Characterisation of the Fpr2 null mouse

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PhD Thesis

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I, Neil Dufton, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated and appropriately referenced.

Signed: …….

Date: …….

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Dedicated with love to Paul and Judith

“I do not seek. I find.”

Pablo Picasso

Abstract

A novel Fpr2$^{-/-}$ mouse colony was used to explore the biology of Fpr2, a GPCR related to the human FPR2/ALX receptor that recognises lipoxin A_4 (LXA_4) annexin A1 (AnxA1) and serum amyloid A (SAA). Southern blotting, PCR and radio-ligand binding confirmed receptor deletion in the mouse Fpr2$^{-/-}$ colony.

A GFP target/reporter strategy was employed in generating this novel transgenic to monitor promoter activity in living cells. This study revealed a propensity of Fpr2 for granulocytes, as well as a distinct role in macrophage (Mϕ) maturation. Characterisation of Fpr2$^{-/-}$ Mϕ revealed selective ERK phosphorylation triggered by the AnxA1-derived peptide Ac2-26, W peptide and Compound 43 (C43). Despite this Fpr-dependent signalling cascade via ERK, it was not a functional prognostic for cell migration *in vitro* or *in vivo.* Formyl peptide (*f*MLP) and serum amyloid A (SAA) chemotactic action was attenuated in Fpr2$^{-/-}$ Mϕ, as well as the pro-phagocytic effects of Ac2-26 and LXA_4.

There was no observable naïve phenotype associated with Fpr2 depletion. To investigate the patho-physiology of Fpr2, acute and chronic inflammatory models were investigated *in vivo* to dissect different aspects of the receptor during disease progression. Notably Fpr2$^{-/-}$ mice exhibited stimulus specific discrepancies in inflammatory response. An acute IL-1β-induced air pouch model

revealed predominantly anti-migratory pharmacology of Fpr2 ligands, with a notable exception of SAA, discovered to be anti-migratory in the absence of Fpr2. Analysis of the full time-course of the zymosan peritonitis pointed to a subtle role for Fpr2 in neutrophil and monocyte migration as well as Mϕ maturation. Of interest, exudate levels of SAA were augmented in $Fpr2^{-/-}$ mice revealing complex regulatory receptor/ligand circuits active during on-going inflammatory reactions. Finally, $Fpr2^{-/-}$ mice displayed pronounced arthritic responses upon treatment with the K/BxN arthrogenic serum, in comparison to their wild type controls. We conclude that Fpr2 can serve varied regulatory functions during the host response to inflammatory insult.

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Abbrevations

-/-	Knockout
$A\beta_{42}$	42 amino acid peptide amyloid-β
Abs	Antibodies
Ac2-26	Acetylated peptide from residues 2-26
AD	Alzheimer's disease
Ag	Antigen
AhR	Aryl hydrocarbon receptor
AMP	Ampicillin
ANOVA	Analysis of variance
AnxA1	Annexin-A1
APC	Antigen presenting cell
ApoA1	Apolipoprotein
APS	Ammonium persulfate
BCA	Bicinchoninic acid
BMMϕ	Bone marrow derived macrophages
BOC	N-t-butoxycarbonyl-Phe-$_D$-Leu-Phe-$_D$-Leu-Phe
bp	Base pairs
BSA	Bovine serum albumin
C43	Compound 43
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAMs	Cell adhesion molecules
cDNA	Complementary DNA
Ci	Curies
CLA-1	CD36 and LIMPII Analogous-1
CMC	Carboxymethyl cellulose
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disorder
COX	Cyclooxygenase
CRP	C-reactive protein
d	Day
DC	Dendritic cell
dLN	Draining lymph node
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EC	Endothelial cells
E.coli	Escherichia coli
ECL	Enhanced chemiluminescencs

EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem cell
FACS	Fluorscence-activated cell sorter
FCS	Foetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
*f*MLF	Formyl-methionyl leucyl phenylalanine
FPR	Formyl-peptide receptor
FPR-rs	FPR-related sequence
FPR2/ALX	Formyl-peptide receptor 2/aspirin-triggered lipoxin receptor
FSC	Forward-scattered light
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid
GFP	Green Fluorescent Protein
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GPCR	G-protein couple receptor
Gr-1	Granulocyte-differentiation antigen-1
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
h	Hour
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HEK-293	Human Embryonic Kidney 293
HIV-1	Human immunodeficiency virus-1
HMG-CoA	3-hydroxy-3methylglutaryl coenzyme A
hrAnxA1	Human recombinant annexin-A1
HPA	Hypothalamo-pituitary-adrenocortical
HRP	Horse-radish peroxidase
IgG	Immunoglobulin G
IL	Interleukin
in Hg	Inches of mercury
i.p	Intra-peritoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
i.v	Intra-venous
JAMs	Junctional adhesion molecules
kb	Kilo bases
KC	Keratinocyte-derived Cytokine
kDa	Kilo dalton
LB	Lennox L broth base
LDL	Low density lipoprotein
LFA-1/CD2	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide

LT	Leukotriene
LXA_4	Lipoxin A_4
M-CSF	Macrophage-colony stimulating factor
Mϕ	Macrophage
MFI	Median fluorescence intensity
MCP-1	Monocyte chemoattractant protein-1
MPO	Myeloperoxidase
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NK	Natural killer cells
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NS	Non-significant
NSAIDs	Non-steroidal anti-inflammatorys
OmpA	Outer membrane protein A
P	Probability
p.o	Per os
PAF	Platelet-activating factor
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBC	Phosphate-buffered saline with Ca^{2+} and BSA
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformyladehyde
PG	Prostaglandin
PI3K	Phosphoinoside 3-kinase
PKC	Protein kinase C
PL	Phospholipase
PMN	Polymorphonuclear
PR3	Proteinase 3
Pred	Prednisolone
PRR	Pattern Recognition Receptor
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene difluoride
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE-Cy5	Cyanin 5
RPMI	Roswell park memorial institute medium
RT-PCR	Reverse transcription PCR
SAA	Serum amyloid A
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean

SSC	Side-scattered light
STAT	Signal transducers and activators of transcription
TCR	T-cell receptor
TEMED	N,N,N',N'-Tetramethyethylene
TLR	Toll-like receptor
TM	Transmembrane domains
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TTBS	Tris-Tween Buffered Saline
TX	Thromboxane
W-petide	Trp-Lys-Tyr-Met-Val-D-Met peptide
WT	Wild type
VLA4	Very-late antigen 4

Chapter 1.

Introduction

1.1 Inflammation

To introduce such a complex and wide-ranging subject I will begin this thesis with a contemporary definition of inflammation:

inflammation *n.* Acute inflammation is the immediate defensive reaction of tissue to any injury, which may be caused by infection, chemicals, or physical agents. It involves pain, heat, redness, swelling, and loss of function of the affected part. Blood vessels near the site of injury are dilated, so that blood flow is locally increased. White blood cells enter the tissue and begin to engulf bacteria and other foreign particles. Similar cells from the tissues remove and consume the dead cells, sometimes with the production of pus, enabling the process of healing to commence. In certain circumstances healing does not occur and chronic inflammation ensues. *Concise Medical Dictionary. Oxford University Press, 2007*

1.1.1 A brief history of inflammation

The first recorded observation of the inflammatory process in human pathology was first categorised by Greek philosophers, such as Aristotle, who suggested that disease was an imbalance of the four humours; earth, fire, wet and cold. This theory was further expanded by Cornelius Celsus, a medical writer of first century A.D., who suggested cardinal signs of inflammation designated heat, redness, swelling and pain succinctly summarised by its

strict translation from latin, *inflammare*; to set fire (Scott *et al.*, 2004). A 19th century pathologist Rudolf Virchow added a fifth and final attribute termed loss of function, anthropomorphised below by five roman characters (Figure 1.1).

