reported a number a grade 3 (severe) adverse events including pneumonia and neutropenia [452] which may limit clinical use, particularly in the elderly population. In contrast, Statins, a class of drugs widely prescribed to treat hypercholesterolemia, are safe in most patients and relatively well tolerated. This makes them an attractive intervention to improve neutrophil function in the elderly.

Existing population-based retrospective studies already suggest statin therapy may improve innate immune function *in vivo* as well as *in vitro* as reduced rates of morbidity and mortality from bacterial pneumonia have been observed in elderly patients when on statin therapy prior to admission into hospital [393,395,396]. However, *in vivo* pleiotropic effects of statins, specifically on neutrophil anti-microbial functions with advancing age, are yet to be elucidated.

Based on data presented in chapter 5, it was hypothesised that *in vivo* simvastatin therapy in older adults would restore neutrophil anti-microbial functions to levels comparable to the young. This hypothesis was tested by carrying out a double-blind placebo controlled trial investigating the effects of 80mg/day Simvastatin on neutrophil anti-microbial functions. The primary outcome was neutrophil chemotaxis in response to IL8 and fMLP. Secondary outcomes were other neutrophil migratory parameters (chemokinesis, persistence and chemotactic index, percentage cells adhering, migrating and time to initiation of migration),

neutrophil phagocytosis of *S.aureus* and *E.coli* bioparticles, reactive oxygen species production in response to IL8 and fMLP, patient safety and drug tolerability.

Work presented in this chapter was completed in partnership with Dr. Jaimin Patel, under the clinical supervision of Dr Elizabeth Sapey and Dr David Thickett, University Hospital Birmingham, Birmingham, UK

6.2 Methods

In order to assess the effect of statin treatment on neutrophil function *in vivo*, a randomised double blind, placebo-controlled, crossover study was used to assess whether a daily dose of 80mg Simvastatin taken orally for two weeks was sufficient to correct any age-related defects in neutrophil functions namely migration, phagocytosis and superoxide production.

6.2.1 Study Design

Power analysis suggested that a sample size of 20 was adequate. 22 healthy older (age > 60) volunteers were recruited into the study to allow for non-completion and were randomised to receive either a matched placebo or 80mg Simvastatin daily for 2 weeks in a cross–over, double-blinded, placebo controlled trial. This followed a two week wash out period (chosen to allow a new population of blood neutrophils to be released) and then subjects received the alternative treatment, again in a double blinded fashion. Simvastatin and placebo were both manufactured by Bilcare Ltd to GMP standards and the trial received ethical approval, Medicines and Healthcare Products Regulatory Agency (MHRA) approval and was sponsored by the University of Birmingham in accordance with national regulations for Clinical Trials of an investigational medicinal product [453]. Neither medical nor laboratory staff involved in the trial were aware of the identity of the drug being taken until all analysis was competed and the trial database unblinded. All assays were performed by a single analyst.

Subjects were recruited from the 1000 Elders Cohort, a research cohort of healthy older people and written informed consent was obtained (REC 11/SC/0356). Subjects attended the Wellcome Trust Clinical Research Facility (WTCRF) for 4 visits over a 6-8 week period.

Visit 1: Subject eligibility for the study was assessed by fulfilment of all inclusion criteria and none of the exclusion criteria (see **Table 6.1**). In order to assess liver function (LTFs), thyroid function (TFTs), creatine kinase (CK), urea and electrolyte levels (U&Es), and baseline cholesterol a peripheral blood sample was collected from each participant. Results were

reviewed by the trial physicians (Sapey and Thickett) to ensure all criteria were met and there were no contraindications to inclusion.

Visit 2: Inclusion and exclusion criteria were reassessed and patients were randomised to receive either placebo or simvastatin. A 35ml peripheral blood sample was collected from each subject to measure neutrophil function (migration, phagocytosis and superoxide production) immediately prior to simvastatin/placebo administration.

Visit 3: Following completion of the first 2 weeks of therapy, subjects returned to the WTCRF where a 35ml blood sample was collected to allow measurement of neutrophil function (migration, phagocytosis and superoxide production) and assessment of relevant health parameters (clinical review, LFTs, TFTs, U&Es, CK and full blood count) following simvastatin or placebo administration to ensure there were no adverse effects of the treatment. Cholesterol levels were assessed but were not made available to trial personnel. Subjects were then prescribed the alternative treatment (80mg Simvastatin or placebo) and asked to begin taking these tablets in 14 days time to allow a 2 week wash out period.

Visit 4: After completion of the second treatment, subjects returned to the WTCRF where their health status and cholesterol levels were assessed as before, and a 35ml blood sample was collected to measure neutrophil function following the second set of treatment. Subjects were asked to return all medication packaging and a treatment diary to prove compliance.

Table 6.1 Inclusion and Exclusion Criteria for Statin Clinical Trial

Inclusion Criteria	Exclusion Criteria
1. Age ≥ 60 years	1. Unable to provide written consent
2. Mentally competent to participate	2. History of significant chronic illness including Diabetes, COPD, Asthma, TB, Bronchiectasis, Malignancy, Auto immune disease and Cardiovascular Disease
3. Able to provide written informed consent	3. Clinical evidence of acute viral or bacterial
4. MRC dyspnoea scale < 2	4. Any regular therapies
5. Normal Lung Function (FEV1 > 80% predicted, FVC > 80% predicted, normal TLC, normal KCO)	5. Pregnancy (requirement for a negative pregnancy test in young females)
6. Normal peripheral oxygenation (assessed by pulse oximetry with oxygen saturation above 92%)	6. Any contradictions for statin therapy
7. Medication Free	
8. No medical evidence of acute infection or chronic illness	
9. Normal liver and kidney function as assessed by peripheral blood liver function, urea, creatine kinase, thyroid function tests and urinary dipstick	

MRC, Medical Research Council; FEV, Forced Expiratory Volume; FVC, Forced Vital Capacity; TLC, Total Lung Capacity; KCO, Gas Transfer Coefficient; COPD, Chronic Obstructive Pulmonary Disease; TB, Tuberculosis

6.2.2 Measurement of Neutrophil Function

Neutrophil migration was measured as described in section 2.3 in response to 100nM IL8 and 10nM fMLP; phagocytosis was measured as described in section 2.5 and superoxide production was measured as described in section 2.6.

Additional migratory parameters were determined as described below:

Neutrophil adherence (%): the number of adherent cells present within the field of view divided by the total number of cells in the field x100.

Proportion of migrating cells (%): the number of migrating cells present within the field of view divided by the number of adhered cells in the field x 100

Time to first movement (seconds): average time taken to migrate 5μm (half the average body length of a polarised cell) and presented in 20s intervals due to time delay between frames.

6.2.3 Rational for choice of drug and dose

The *in vitro* data collected had suggested a positive effect on neutrophil migration with simvastatin, but other classes of statins have not been tested. It was therefore decided to utilise simvastatin. Although there is a large amount of data suggesting statins may be beneficial in animal models of infection and acute lung injury, only a single study has compared 2 doses of simvastatin (5 or 20 mg/kg given intraperitoneally 24 hours before and concomitantly with the injury to induce lung injury) and only the higher dose was effective in attenuating lung injury [454]. In a double-blind placebo-controlled study, Steiner *et al* exposed healthy volunteers to low dose intravenous endotoxin, which produces a detectable systemic inflammatory and pro-coagulant response without adverse effect [455]. Simvastatin 80mg for 4 days before endotoxin challenge inhibited these processes [455]. No other clinical studies have been published demonstrating that a lower dose is effective. Furthermore, in a study where 2265 patients following an acute coronary syndrome were randomised to receive 80 mg simvastatin, myopathy (CK >10 times the upper limit of normal associated

with muscle symptoms) occurred in only 0.4% and rhabdomyolysis (CK > 10000 units/L with or without muscle symptoms) in 0.13% after 24 months treatment [456].

14 days therapy had been selected based on two factors. Firstly, 80mg Simvastatin once daily achieves steady-state plasma concentrations, as demonstrated by a study of healthy volunteers (with no reported adverse events in this healthy group) [457]. Secondly, 14 days will allow all circulating neutrophils to be exposed to the simvastatin, based on studies of average neutrophil maturation and lifespan of approximately 11 days [124].

In light of these data, it was proposed that Simvastatin 80mg was the only choice where both *in vitro* studies and clinical data supported efficacy while maintaining an acceptable safety profile.

6.3 Results

To assess the *in vivo* effects of simvastatin on neutrophil function, 20 healthy volunteers aged over 60 years (range 60-94 years) were recruited to the study and given either placebo or 80mg/day simvastatin for two weeks after which neutrophils were isolated from whole blood. The trial was unblinded following all data analysis in an "intention to treat" manner.

Demographics and health status at enrolment of all patients who completed the trial are shown in **Table 6.2**. During the study, there were no serious adverse events (SAE). Adverse events (AE) consisted of 1 subject who developed a migraine whilst on placebo (although this patient reported a medical history of migraines) and 2 subjects who developed generalised aches whilst on the statin however none of these patients felt these side-effects were severe enough to withdraw from the trial, with all patients successfully completing the study protocol. 1 additional patient presented with a raised Creatine Kinase (CK) after taking the statin however, this was asymptomatic and did not result in this patient withdrawing from the trial. Upon consulting their GP, their CK levels had returned to baseline two weeks after completing the study protocol. 1 patient developed a chest infection whilst taking the placebo and subsequently withdrew from the trial before taking the statin, with one further patient withdrawing from the trial due to reports of negative side-effects of simvastatin appearing in the media during the course of the trial. 19 out of the 20 patients included in the study took the full statin course, with only 1 patient failing to take 1 tablet during the middle of the statin course.

Table 5.2 Subject Characteristics upon Enrolment

	Health Status at Enrollment
n completed (total)	20 (23)
Age, mean (range), years	71.9 (60-94)
Gender, n (%)	
Male	9 (43)
Female	12 (57)
Smoker	
Never	13
Ex, n (PYH)	6 (21.5)
Current, n (PYH)	2 (21.5)
FEV1 , mean ± SD (L)	2.67 ± 0.7
FEV1pp, mean ± SD (L)	115.5 ± 19.1
FVC, mean ± SD (L)	3.573 ± 0.8
FVCpp, mean ± SD (L)	123.25 ± 21.9
Ratio , mean ± SD	74.7 ± 9.1
BMI, mean ± SD	26.04 ± 3.7
Heart Rate, median (IQR) bpm	67 (64.5-72.0)
Blood Pressure, median (IQR) mmHg	
Systolic	142 (132-149)
Diastolic	82 (74-90.5)
Oxygen Sats, median (IQR) %	97 (96-98)
Urea, median (IQR) mmol/L	5.3 (4.4-6.5)
Creatinine, median (IQR) µmol/L	79 (69.5-80.5)
eGFR, median (IQR) ml/min/1.73m^2	78 (66-85.5)
Bilirubin, median (IQR) µmol/L	8 (7-10)
Alkaline Phosphatase, median (IQR) IU/L	70 (60-78.5)
Alanine Transferase, median (IQR) IU/L	17 (15-22)
TSH, median (IQR) mU/L	1.9 (1.1-3.7)
FreeT4, median (IQR) pmol/L	15 (14.1-16.8)
Creatine Kinase, median (IQR) IU/L	80 (59.5-134.5)

Demographics and health status of study participants at visit 1. Participant health status was determined on the basis of lung function and biochemical blood tests and assessed by medical staff at the QE hospital. Upon determination of health status, qualifying participants were then enrolled into the trail and subsequent visits arranged. PYH, Pack year history; FEV1, Forced Expiratory Volume; FVC, Forced Vital Capacity; pp, percentage predicted; BMI, Body Mass Index; IQR, Interquartile Range; eGFR, estimated Glomerular Filtration Rate; TSH, Thyroid Stimulating Hormone.

6.3.1 80mg/day Statin Therapy does not affect the health status of older adults

Throughout the trial, the health status of each volunteer was monitored to ensure there were no adverse side effects related to statin administration. There was no significant change in parameters used to quantify health status following prescription of 80mg/day simvastatin compared to placebo. See **Table 6.3**.

6.3.2 Statin administration reduced serum cholesterol

To ensure tablets taken during this trial had therapeutic value, serum cholesterol was measured at baseline and after taking both placebo and statin as a marker of compliance. Serum cholesterol values remained unchanged following placebo but were significantly reduced following statin administration (baseline vs. placebo, median (IQR); 5.7 (5.2-6.4) vs. 5.8 (5.1-6.1) mmol/L $p=0.1$; baseline vs. statin, median (IQR); 5.7 (5.2-6.4) vs. 3.8 (3.6-4.4) mmol/L $p<0.001$). See **Figure 6.1**.

Table 6.3 Health status of Study Participants throughout the Trial Protocol

	Baseline	Placebo	Statin	p value		
				B v P	B v S	P v S
Heart Rate, median (IQR) bpm	67 (64.5-72.0)	73 (65-78)	72 (65.3-79.8)	0.023	0.025	1.000
Blood Pressure, median (IQR) mmHg						
Systolic	142 (132-149)	136 (122-145)	139.5 (127.8-144.8)	0.155	1.000	0.758
Diastolic	82 (74-90.5)	81 (73.5-85)	77 (68-82)	0.757	0.214	1.000
Oxygen Sats, median (IQR) %	97 (96-98)	97 (97-98)	97.5 (96-98)	0.254	0.429	1.000
Urea, median (IQR) mmol/L	5.3 (4.4-6.5)	5.7 (5.1-6.2)	5.6 (4.9-6.8)	1.000	0.902	1.000
Creatinine, median (IQR) µmol/L	79 (69.5-80.5)	74.5 (64-81.3)	74.5 (68.5-80)	1.000	1.000	0.668
eGFR, median (IQR) ml/min/1.73m^2	78 (66-85.5)	80.5 (68-89)	79 (66.3-86.8)	0.845	1.000	0.614
Bilirubin, median (IQR) µmol/L	8 (7-10)	7 (6.5-10)	7 (2.3-10.8)	1.000	1.000	1.000
Alkaline Phosphatase, median (IQR) IU/L	70 (60-78.5)	74 (58.5-83.50	69.5 (56.8-79.5)	0.427	1.000	0.314
Alanine Transferase, median (IQR) IU/L	17 (15-22)	17 (13-21)	17 (15-21.5)	1.000	1.000	0.942
TSH, median (IQR) mU/L	1.9 (1.1-3.7)	1.9 (0.9-5.3)	1.81 (1.1-6.2)	0.889	1.000	1.000
FreeT4, median (IQR) pmol/L	15 (14.1-16.8)	15.5 (12.9-17.2)	15.9 (13-16.5)	1.000	1.000	1.000
Creatine Kinase, median (IQR) IU/L	80 (59.5-134.5)	78 (56.5-104)	74.5 (64.8-115.25)	1.000	0.928	0.909

Participant health status was determined on the basis of biochemical blood tests and assessed and monitored though out the trial by medical staff at the QE hospital. Upon determination of health status, qualifying participants were then enrolled into the trail and subsequent visits arranged. IQR, Interquartile Range, eGFR, estimated Glomerular Filtration Rate, TSH, Thyroid Stimulating Hormone.

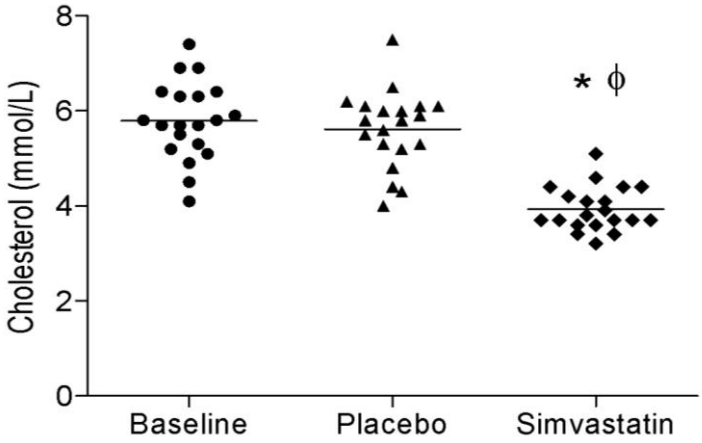


Figure 6.1 Serum Cholesterol levels Throughout Trial Protocol

Serum Cholesterol was measured at baseline and following prescription of both placebo and simvastatin to confirm compliance with trial protocol. Data sets were normally distributed and significance assessed by repeated measured ANOVA. *p<0.05 baseline vs. simvastatin; φ p<0.05 placebo vs. simvastatin, Data sets are from 20 independent experiments with bar showing the mean for each group.

6.3.3 In vivo statin therapy restores neutrophil migration in the healthy elderly

Neutrophil migration was measured in response to 100nM IL8 and 10nM fMLP at baseline and following prescription of placebo or 80mg/day simvastatin for two weeks. Statistical significance was measured using the Wilcoxon Signed-Rank test and p values manually adjusted for Bonferroni correction and therefore significance was accepted at $p \leq 0.017$ with the exception of neutrophil persistence which was normally distributed and therefore assessed using a repeated measured ANOVA with significance accepted at $p < 0.05$.

Following a two week prescription of 80mg/day of simvastatin, chemokinesis was reduced in the basal state (in the presence of buffer alone) compared to baseline (mean ± SEM; 2.92 ± 0.1 vs. 2.6 ± 0.1, p = 0.16) but not when compared to placebo (placebo vs. simvastatin, mean ± SEM; 2.78 ± 0.15 vs. 2.6 ± 0.12, p = 0.184). In the presence of IL8, chemokinesis following simvastatin treatment was unaffected when compared to both baseline and placebo values (baseline vs. simvastatin, mean ± SEM; IL8: 4.05 ± 0.13 vs. 3.94 ± 0.17 p = 0.297; placebo vs. simvastatin, mean ± SEM; 4.06 ± 0.13 vs. 3.94 ± 0.17, p = 0.221). Chemokinesis towards fMLP was also unaffected by simvastatin therapy when compared to baseline (baseline vs. statin, mean ± SEM; 4.12 ± 0.12 vs. 3.71 ± 0.18, p = 0.025) or placebo (4.06 ± 0.15 vs. 3.71 ± 0.18, p = 0.091) (see **Figure 6.2**).