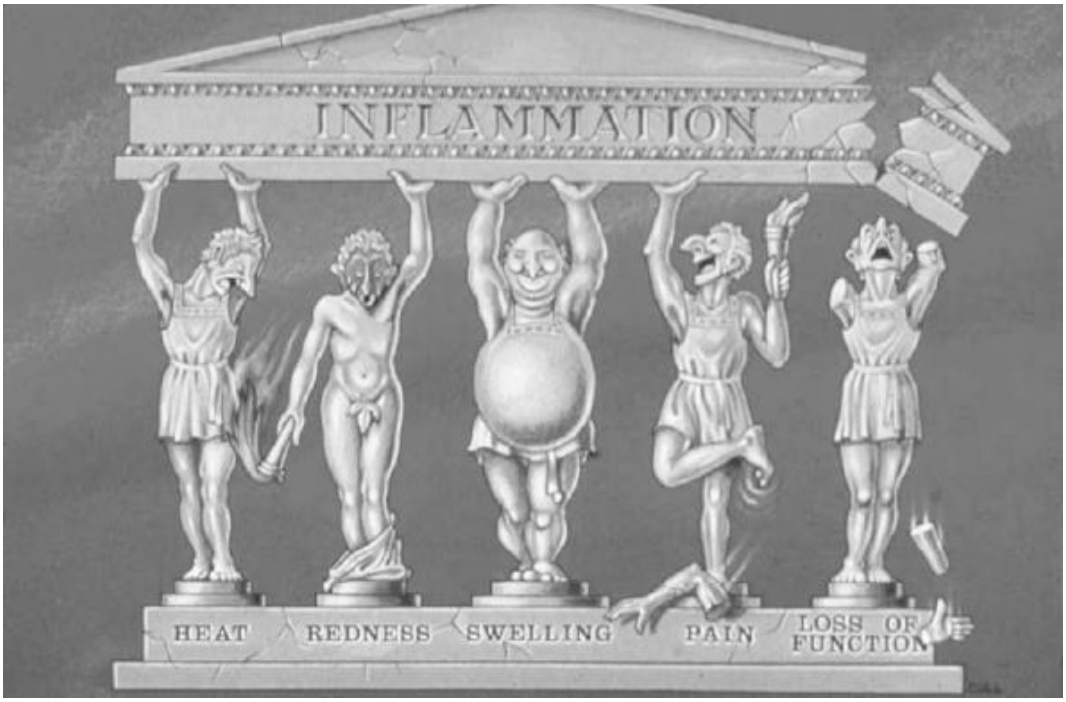


Figure. 1.1. Cartoon illustrating the five well characterised symptoms of the inflammatory process. This depiction was originally commissioned by Professor D Willoughby and Professor W Spector at the Medical illustration department, St. Bartholomew's Medical College.

This rudimentary appreciation for the complex processes of inflammation was revolutionised by William Harvey (1578-1657) who first described the circulatory system in accurate detail (Singer, 1928). This huge leap in anatomical understanding became the foundation of modern physiology and medical rational, subsequently leading to considerable scientific progression, which closely paralleled technological advances in microscopy. Pragmatists such as Rudolph Virchow (1821-1902) and Julius Cohnheim (1839-1884) applied these analytical techniques to investigate the constituents of blood

and pus identifying both the presence leukocytes and the roles they might play in shaping inflammatory processes.

1.2 Immunological processes of inflammation

The biology of mammalian inflammation is composed of two distinct mechanisms termed innate and acquired or adaptive immunity. The prevalence of each arm of immunity is dependent on disease pathology, genetics and environmental stimuli. Therefore for practicality and simplicity I will describe immune responses attributed to a spontaneously resolving pathology used experimentally throughout my thesis (Figure 1.2).

The overall inflammatory process can be compartmentalised into three phases; onset, transition and resolution. The initial response in the onset phase is orchestrated by the release of inflammatory mediators from both local tissue and resident cells. This induces leukocyte migration to the site of inflammation, which peaks during the transition phase. Finally the balance of pro-resolution mediators outweighs the pro-inflammatory, resulting in clearance of apoptotic cells and pathogens by phagocytosis. This tightly regulated sequence of events acts to re-establishing tissue homeostasis, however dys-regulation of any given phase can lead to chronic inflammation and potentially result in disease pathologies (Figure 1.2).

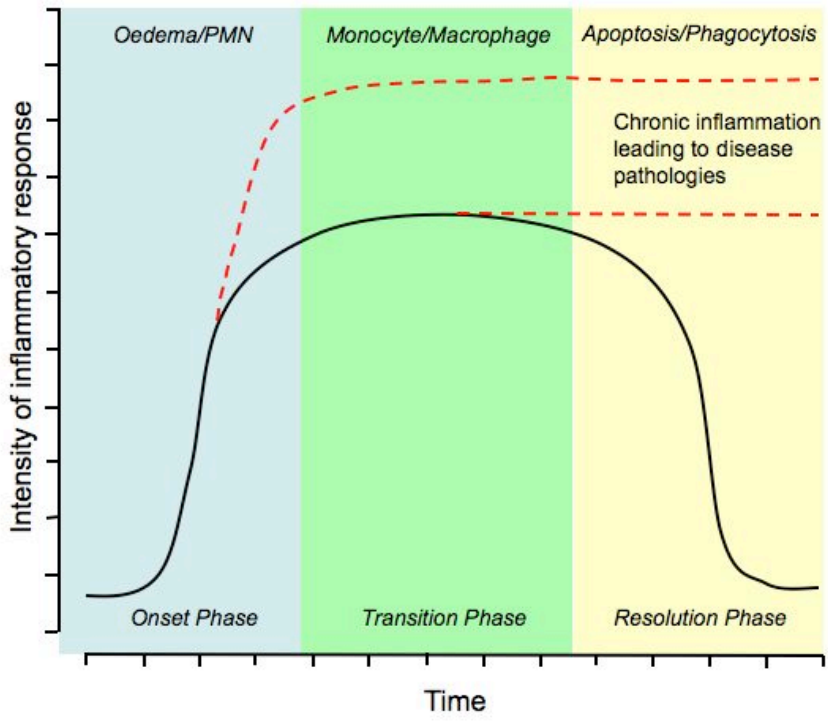


Figure 1.2 A schematic of a classical acute inflammatory time-course. The inflammatory process follows some very generic mechanistic profiles consisting of three discernable phases. The 'onset phase' characterised by release of inflammatory mediators and rapid influx of granulocytes (PMN). The 'transition' from pro- to anti-inflammatory is orchestrated by the infiltration of monocytes and subsequent differentiation towards macrophages. Clearance of apoptotic leukocytes and inflamogens by phagocytic cells are the main hallmarks of the 'resolution phase'. The disregulation of either the onset/acute phase or an inability to actively clear inflamogens can lead to chronic inflammation and potentially disease pathologies. Adapted from (Serhan *et al.*, 2007).

1.2.1 Innate Immunity

The innate immune system is an ancient evolutionary trait that is present in the majority of multi-cellular organisms. The acute response can be provoked by a myriad of stimuli including physical injury, infection, ischemia, auto-antibodies and thermal exposure. Each stimulus evokes a characteristic pattern of responses, for example bacterial or viral pathogens are recognised by pathogen-associated molecular patterns (PAMPs) to provoke the 'classical' inflammatory cascade described.

1.2.1.1 Inflammatory mediators

There is a multitude of inflammatory mediators involved in the initiation of any given inflammatory cascade, many beyond the scope of this thesis. Among the most documented are kinins, lipid-derived eicosanoids (prostaglandins and leukotrienes), cytokines, acute phase proteins and complement pathway.

The inflammation cartoon (Figure 1.1) illustrates symptomatic characteristics of distinct aspect of inflammation that can be attributed to a particular process in this cascade. The onset phase begins within seconds with pain and swelling, often the most immediate reaction, to inflammatory insult (Figure 1.2). Pre-stored mediators such as histamine, tumor necrosis factor-α (TNF-α) and rapidly synthesised platelet-activating factor (PAF), are released by damaged tissue and resident leukocytes, particularly mast cells, initiating a broad cascade of local and systemic responses.

Pain is predominantly mediated by kinins, e.g bradykinin, rapidly cleaved from precursor protein in the plasma and act on the BK receptor (Cortright *et al.*, 2004). The resultant local release of neuropeptides, e.g. substance P and calcitonin gene-related peptide, from small diameter sensory neurons are prominent mediators linking nociception and neurogenic inflammation (Richardson *et al.*, 2002). This pathway is extenuated in a cumulative manner by synthesis of prostaglandins (PG) inducing potent hyperalgesia (Higgs, 1980). The complex roles of pain, both nociceptive and more recently neuropathic pathways, in acute and chronic pathologies are still to be fully elucidated (Couture *et al.*, 2001).