Chemotaxis was unaffected by simvastatin treatment in the basal state (mean ± SEM; baseline vs. simvastatin, 0.19 ± 0.07 vs. 0.21 ± 0.07, p = 0.174; placebo vs. simvastatin, 4.06 ± 0.13 vs. 3.93 ± 0.17, p = 0.184). There was a trend towards increased chemotaxis with statin therapy when migrating towards IL8 (baseline vs. simvastatin, mean ± SEM; 0.96 ± 0.13 – 1.28 ± 0.16, p = 0.020; placebo vs. baseline, mean ± SEM; 0.86 ± 0.17 vs. 1.28 ± 0.16, p = 0.022). Migration towards fMLP was significantly increased when compared to both baseline and placebo values (baseline vs. simvastatin, mean ± SEM; 0.766 ± 0.15 vs. 1.09 ± 0.09, p = 0.013; placebo vs. simvastatin, mean ± SEM, 0.55 ± 0.13 vs. 1.09 ± 0.09, p =

0.001). There was no significant difference in chemotaxis following placebo treatment compared to baseline (see **Figure 6.3**).

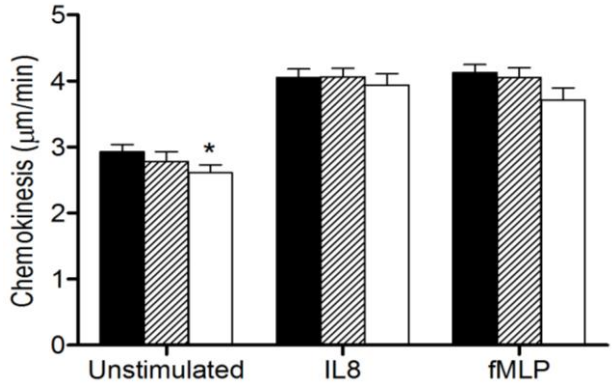


Figure 6.2 Effect of Simvastatin on Neutrophil Chemokinesis in response to IL8 or fMLP. Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Chemokinesis was measured in response to 100nM IL8 or 10nM fMLP. Data sets were non-normally distributed and statistical significance assessed by Wilcoxon-Signed Rank Test with significance accepted at $p<0.017$ to adjust for Bonferroni correction. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; #$p<0.017$ compared to placebo

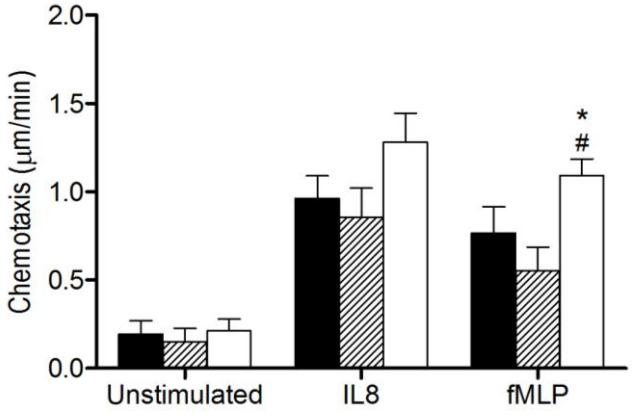


Figure 6.3 Effect of Simvastatin on Neutrophil Chemotaxis in response to IL8 or fMLP.

Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Chemotaxis was measured in response to 100nM IL8 or 10nM fMLP. Data sets were non-normally distributed and statistical significance assessed by Wilcoxon-Signed Rank Test with significance accepted at $p<0.017$ to adjust for Bonferroni correction. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; #$p<0.017$ compared to placebo

Persistence, a measure of cell orientation toward the chemotactic gradient, was unaffected in the basal state following simvastatin treatment (baseline vs. simvastatin, mean ± SEM; 0.24 ± 0.03 vs. 0.25 ± 0.02, p = 1.000; placebo vs. statin, mean ± SEM, 0.17 ± 0.02 vs. 0.25 ± 0.02, p = 0.213). In response to a gradient of IL8, persistence was significantly increased following simvastatin treatment when compared to baseline (baseline vs. simvastatin, mean ± SEM; 0.26 ± 0.03 vs. 0.41 ± 0.04) but not when compared to placebo (placebo vs. simvastatin, mean ± SEM; 0.31 ± 0.03 vs. 0.41 ± 0.04, p = 0.106). Persistence toward fMLP was unaffected by statin therapy when compared to baseline (baseline vs. simvastatin, mean ± SEM; 0.25 ± 0.03 vs. 0.30 ± 0.03 p = 0.983). Following placebo treatment, persistence was unaffected in the basal state and when migrating towards IL8 but was significantly increased when compared to baseline and following simvastatin therapy when migrating toward fMLP (baseline vs. placebo, mean ± SEM; 0.25 ± 0.03 vs. 0.4 ± 0.02, p = 0.001; placebo vs. simvastatin, mean ± SEM; 0.4 ± 0.02 vs. 0.29 ± 0.03, p = 0.044) (see **Figure 6.4**).

In the basal state, the chemotactic index (CI), an overall measure of migratory accuracy, was unaffected by simvastatin therapy. When migrating towards IL8, CI was significantly increased when compared to placebo (placebo vs. simvastatin, mean ± SEM, 0.16 ± 0.03 vs. 0.24 ± 0.03, p = 0.007) but not when compared to baseline (baseline vs. simvastatin, mean ± SEM; 0.15 ± 0.02 vs. 0.23 ± 0.03, p = 0.091). In response to fMLP, CI was significantly increased following simvastatin treatment when compared to both baseline and placebo (baseline vs. simvastatin, mean ± SEM; 0.12 ± 0.02 vs. 0.20 ± 0.03, p = 0.015; placebo vs. simvastatin, mean ± SEM; 0.09 ± 0.03 vs. 0.20 ± 0.03, p = 0.001). There was no significant difference following placebo treatment compared to baseline (see **Figure 6.5**).

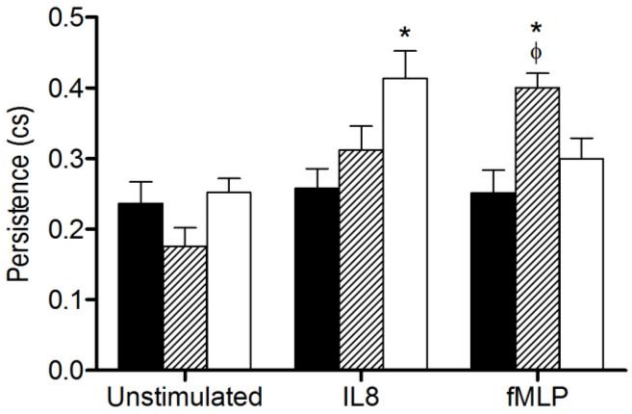


Figure 6.4 Effect of Simvastatin on Neutrophil Persistence in Response to IL8 or fMLP. Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Persistence was measured in response to 100nM IL8 or 10nM fMLP. Data was normally distributed and significance assessed by repeated-measured ANOVA with significance accepted at $p<0.05$. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; Φ $p<0.017$ compared to statin

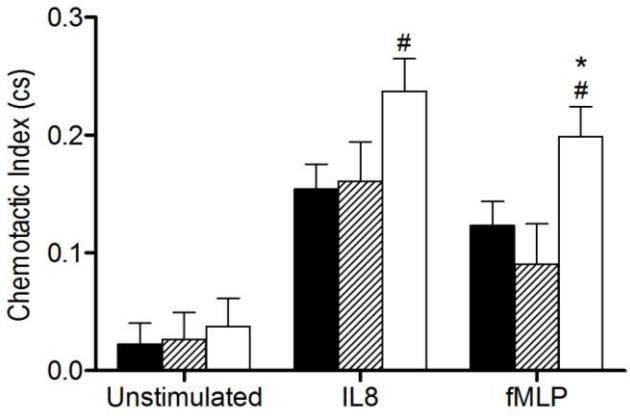


Figure 6.5 Effect of Simvastatin on Neutrophil Chemotactic Index in response to IL8 or fMLP. Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Chemotactic index was measured in response to 100nM IL8 or 10nM fMLP. Data sets were non-normally distributed and statistical significance assessed by Wilcoxon-Signed Rank Test with significance accepted at $p<0.017$ to adjust for Bonferroni correction. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; #$p<0.017$ compared to placebo

Additional parameters pertaining to neutrophil migration were also collated, namely percentage of cell adhered to the coverslip (% adherent), the percentage of those adherent cells that initiated migration (% migration) and the time to first movement defined as the average time taken to migrate five micrometres (chosen to represent half the average body length of a polarised cell).

In the basal state, the proportion of adherent cells was significantly reduced following placebo treatment compared to baseline (baseline vs. placebo, mean ± SEM, 79.15 ± 1.57 vs. 73.35 ± 1.88, $p < 0.001$) and further reduced following simvastatin therapy (baseline vs. simvastatin, mean ± SEM, 79.15 ± 1.57 vs. 67.25 ± 2.81, $p < 0.001$; placebo vs. simvastatin 73.35 ± 1.88 vs. 67.25 ± 2.81, $p = 0.017$). In the presence of IL8, % adherent cells was significantly reduced following simvastatin therapy when compared to both baseline and following placebo therapy (baseline vs. placebo, mean ± SEM; 90.85 ± 1.06 vs. 73.4 ± 1.79, $p < 0.001$; placebo vs. simvastatin, mean ± SEM, 89.1 ± 1.36 vs. 73.4 ± 1.79, $p < 0.001$). In response to fMLP, % adherent cells was unchanged when compared to baseline (baseline vs. simvastatin, mean ± SEM, 85.63 ± 2.27 vs. 82.8 ± 1.70, $p = 0.097$) while there was a trend towards reduced adherence following statin therapy compared to placebo (placebo vs. simvastatin, mean ± SEM, 87.45 ± 1.7 vs. 82.8 ± 1.70, $p = 0.029$). There was no effect on % adherence following placebo treatment when exposed to either IL8 or fMLP (see **Figure 6.6**).