This initial unpleasant reaction is quickly followed by vasodilatation, induced largely by histamine, PG and PAF, resulting in heat and redness as blood flow to the site of inflammation and cell metabolism increase. Vasodilation and activation of vascular endothelium encourage vascular permeability, allowing the tissue to become permissive to vascular fluid (oedema; swelling) and infiltrating leukocytes. There are complex interplay between cellular activation by inflammatory mediators and more specific cytokines and chemokines that allow homing of circulating leukocytes to the site of inflammation (Okayama *et al.*, 2006).

1.2.1.2 Leukocyte Migration

The migration of leukocytes is an exquisitely regulated process, which despite extensive study is still not fully characterised. In this hypothetic model of transmigration, there are three protagonists; resident/tissue specific cells, endothelial cells (EC), and circulating cells. Each population orchestrate the overall process in sequentially fashion culminating in leukocyte influx.

Resident cells, both leukocytes and lymphocytes, respond to locally released chemical mediators by secreting a number of chemoattractant factors (Ajuebor *et al.*, 1999) crucial for the homing of circulating leukocytes from peripheral blood. Inflammatory mediators, such as PG and PAF, are potent activators of the vascular endothelium inducing selective up-regulation of selectins, integrins and junctional adhesion molecules (JAMs). Each

molecular plays a distinct role during leukocyte migration being prevalent mediators of leukocyte rolling, adhesion, and diapedesis respectively (Figure 1.3).

Leukocyte rolling is regulated by selectin expression on both circulating leukocytes, termed L-selectin, and inflamed vascular EC, E- and P-selectin. During non-inflammatory condition leukocytes instigate frequent and transient interactions with the endothelium, predominantly via L-selectin, with very high on-/off- rates allowing the leukocyte to survey the vasculature for further activation markers, such as chemokines and integrins. Selectins preferentially bind glycosylated ligands; during inflammation P-selectin glycoprotein ligand-1 (PSGL-1) plays a dominant role in capture and rolling as a ligand for all three selectins (Ley *et al.*, 2007).

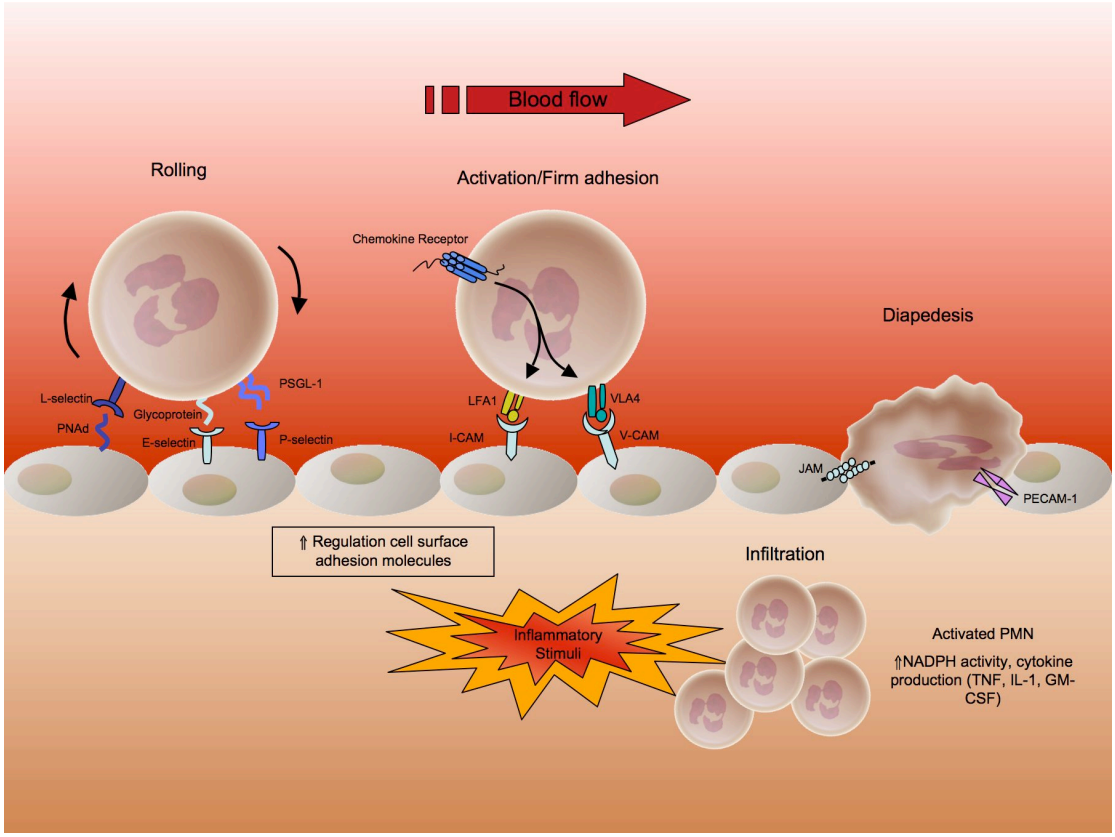


Figure 1.3 Leukocyte migration cascade. Following an inflammatory stimulus there is a well-characterised sequential response by both EC and circulating leukocytes. Rolling is largely mediated by L-selectin, expressed by leukocytes, and E- and P-selectin, expressed on inflamed EC, which bind corresponding glycoproteins. The activation of leukocytes, particularly via inside-out signalling of chemokines, results in integrin expression and activation inducing firm adhesion to the EC. Finally conformational changes of the leukocyte and interactions of JAMs and CAMs permit diapedesis of leukocytes. Adapted from Walzog et al. (2000).

Chemokines, a name derived from chemo(tactic cyto)kines e.g IL-8, are involved in both recruiting leukocytes from the periphery and local activation of migrating leukocytes. This secondary process of activation 'fine-tune' the positioning of a rolling leukocyte on the endothelium by inducing inside-out signalling (Abram *et al.*, 2009), resulting in rapid modulation of integrin affinity such as lymphocyte function-associated antigen 1 (LFA1) and very-late antigen 4 (VLA4) (Figure 1.3). These integrins recognise cell adhesion molecules (CAMs) on the EC surface to induce firm adhesion to a particular site on the endothelium.

Diapedesis of the activated leukocyte across the endothelium is the final step in the cascade prior to reaching the site of inflammatory insult. This is a relatively young field of great interest and complexity. It has been revealed that a number of conformational changes by both the migrating leukocyte and EC are required for diapedesis to occur. Although the specific processes are under debate integrins and an emerging family of JAMs play considerable roles in mediating this fnal step of egression (Ley *et al.*, 2007).

In this hypothetical model (Figure 1.2) circulating polymorphonuclear (PMN) cells, neutrophils, basophils and eosinophils, are the first leukocytes to respond to the cascade of inflammatory mediators. Neutrophils, the most abundant of the PMN, are matured within bone marrow prior to being released into the circulation. They have a short lifespan, from 7 h up to 2 days during disease pathology, can be rapidly mobilised during stress, accumulating at the site of inflammation within the first hours of insult (Smith, 1994).

Neutrophils contain numerous cytosolic granules containing proteases, cathepsin and elastase (Witko-Sarsat *et al.*, 2000). PMN infiltrate therefore has gained a notorious reputation for mediating a non-specific and over-zealous response, thought to be potentially detrimental to resolution. This notion is mistaken with PMN shown, by depletion (Smith, 1994), to play an essential role in stemming systemic inflammation, infection and chronic disease (Nathan, 2006a). Neutrophils are capable of phagocytosis and processing antigen by multiple receptors, which both direct the cell towards

apoptosis for engulfment by phagocytes (Haslett *et al.*, 1994; Section 1.2.1.3), and also shape the release of cytokines and chemokines to regulate the inflammatory response (Kantari *et al.*, 2008).

Following the initial neutrophil influx, described above, mononuclear cells, monocytes and lymphocytes, begin to migrate to the site of inflammation, resulting in a peak of infiltrating cell numbers through the transition phase (Figure 1.2).