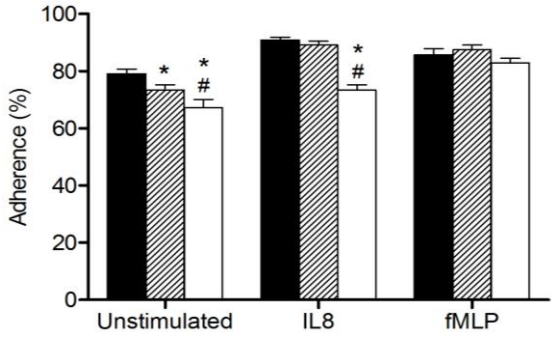


Figure 6.6 Effect of Simvastatin on Neutrophil Adherence when Migrating towards IL8 and fMLP. Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Adherence was measured in response to 100nM IL8 or 10nM fMLP. Data sets were non-normally distributed and statistical significance assessed by Wilcoxon-Signed Rank Test with significance accepted at $p<0.017$ to adjust for Bonferroni correction. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; #$p<0.017$ compared to placebo

The proportion of cells migrating (% migration) was unaffected by simvastatin in the basal state when compared to baseline or placebo (baseline vs. simvastatin, mean ± SEM, 26.9 ± 1.34 vs. 23.65 ± 1.60, p = 0.105; placebo vs. simvastatin, mean ± SEM, 22.3 ± 1.85 vs. 23.65 ± 1.60, p = 1.000). When migrating towards IL8, the proportion of migrating neutrophils was significantly increased following simvastatin therapy compared to baseline (baseline vs. simvastatin, mean ± SEM, 48.35 ± 1.57 vs. 65.6 ± 4.48, p = 0.002) but not compared to placebo (placebo vs. simvastatin, mean ± SEM; 74.45 ± 3.94 vs. 65.6 ± 4.48, p = 0.882). In response to fMLP, the proportion of migrating cells was significantly increased following simvastatin therapy when compared to both baseline and placebo (baseline vs. simvastatin, mean ± SEM; 56.7 ± 1.82 vs. 47.33 ± 1.68, p < 0.001; placebo vs. simvastatin, mean ± SEM,; 56.35 ± 1.52 vs. 47.33 ± 1.68, p = 0.002). Following placebo treatment, the proportion of migrating cells was significantly increased in response to IL8 compared to baseline (baseline vs. placebo, mean ± SEM; 48.35 ± 1.56 vs. 74.45 ± 3.94, p < 0.001) but unaffected in the basal state or when migrating towards fMLP (see **Figure 6.7**).

In the basal state, the time to first movement was unaffected by simvastatin treatment when compared to both baseline and following placebo (baseline vs. simvastatin, mean ± SEM, 114 ± 4.83 vs. 121 ± 3.40, p = 0.086; placebo vs. simvastatin, mean ± SEM, 122 ± 4.32 vs. 121 ± 3.40, p = 448). When migrating towards IL8 and fMLP, this was significantly increased following simvastatin therapy when compared to both baseline and placebo (baseline vs. simvastatin, mean ± SEM; IL8: 95 ± 3.51 vs. 113 ± 3.33, p = 0.003; fMLP: 87.37 ± 2.74 vs. 110 ± 4.35, p < 0.001; placebo vs. simvastatin, mean ± SEM; IL8: 92 ± 2.25 vs. 113. ± 3.33, p < 0.001; fMLP: 89 ± 2.70 vs. 110 ± 4.35, p = 0.003). There was no effect on the time to first movement following placebo treatment when exposed to either IL8 or fMLP or in the basal state (see **Figure 6.8**).

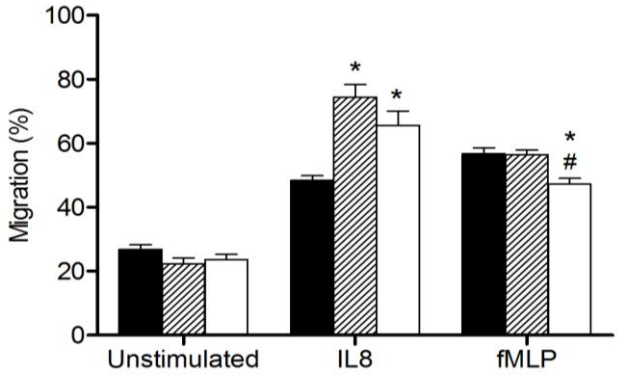


Figure 6.7 Effect of Simvastatin on Percentage of Migrating Neutrophil in response to IL8 and fMLP. Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Proportion of migrating neutrophils was measured in response to 100nM IL8 or 10nM fMLP. Data was normally distributed and significance assessed by repeated-measured ANOVA with significance accepted at $p<0.05$. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; #$p<0.017$ compared to placebo

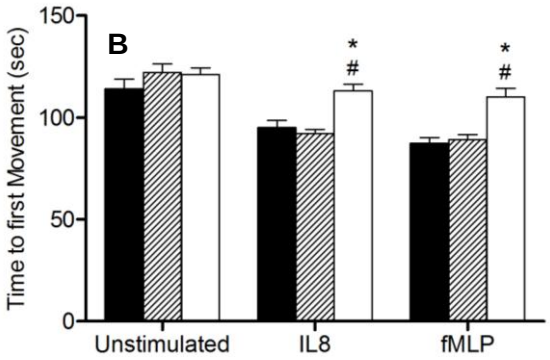


Figure 6.8 Effect of Simvastatin on Neutrophil Time to First Movement in response to IL8 and fMLP. Migration of neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. Initiation Time was measured in response to 100nM IL8 or 10nM fMLP Data sets were non-normally distributed and statistical significance assessed by Wilcoxon-Signed Rank Test with significance accepted at $p<0.017$ to adjust for Bonferroni correction. Data are mean ± SEM (n=20). *$p<0.017$ compared to baseline; #$p<0.017$ compared to placebo

6.3.4 In vivo statin therapy has no effect on neutrophil phagocytosis in the healthy elderly

Neutrophil phagocytosis was measured in response to *S.aureus* and *E.coli* bioparticles at baseline and following prescription of placebo or simvastatin and the phagocytic index (PI) calculated. Changes in Phagocytic Index (mean ± SEM) between treatment groups are presented in **Figure 6.9**.

There was no significant difference following statin prescription compared to baseline at any time point nor was there any difference between baseline and placebo at any time point. There was a significant effect for placebo treatment at 30 minutes when compared to statin treatment (placebo vs. statin, mean ± SEM, 54.09 ± 9.04 vs. 31.56 ± 6.39, p=0.046); this effect was lost at later time points.

Upon co-incubation with *E.coli* bioparticles for 30-minutes, there was a significant increase in the phagocytic capacity of neutrophils isolated following placebo prescription when compared to baseline (Baseline vs. placebo, mean ± SEM; 19.2 ± 4.3 vs. 33.21 ± 4.5, p=0.042). There was no difference observed in the phagocytic capacity of neutrophils in response to *E.coli* bioparticles at any subsequent time points or following prescription of 80mg/day simvastatin (see **Figure 6.9**).

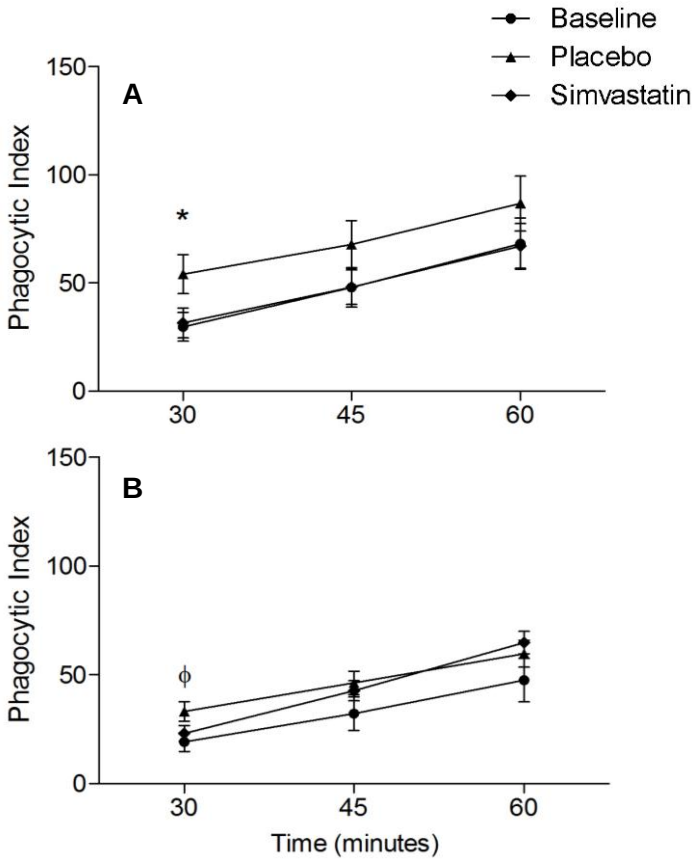


Figure 6.9 Effect of Simvastatin on Neutrophil Phagocytosis of *S.aureus* and *E.coli*.