Monocytes act to strengthen the host inflammatory response and as an inflammatory 'checkpoint' determining the magnitude and duration of the response (Serhan *et al.*, 2007). This immunological switch from PMN to mononuclear cells bridge the initial pro-inflammatory host response with the initiation of pro-resolution mediators and leukocyte phenotypes, contributing to clearance, tissue remodelling and repair. Infiltrating monocytes are particularly important through their ability to differentiate to professional antigen presenting cells (APC), such as macrophage (Mϕ) or dendritic cell (DC) phenotypes (Gordon *et al.*, 2005).

1.2.1.3 Clearance and phagocytosis

The implication of the resolution phase as an active process is an emerging concept that will be one of the themes of my thesis. Recent studies have revealed that numerous endogenous mediators instigate a wide range of processes, from preventing leukocyte migration to inducing apoptosis and

phagocytosis. In this particular model the predominant process to re-establish tissue homeostasis is *via* the apopto-phagocytosis system (Majai *et al.*, 2006).

There are two characteristic pathways for cell death termed apoptosis and necrosis. Necrosis occurs as a response to extreme environmental changes, such as hypoxia or as a result of membrane damage. Subsequently the cells lose membrane integrity and release their cellular contents leading to the initiation of inflammatory machinery (Haslett *et al.*, 1994).

Programmed cell death, apoptosis, occurs under normal physiological conditions with underling roles in cellular turnover and tissue homeostasis. It is therefore a key feature of inflammation to promote apoptosis of activated leukocytes to prevent excessive tissue damage (Majai *et al.*, 2006). During the transition phase, PMN undergo programmed cell death following ingestion of pathogen, up-regulation of pro-apoptotic signals e.g Fas-L (Kantari *et al.*, 2008) or the lack of pro-survival signalling such as ERK-1/2 (Sawatzky *et al.*, 2006). This leads to the exposure of 'eat-me' markers on the apoptotic cell surface, notably phosphatidylserine (PS), which facilitate recognition by Mϕ.

Phagocytosis is triggered by simultaneous interaction of multiple receptors, including Fcγ, complement, scavenger and pattern-recognition receptors (Section 1.3) to recognise pathogens and apoptotic cells. To counter-regulate these processes 'don't eat me' markers have also been identified, notably CD31 and CD47, that prevent the engulfment of viable cells by Mϕ (Kantari *et al.*, 2008). Phagocytic Mϕ not only ingest inflammogens and apoptic

leukocytes but are also capable of producing cytokines, such as IL-6, IL-10, TGF-β (Scannell *et al.*, 2007). IL-6 is a particularly important immunological switch integrating the innate and adaptive immunology (Jones, 2005), a notion that has lead to the definition of pro-resolving mediators (Serhan *et al.*, 2008). The concept of pro-resolving mediators is a contemporary term largely prescribed to the pharmacology of a potent family of lipid mediators derived from arachidonic acid (Flower *et al.*, 2005). Lipoxins (Section 1.5.2.1) and PGD_2 are among the current members to be characterised in their ability to regulate leukocyte recruitment, stimulate apoptosis of PMN and increase phagocytic capacity of Mϕ (Schwab *et al.*, 2006).

Both Mϕ reprogramming and the derivation of regulatory DC produce an anti-inflammatory environment prominent in the final stages of resolution. They are an important link between innate and adaptive immunity with Mϕ shown to migrate to draining lymph nodes (dLN) to instigate antigen (Ag) presentation and the priming of the adaptive immune response (Bellingan *et al.*, 1996).

Poignantly, the dys-regulation of any of these phases or 'checkpoints' can lead to more chronic pathologies, responsible for the fifth and final characteristic of inflammation, loss of function, where persistent inflammation and remodelling of the affected tissue renders it incompetent (e.g chronic obstructive pulmonary disorder (COPD) (Rennard, 1999).

1.2.2 Adaptive Immunity

The importance of adaptive immunity was first characterised by Edward Jenner (1749-1826) who detailed the ability of cowpox to protect milkmaids form the more virulent and deadly smallpox virus. The idea of vaccination has since been arguably the single most successful improvement in medical care, and to the credit and testament of Jenner, smallpox was eradicated in 1980 (Akira, 2009).