Phagocytic Index of neutrophils isolated from old donors at baseline (black circles) and after prescription of placebo (black triangles) or 80mg/day simvastatin (black diamonds) for two weeks. Phagocytosis was measured in response to (A) *Staphylococcus aureus* and (B) *Escherichia coli* for 30, 45 or 60 minutes. All data sets are normally distributed and statistical significance measured by repeated measured ANOVA with significance accepted at $p<0.05$. Φ$p<0.05$ Placebo vs. Statin; *$p<0.05$ Baseline vs. Placebo. Data are mean ± SEM (n=20). PI: Phagocytic Index.

6.3.5 In vivo statin therapy has no effect on ROS production in the healthy elderly

Production of reactive oxygen species (ROS) was measured by neutrophils isolated from healthy elderly donors at baseline and following placebo or simvastatin therapy using PMA as a positive control. There was a significant increase in the amount of ROS produced in response to 2.5μM fMLP and 25nM PMA but not 1.25nM IL8 when compared to unstimulated neutrophils (unstimulated vs. stimulated, mean ± SEM; fMLP: 47.75 ± 6.84 – 191.168 ± 41.43, $p= 0.009$; PMA: 47.75 ± 6.84 – 342.84 ± 51.75, $p < 0.001$). There was however, no significant difference in the amount of ROS generated by neutrophils isolated from donors following prescription of placebo or simvastatin for two weeks compared to baseline or placebo (see **Figure 6.10**).

In summary, a daily dose of 80mg Simvastatin for two weeks, improved neutrophil chemotaxis, reduced neutrophil adherence to albumin, reduced the percentage of cells that migrate but increased the time taken to initiate movement. In addition, 80mg/day Simvastatin did impact upon phagocytosis or ROS production.

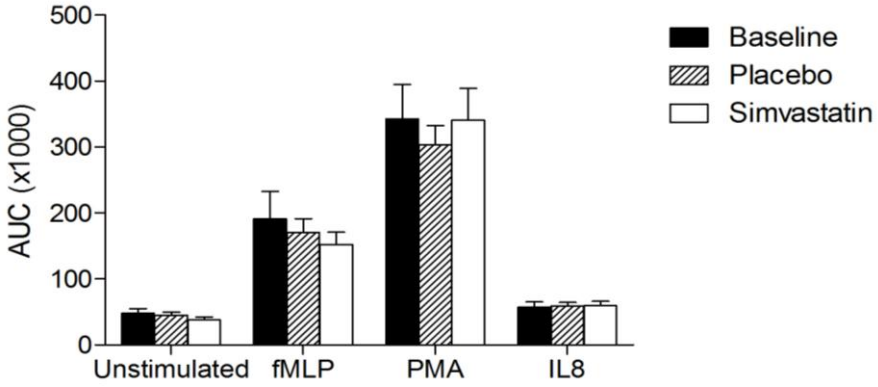


Figure 6.10 Effect of Simvastatin on Neutrophil ROS Production in Response to fMLP, PMA and IL8.

Production of Reactive Oxygen species (ROS) by neutrophils isolated from old donors at baseline (black bars) and after prescription of placebo (hashed bars) or 80mg/day simvastatin (white bars) for two weeks. ROS production was measured in response to 2.5μM fMLP, 25nM PMA or 1.25μM IL8 in the presence of 100μM luminol. Data are mean ± SEM (n=20). AUC: area under the curve.

6.4 Discussion

Statins are a family of drugs widely prescribed in the general population to lower serum cholesterol. Simvastatin, the most commonly prescribed member of this family, is relatively well tolerated, safe to prescribe and inexpensive to manufacture as it is off patent. The previous chapter demonstrated the *in vitro* ability of simvastatin to restore aberrant neutrophil migration in neutrophils from the healthy elderly to levels comparable to that seen in young donors. This medication was therefore used in a clinical trial as a potential intervention to overcome immune-senescence in the healthy elderly population. The data demonstrate for the first time the ability of simvastatin to improve neutrophil migration but not ROS production in response to both intermediate (IL8) and end-point (fMLP) chemoattractants, or unopsonised phagocytosis of *S.aureus* or *E.coli* bioparticles in a small cohort of older adults.

Little work exists on the *in vivo* effects of simvastatin of neutrophil function in the healthy elderly with this report forming the most complete assessment to date. The primary endpoint of this trial was the improvement of neutrophil chemotaxis in response to IL8 and fMLP. A number of secondary endpoints were also modified by simvastatin including neutrophil chemokinesis, persistence and chemotactic index along with percentage adherence, percentage cells migrating and time to first movement. Other secondary end-points were not improved including phagocytosis and ROS production. Overall, prescription of simvastatin improved migratory dynamics without negatively impacting on other neutrophil functions and would therefore appear to be a safe intervention with which to improve neutrophil migration in older adults.

The only other clinical trial to date investigating the effects statins on neutrophil migration found migration to be reduced following a two week prescription of 40mg atorvastatin [458], while data presented here describe a beneficial effect of simvastatin on neutrophil migration in the healthy elderly. Comparisons between these two studies should however be drawn with caution for a number of reasons: firstly, different statins were used. It is currently unclear

whether actions on cell behaviour are a class effect or limited to certain statins. Secondly the statins used were at different doses. Although within the clinical range, the concentration of simvastatin used in this thesis was 80mg/day whereas atorvastatin was used at 40mg/day, the mid-range dose. Mita *et al* have suggested differential effects across the statin family: in an open-label prospective crossover trial, pravastatin was found to have a more favourable effect on pancreatic β-cell function compared to Atorvastatin in patients with type-2 diabetes [459]. This suggests that different statins have the ability to differentially regulate cell function perhaps owing to their different chemical structures and doses used. Secondly, studies performed by Kinsella *et al* measured neutrophil function in 15 male healthy volunteers who ranged from 25 – 60 years [458], therefore utilizing a different cohort in both age and gender compared to studies presented here. Thirdly, discrepancies in methodology may explain the differences observed; Kinsella *et al* quantified neutrophil migration in response to 10nM fMLP using a modified Boyden chamber assay in which neutrophils migrated across a human pulmonary artery endothelial cell (HPAEC) monolayer grown on a transwell filter, as compared to albumin coated glass coverslips used in our studies. The former method does not allow for chemokinesis to be measured independently of chemotaxis as defined in this study and in our studies it was only the latter that was improved by simvastatin.

Mechanisms underlying the ability of simvastatin to modulate neutrophil migration remain unknown, however modulation of adhesive contacts may provide some explanation. Cellular adhesion to the substratum constitutes a major component of forward movement by providing traction and driving cellular motility during migratory processes. Here it was demonstrated that there was a reduced percentage of adherent cells following simvastatin therapy, both in the basal state and in response to IL8. Weitz-Schmidt *et al* reported a novel binding site within the structure of the β2 integrin LFA1 to which simvastatin and lovastatin have been shown to bind subsequently preventing integrin activation [460]. In the model used in this study, neutrophils migrate across albumin-coated surfaces (a surrogate for ICAM-1) utilizing β2 integrins for traction, a process which is therefore susceptible to

modulation by simvastatin and lovastatin through allosteric binding to β2 integrins. Whether or how this could contribute to improved directional migration is unclear.

The time to first movement was also significantly increased while the % of migrating cells was reduced in response to IL8 and fMLP following statin therapy. This suggests that in the presence of statins, as well as improving migratory accuracy (evidenced by increased chemotaxis and chemotactic index), statins are also able to improve the efficiency with which neutrophils from old donors respond i.e. fewer cells respond to a chemotactic gradient but of those that do, migration occurs more accurately. This may be a result of simvastatin reducing baseline activation (demonstrated as a reduced chemokinesis in the basal state) allowing greater amplification of activatory signals upon meeting an inflammatory stimulus i.e. a chemokine gradient. Within the context of infection and inflammation, this may correct the hypothesised “delayed arrival” at the site of infection driven by poor accuracy, and also reduce the increased ‘bystander’ tissue damage postulated to occur in the elderly, by reducing “wandering” and ultimately improving patient outcomes.

In some instances, data presented in this study also shows a significant effect following prescription of placebo for two weeks. Reasons underlying this are unclear however it may stem from the experimental design used as 50% of participants received the placebo after previously taking simvastatin for two weeks. Although a two week wash-out period was deemed to be sufficient time to allow for complete renewal on neutrophils within the bone marrow, it is plausible that some neutrophils exposed to the statin during treatment 1 still remained and were contributing to migratory dynamics when measured following placebo prescription. Based on data collected here, we are unable to conclusively test this hypothesis due to the small number of subjects randomised to the group which received the statin first.

CHAPTER 7

DISCUSSION

7.1 Ageing and neutrophil migration

We are currently living through a demographic shift in the population, with the proportion of those aged over 65 years soon to outnumber those aged under 16 years for the first time in history [2]. However, although we, as a population, are living longer, we are not living healthier with increments in health-span lagging significantly behind increments in lifespan [5]. This severely compromises the quality of life of older adults with an increased incidence of infectious diseases and chronic inflammatory conditions [9,10,15,26], both associated with poorer clinical outcomes and ultimately increasing burdens on the provision of hospital and community based healthcare.

Natural ageing is accompanied by wide spread immune senescence evident within both the adaptive and innate compartments of the immune response (reviewed in [137,414]). Adaptive immune senescence is widely documented encompassing observations of a reduced vaccine response [63-65], including a lesser ability to produce a protective antibody titer [66], an inverted $CD4^+$:$CD8^+$ T cell ratio [33] and a reduced output of naïve T cells from the thymus (reviewed in [461]).

In comparison, our knowledge of innate immune senescence is not as well advanced in particular with reference to neutrophil senescence. The effects of age on neutrophil migration, the process by which these cells migrate out of the blood toward to site of infection, was first investigated in 1978 [372] however in the intervening 35 years, no clear picture has emerged. Here a body of data describe a robust ‘old-migratory’ phenotype in healthy older adults, characterised by a maintained speed of migration but with reduced directional speed and overall accuracy of migration [439]. This phenotype was characteristic of neutrophils from older adults over the age of 60 years, was present in response to a number of physiological chemoattractants encompassing multiple stages of the inflammatory response and is therefore not stimuli specific. Related data suggested this defect does not

"correct" during infective episodes, data presented here (and provided by Dr. Elizabeth Sapey) showed worsening migration towards IL8 during pneumonia infection in older adults.

7.2 Reduced Neutrophil chemotaxis and health

The age-related reduction in chemotactic accuracy could have implications both when older people are uninfected and during acute infective events. Poor migratory accuracy could limit an effective response to invading bacteria, but may also be associated with increased bystander tissue damage. While there remains debate as to whether neutrophils need to utilise neutrophil elastase to migrate [462,463], there is clear evidence to suggest elastase is released upon migration [223,239,464]. There is also evidence to support an area of obligate tissue damage directly next to neutrophils following neutrophil elastase release [239]. The 'old-migratory' phenotype presented here may lead to increased "wandering" during interstitial migration, if this were the case, and elastase were released throughout the extended migratory period, then increased tissue damage would occur as a result of the circuitous route of migration.

While this thesis has no direct evidence to support this proposal, the data provide indirect evidence which is in keeping with this hypothesis. Neutrophils from healthy elderly donors with no clinical symptoms or signs of acute or chronic infection exhibited increased expression of CD63, an azurophil specific granule specific marker on their surface with concomitant increases in the concentrations of the neutrophil elastase-specific fibrinogen breakdown product $A\alpha Val^{360}$ in the plasma. CD63 was measured on neutrophils using a protocol to minimize experimental activation, and the increased expression is consistent with mobilisation of primary granules to the cell surface and degranulation in the basal state in the elderly. $A\alpha Val^{360}$ is a well-validated systemic footprint of elastase activity [406] and since there is no evidence of there being more circulating neutrophils with old age [130] and the propensity for apoptosis of neutrophils from the elderly is increased in the basal state (as

measured in this study) [465] we propose that this equates to more elastase being released per cell, although this would need to be confirmed. Altered neutrophil migration may also have implications in health, as transient movements into tissue as the cells circulate will generate tissue damage and an inflammatory response. Therefore the reduced chemotactic accuracy could contribute to inflamm-ageing.

Alternatively, inflamm-ageing may be a driver of neutrophil senescence and not a consequence. Although adults included in this study were considered healthy, a typical "western" lifestyle with low physical activity, a calorie-rich but nutrient-poor diet and endemic obesity may impact on results. In 2011, 25.9% of women and 23.6% of men in England were considered to be obese (a body mass index > 30kg/m^2) and 35.8% of women and 35.5% of men in the United States [466]. Obesity has been reported to be associated with increased systemic inflammation (Inflamm-ageing), particularly TNFα and IL6 [467,468] secreted by an abnormal accumulation of macrophages present within white adipose tissue, the magnitude of which is proportional to adipocyte size and body index [468]. Inflamm-ageing may affect cellular function by inducing a state in which immune cells, in this case neutrophils, are exposed to chronic low level activation reducing their ability to respond to activatory signals during infection, thereby compromising function. There are few studies of neutrophil function with age in populations where obesity is less endemic. In Japan, where only 2.3% of men and 3.4% of women were considered to be obese in 2000 [466] and the proportions of centenarians equates to 53/100,000 of the population [469], neutrophil immune-senescence has not been associated with BMI (although none of the participants in the relevant study were obese), but instead correlated with stressful life events [470]. Psychological stress has also been associated with systemic inflammation and subsequent immune-senescence [471], once again supporting the hypothesis that inflammation drives immune dysfunction, perhaps including the 'old-migratory' phenotype. It would be informative to repeat these studies in elderly adults from communities with extended longevity and less 'negative' lifestyle practices (such as traditional Japanese diet consisting mainly of fish and protein

[472] and low psychological stress to try and determine if alterations in neutrophil behaviour are an intrinsic, unalterable process of ageing of whether environmental factors are key.

In addition, this thesis only tested neutrophil functions in a cohort of healthy older adults on a single occasion. Although there was a negative relationship between neutrophil directional migration and accuracy of migration with increasing age, we can only hypothesise that neutrophil function declines within an individual over time due to the cross-sectional nature of the study. It would be of value to prospectively and longitudinally measure neutrophil function in healthy older adults to see if a decline in migratory dynamics occurs as they age.

7.3 Potential technical study limitations

The chosen migratory assay has enabled us to adequately differentiate between chemokinesis and chemotaxis revealing differences that up until now have remained largely elusive. However, there are limitations associated with our methods. Firstly any protocol utilised to isolate neutrophils will result in a degree of neutrophil activation, although Percoll (as used in this thesis) has been shown to induce minimum neutrophil activation [473]. There is a clear difference between migratory dynamics of “older” and “younger” cells which were prepared using the same technique, reducing the possibility of cellular activation as a driving factor behind the ‘old-migratory’ phenotype. Secondly, the study used an albumin coated surface to overcome hydrostatic forces of glass alone, which in itself may impact on results. Albumin is a surrogate for ICAM-1 [474] an adhesion marker highly expressed on the endothelium facilitating extravasation from the blood stream and also on fibroblasts present within the interstitium upon initiation of inflammation [475-477]. ICAM-1 is recognised by LFA-1/Mac-1 expressed on the neutrophil surface however, neutrophils lacking CD18 are still capable of migration (albeit with a 50% reduction in efficiency [478]) implying migration can occur independently of CD18 ligation with ICAM-1. Neutrophils are also thought to utilise VCAM-1 (which is bound by VLA-4, [478]) to provide traction during migratory processes. It

would therefore be of value to repeat initial migration studies using different surfaces to determine a role for ICAM-1 and/or CD18 in the development of the 'old-migratory' phenotype as observed here. Thirdly, this assay utilises shallow gradients and migratory measurements are taken in 2D. This is not physiological and does not replicate the complexity inherent within a neutrophils journey from the blood to areas of infected or inflamed tissue. A more physiological *in vitro* model would be to establish a 3D system where cells transmigrate through endothelium, into a collagen based gel (forming the interstitum).

7.4 Mechanisms of reduced chemotaxis with age

Following the initial observation of reduced chemotaxis in old donor cells, this thesis went on to investigate potential mechanisms which may underlie this migratory defect. Phosphatidylinositol-3-kinase (PI3Kinase) and its lipid product phosphatidylinositol - 3,4,5 - triphosphate (PIP_3) are important components of the signalling network present within the leading edge of migrating cells. PI3Kinase-dependent PIP_3 production is confined to the leading edge [346] where it initiates a signalling cascade resulting in the localised assembly of F-actin protrusions driving the forward motility of the plasma membrane [315] through recruitment and activation of a number of downstream effectors including small-GTPases, WASP family members and actin nucleating proteins (reviewed in [479]).

The data presented here demonstrated constitutive, basal activation of PI3Kinase in neutrophils isolated from old donors while, in those isolated from young donors this activity was transient. The antibody utilised has the advantage of measuring PI3Kinase regulatory subunit phosphorylation, and thus is a surrogate of kinase activity. However, it only measures Class IA activity (p85) and therefore it is not possible to comment on Class IB activity; studies of the p110 subunit would be needed in order to clarify this. In addition,

increased PI3Kinase activity might not have influenced migration, and alternative elements involved in directional migration could be the key effectors.

To explore this and try to confirm the importance of dysregulated PI3Kinase, the study utilised isoform selective inhibitors and repeated the migratory assays. PI3Kinase-γ is the main isoform responsible for PIP_3 production at the leading edge in response to GPCR stimulation, while PI3Kinase-δ is responsible for the localised production in response to tyrosine kinase receptor stimulation [256]. The data demonstrated PI3Kinase-γ and –δ to be the most relevant isoforms in the generation of the 'old-migratory' phenotype with inhibition of these isoforms able to restore migratory parameters of neutrophils isolated from old donors to levels comparable to those observed in neutrophils isolated from young donors [439]. Crucially inhibition of the phosphatase SHIP which regulates PI3Kinase phosphorylation and activity, further reduces neutrophil chemotactic accuracy in "old" cells (personal communication, Dr. Elizabeth Sapey), further supporting the suggestion that aberrant migration is driven by constitutive activation of the PI3Kinase pathway.

The mechanisms driving up-regulation of PI3Kinase activity remain to be determined. Activation of PI3Kinase was measured through phosphorylation of the p85 regulatory subunit which is responsible for the regulation of PI3Kinase-α, -β and -δ isoforms, while PI3Kinase-γ is under the control of a p101 regulatory subunit [480]. The ability of PI3Kinase-γ selective inhibitors to restore migratory dynamics as well as the δ isoform inhibitor would suggest this isoform is also dysregulated as a consequence of natural ageing. Dysregulation of class IA PI3Kinase could suggest dysregulated integrin signalling with increasing age as these isoforms are activated downstream of tyrosine kinase receptors including members of the integrin family [481]. However, inhibition of SHP-1, a tyrosine kinase specific protein phosphatase, does not alter the migratory phenotype of older adults (Dr. Adam Usher, personal communication). Class IB PI3Kinase is under the control of the Gβγ subunit of GPCRs [280], the activity of which may be influenced by an increased pro-inflammatory

micro-environment (a result of inflamm-ageing) leading to sustained GPCR activation and subsequent aberrant signal transduction (see **Figure 7.1**). The apparent combined dysregulation of PI3Kinase-γ and –δ isoforms may have a number of explanations. Firstly, in the model used here, both PI3Kinase-γ and –δ isoforms are activated due to the adhesive nature of the assay (via integrins) and exposure to pro-inflammatory chemokine gradients respectively. Secondly, there is emerging evidence of redundancy within the class 1A and 1B isoforms, with different isoforms sharing activity streams previously considered to be separate [482,483]. Finally, although inhibitors used in this thesis are selective for the different isoforms of PI3Kinase, it is unclear whether they are also isoform specific. Measuring migratory dynamics and PI3Kinase activity of young neutrophils pre-incubated with plasma or serum from elderly donors may reveal environmental factors influencing PI3Kinase activity (and by extension the migratory phenotype). This would also determine if the phenotype observed here is a consequence of intrinsic age-related alterations or is modulated thought environmental factors.

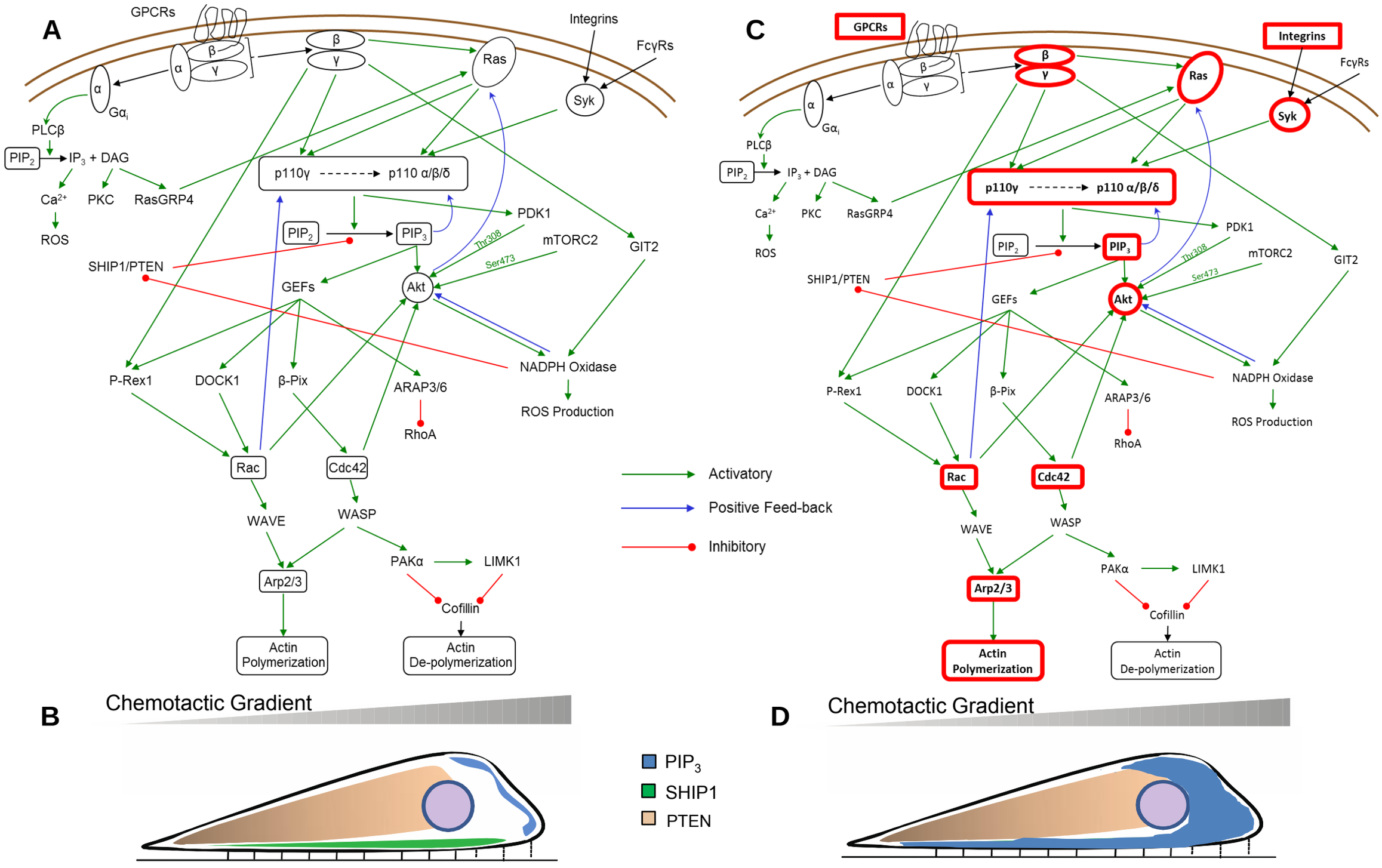


Figure 7.1 Proposed Age-Related Alterations in Leading Edge Signalling.
Homeostatic signalling pathways within the leading edge of neutrophils isolated from young donors (A) lead to compartmentalized PIP_3 at the leading edge (B), however, within the elderly (C), inflamm-ageing (chronic pro-inflammatory micro-environment) leads to sustained stimulation of GPCRs resulting in basal PI3K activity and enhanced PIP_3 production which is no longer compartmentalised within the leading edge (D) thus affecting the spatial and temporal localization of down-stream signalling molecules ultimately compromising actin nucleation.
Part (B) and (D) adapted from [281].

7.5 Correcting the age-related decline in chemotaxis

If increased PI3Kinase activity were to be a consequence of the altered micro-environment i.e. inflamm-ageing, interventions aimed at reducing systemic inflammation could be effective in improving neutrophil migration. For example increasing physical activity may be effective as exercise is known to reduce inflammation [484,485], reducing BMI could also help as adipose tissue is a major source of inflammatory cytokines [486]. Interestingly, by stratifying 200 older adults according to their levels of physical activity, the migratory dynamics of those who were in the lowest decile for physical activity were significantly lower than those who were the most physically active (David Bartlett, personal communication). This suggests modulation of physical activity may improve migratory outcomes, although lifestyle interventions are notoriously difficult to implement with poor levels of compliance.

The alternative to this is pharmacological intervention, however drug development can take anywhere up to 20 years from conception to treatment therefore the use of PI3Kinase inhibitors may take many years to implement. Such drugs may also be expensive and not suitable for long term use. In contrast the data reported here showed the beneficial effects of simvastatin on neutrophil migratory function. In recent years statins have received a great deal of attention due to their pleiotropic, anti-inflammatory properties that occur outside of their ability to lower serum cholesterol for example, in a number of retrospective cohort studies and meta-analyses, statins have been show to confer a survival advantage to bacterial pneumonia patients when taking a statin prior to admission [390-396]. Work published by other groups have previously demonstrated the ability of statins to modulate neutrophil migration in younger cohorts [398] however, work presented here is the first in which statins have been shown to restore migration in a cohort made up exclusively of older adults (age ≥ 60 years). Mechanisms driving this phenomenon have been previously attributed to the inactivation of GTPases by inhibiting their isoprenylation and subsequent

insertion into the membrane [398]. This study did not test this mechanism but did not support changes in either the phospholipid composition or cholesterol content of the plasma membrane as contributory factors to statin-mediated modulation of migratory dynamics.

Stains are a class of drugs relatively well tolerated within the population that are widely prescribed to treat hypercholesterolemia or reduce cardiovascular risk. The ability of simvastatin to restore neutrophil migration in the healthy elderly was shown here both *in vitro* and *in vivo*. Restoration of neutrophil migration in the elderly by simvastatin, potentially through inhibition of RhoA [398], downstream of PI3Kinase-γ and –δ isoforms could imply broader dysregulation of signalling pathways involved in the control of migration (see **Figure 7.1A**), or even other neutrophil functions which rely upon small GTPases. Upstream of PI3Kinase is the small GTPase Ras to which all isoforms containing a p110 catalytic subunit (i.e. all class I isoforms) are sensitive and the action of which is essential for full activation of both p110γ and p110δ isoforms [256,487,488]. Statins would also inhibit Ras function and this could explain their beneficial effect on migration. In addition, the small GTPases, RhoA, Rac and Cdc42 which are classically thought to exist downstream of PI3Kinase, are also capable of positive feedback onto PI3Kinase activity (see **Figure 7.1A**). As the inhibitory activity of statins is generic to all isoprenylated proteins, simvastatin may be able to simultaneously affect both Rho-family-GTPases and PI3Kinase/Akt signalling. One surprising aspect of the results in this context was that other neutrophil functions affected by ageing, specifically phagocytosis, were not improved by the stain intervention. As phagocytosis also involves small GTPases [489,490] it was anticipated that this would also be improved. However, the decline in phagocytosis with age appears to be due largely to reduced CD16 expression [138] and thus affecting signalling events may not overcome this issue. Determination of small GTPase activity before and after statin treatment of neutrophils from old donors would significantly enhance our understanding of the mechanism driving the pleiotropic effects of statins and the age-related defect in migration. Such studies were

attempted during the latter stages of the thesis, but significant variability was found in the data making firm conclusions difficult to draw.

In addition to the inhibition of small-GTPases, there are a number of additional potential mechanisms through which statins may act. Endothelial dysfunction is a common feature of natural ageing [491], a process in which there is a systemic imbalance between vasodilating agents, such as nitric oxide, and vasoconstricting agents, such as endothelin -1, thus favouring the vasoconstricting state [449]. This results in a pro-inflammatory environment characterised by reduced nitric oxide (NO) generation, up-regulation of adhesion molecules such as V-CAM1 and ICAM-1 and generation of chemokine molecules such as MCP-1 [491]. Statins have been shown to inhibit this process restoring the balance between vasoconstriction and vasodilation essential for proper endothelial function. Here the ability of statins to activate a signalling pathway in the endothelium involving PI3Kinase, Akt and endothelial nitric oxide synthase (eNOS) [492] was key to their mode of action and results in increased nitric oxide (NO) production. In a murine model of endothelial activation induced by thrombin, intraperitoneal injection of rosuvastatin prior to thrombin injection resulted in increased release of NO from the vasculature and a 70% reduction in endothelial expression of P-selectin thus reducing leukocyte adherence and transmigration [493]. In the healthy elderly, where inflamm-ageing, the systemic sub-clinical elevation of pro-inflammatory cytokines is common, statins may act to limit inappropriate activation of the endothelium by IL6 and/or TNFα indirectly preventing inappropriate extravasation of leukocytes including monocytes and neutrophils. Thus statins may be having quite different effects on endothelial and neutrophil PI3Kinase activity.

The anti-inflammatory effects of statins have also been shown to result from reductions in the circulating levels of pro-inflammatory mediators: statin therapy correlates with reductions in CRP in a number of clinical trials [403,494], while monocyte expression of TNFα and IL-1β in previously hyperlipidaemic patients following an 8-week course of 20mg/day of simvastatin has been shown to be significantly reduced [495]. Interestingly, CRP has also been shown to

decrease eNOS activity [496,497] heightened levels of which, as part of the inflamm-ageing phenotype, may further exacerbate endothelial dysfunction in the elderly.

Work presented in this thesis invites the proposition that simvastatin has therapeutic potential in reducing rates of morbidity and mortality from bacterial diseases in the elderly population, possibly by modulating neutrophil migration. Throughout this thesis, only the effects of a single statin (at a single dose) were considered. Although undoubtedly critical to mounting an effective immune response, neutrophils do not act in isolation. The effects of statins on T cell function have been well studied with reports documenting suppression of the acute inflammatory response by favouring a Th2 mediated response, potentially protecting against Th1 driven diseases such as rheumatoid arthritis [498,499]. Additional reports also document the opposing actions of simvastatin and atorvastatin on expression of HLA-DR and the immune response to bacterial super-antigens [500].

Lipophilic statins (including simvastatin, atorvastatin, lovastatin and fluvastatin) have also been shown to compromise natural killer (NK) cell degranulation and cytotoxicity through inhibition of conjugate formation between NK and target cells by inhibiting LFA-1 [501]. Further exploration of the most effective statin at mediating restoration of neutrophil migratory parameters, may lead to an intervention able to reduce the rates of morbidity and mortality from bacterial infections in the elderly. However such studies must also establish a therapeutic regime that does not compromise other immune functions, that could for example reduce NK cell function and increase susceptibility to viral infections to which older adults are already susceptible [9].

7.6 Future Work

This thesis has provided a detailed description of neutrophil immune-senescence with a specific focus on neutrophil migratory dynamics, identified mechanisms possibly driving this aberrant migration and highlighted a potential therapeutic intervention to restore neutrophil

migration in the healthy elderly. However, many studies are still required in order to completely understand mechanisms driving migratory senescence, and to efficiently intervene to improve immunity in older adults.

1. Migratory dynamics should be characterised when cells are migrating across components of the extracellular matrix such as fibronectin, collagen, hyaluronic acid and/or laminin as well as through 3D gels/matrices to determine if the migratory phenotype is applicable to interstitial migration.
2. Migratory dynamics should be characterised in response to inflammatory biological secretions, such as sputum from patient with bacterial infections, containing a complex milieu of chemotactic mediators to better characterise migratory defects during clinical inflammation.
3. Expression of chemoattractant receptors should be examined to include expression on activated cells as well as receptor placement with respect to the chemotactic gradient, localisation to lipid rafts and membrane fluidity to determine if receptor localisation contributes to migratory defects.
4. Expression of adhesion receptors on the surface of quiescent and activated neutrophils to examine a role for altered adhesive contacts in migratory dynamics.
5. To assess the effects of ageing on the activity of class 1B PI3Kinase isoforms; on molecules governing PI3Kinase activity (SHIP1 and PTEN) and on the intensity and cellular localisation of the lipid product PIP_3 in migrating neutrophils from old donors. This would generate a more complete picture of the role PI3Kinase plays in generation of the ‘old-migratory’ phenotype.
6. To examine the effects of ageing on other pathways known to be involved in the regulation of migration including mTOR and small GTPase family members.
7. To determine the effects of age on the interaction between small-GTPases, actin nucleating proteins and the actin cytoskeleton and the effects this may have on pseudopod formation, sustainability and retraction on during directional sensing.

8. It would also be pertinent to determine the mechanism by which simvastatin is able to modulate neutrophil migration and to examine in more detail the effects of other statins on neutrophil functions including phagocytosis and ROS production.
9. Pre-incubating neutrophils isolated from young and old donors with plasma or serum from old or young donors prior to measuring migratory dynamics would determine if the defect observed in this work is cell intrinsic or a product of the inflammatory microenvironment.

In conclusion, work presented here describes a generic, robust migratory phenotype characterised by a maintained speed of migration but with reduced migratory accuracy. This phenotype appears to be driven by dysregulated PI3Kinase activity and is amenable to correction through inhibition of PI3Kinase-γ and -δ isoforms and treatment with Simvastatin. Aberrant migration as described here may cause increased collateral damage to otherwise healthy tissue and manifest as delayed arrival to the site of infection and raised systemic inflammation, ultimately compromising host defences and contributing to the increased rates of morbidity and mortality observed in the elderly population.

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Appendix I - Papers Published, Posters, Presentations and Awards won during the completion of this PhD

Published Articles

*Sapey E, ***Greenwood H**, Walton G, Mann E, Love A, Aaronson N, Insall RH, Stockley RA, Lord JM: **Phosphoinositide 3 kinase Inhibition Restores Neutrophil Accuracy in the Elderly: Towards Targeted Treatments for Immune-senescence**: 2013 Blood (In Revision)

*Joint first Authorship

***Greenwood H**, *Bartlett DB: **Meeting Report: British Society for Research on Ageing (BSRA) Annual Scientific Meeting 2012, Aston University, Birmingham**: Longevity and Healthspan 2013, 2(6)

*Joint first Authorship

Sapey E, Stockley JA, **Greenwood H**, Ahmad A, Bayley D, Lord JM, Insall RH, Stockley RA: **Behavioural and Structural Differences in Migrating Peripheral Neutrophils from Patients with Chronic Obstructive Pulmonary Disease**: American Journal of Respiratory and Critical Care Medicine 2011, 183 (9):1176-86

Shaw AC, Samit J, **Greenwood H**, Panda A, Lord JM: **Ageing of the Innate Immune System**: Current Opinion in Immunology 2010, 22(4):507-13

Abstracts

Sapey E, **Greenwood H**, Hazeldine J, Walton G, Thickett D, Lord JM, Stockley RA: **Neutrophil Function and Advancing Age: The Effects of Simvastatin in Health and during Pneumonia**: Thorax 2011, 6:A59

Greenwood H, Mann E, Walton G, Insall RH, Sapey E, Lord JM: **Correction of Aberrant Peripheral Neutrophil Migration in the Healthy Aged Through Inhibition of p110δ isoform of PI3Kinase**: American Ageing Association Annual Meeting, Fort Worth Texas USA, June 2012

Greenwood H, Mann E, Walton G, Sapey E, Lord JM: **Aberrant Neutrophil Migration in the Elderly can be Corrected Through Inhibition of the p110δ isoform of PI3Kinase**: British Society for Research on Ageing (BSRA) Annual Scientific Meeting, Birmingham, UK, 2012

Prizes Won

Greenwood H, Sapey E, Stockley JA, Chahal H, Love A, Aaronson A, Insall RH, Lord JM: **Reduced Neutrophil Migration in the Elderly: A potential cause of Delayed Response to Infection and Resolution of Inflammation**. 1st Prize (Poster) 1st Immuno-senescence Conference, School of Sport and Exercise Science, University of Birmingham 2009

Greenwood H, Mann E, Walton G, Insall RH, Sapey E, Lord JM: **Correction of Aberrant Peripheral Neutrophil Migration in the Healthy Aged Through Inhibition of p110δ isoform of PI3Kinase**, 1st Prize (Poster) British Society for Research on Ageing (BSRA) Annual Scientific Meeting 2012

Travel Grant, College of Medical and Dental Sciences, University of Birmingham 2013, £500

Presentations

Greenwood H: Correction of Aberrant Neutrophil Migration in the Healthy Aged by Inhibition of PI3Kinaseδ, Student Data Blitz American Ageing Association Annual Meeting, Fort Worth Texas USA, June 2012