The ability of the human body to recognise and effectively clear repeated insult by antigens on demand is an evolutionary trait that has become integral to mammalian biology. Very briefly, a foreign antigen is presented to T-lymphocytes by APC such as DC or Mϕ, inducing both the proliferation of effector T-cells, acting immediately at the site of insult, and developing T-memory cells which have the ability to rapidly respond to a secondary insult of a given antigen. Although adaptive immunity is an intricate part of any disease pathogenesis I will not dwell on the multitude of mechanisms involved, as the adaptive arm involved in this hypothetical inflammatory scenario investigated is fairly restricted.

In a resolving model of inflammation resident adaptive immune cells, T/B cells, act to modulate the influx of PMN to the site of insult by the production of cytokines. As alluded to in the case of resident Mϕ, T/B cells migrate to dLN before the repopulation of the inflammatory site by natural killer cells (NK), γ/δ T-cells and memory ($CD4^+/CD25^+$) T-cells during the resolution phase

(Rajakariar *et al.*, 2008). Notably, the inflammatory process in not autonomous, in lymphocyte-deficient ($RAG^{-/-}$) mice there is disregulation of the onset phase although no significant influence on the development on the resolution phase (Rajakariar *et al.*, 2008).

Both aspects of the inflammatory process are clearly entwined and therefore the ability of innate and adaptive immunity to induce a coordinated and integrated response is the key to the pathogenesis and resolution of complex disease pathology.

1.3 Pharmacology of acute inflammation; Pattern Recognition

As mentioned in Section 1.2.1 the processes of inflammation are stimulus specific, with a wide variety of environmental stress factors inducing ancient, pre-determined pharmacological cascades. Over the last two decades considerable research has been invested in the distinct pharmacology associated with PAMPs.

These highly conserved sequences, ranging from bacteria and viral pathogens to nucleic acids of foreign origin, are recognised by pattern recognition receptors (PRR). There are two major classes of PRR termed signalling and endocytic (Jeannin *et al.*, 2008).

1.3.1 Endocytic pattern recognition receptors

Endocytic PRRs are so called for their shared characteristics, promoting attachment, engulfment and destruction of pathogens. As the name would suggest they are found predominantly on the surface of phagocytes and largely consist of scavenger receptors and mannose-binding receptors, which bind carbohydrates expressed on the surface of pathogens and infected cells.

1.3.2 Signalling pattern recognition receptors

A signalling PRR is classified as transducing an innate or adaptive immune response by the production of inflammatory mediators such as cytokines. There are three receptor families that have been identified termed nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), Toll-like receptors (TLR) and formyl-peptide receptors (FPR).

The NLR are a family of cytoplasmic proteins that are able to form oligomers, as there nomenclature suggests, which activate capases, for example caspase-1 which is important in the formation of the inflammasone, and nuclear domains such as NF-κB (Mathews *et al.*, 2008).

The TLR family was famously first discovered in *Drosophila* before being identified in mammals in 1997 by Medzhitov (Akira, 2009). Our understanding of the pharmacology of inflammation has significantly benefited from this discovery of TLR signalling, notably their ability to form either homo- (TLR4)

or hetro- (TLR2/TLR6) dimers in order to produce functional receptors. The TLR family is currently thought to consist of up to 15 membrane-bound receptors, which have a wide, but distinct expression pattern and are capable of recognising an ever increasing array of ligands.

Finally the FPR seven transmembrane G-protein coupled receptor family is an emerging class of PRR. It was initially termed an endocytic PRR, however extensive investigation *in vitro* and *in vivo* (Le *et al.*, 2001b), including within my thesis, have revealed significant roles for FPR family signalling in shaping the immune response. Possibly the most convincing observation that $FPR1^{-/-}$ mice were more susceptible to *Listeria monocytogenes* infection than WT mice *in vivo* (Gao *et al.*, 1999), underlines FPRs role in host defence through pattern recognition. Recently, *N*-formyl-peptides from *Listeria monocytogenes* and *Staphylococcus aureus* were also shown to signal via the FPR family (Southgate *et al.*, 2008). Furthermore both NOD2 and TLR-2 have been shown to up-regulate the expression of Fpr2 in murine microglial cells reinforcing the inclusion of FPR as a PRR (Chen *et al.*, 2008).

1.4 Formyl-Peptide Receptor Family (FPRs)

The FPR receptor family was extensively characterised throughout the 1980's as G-protein coupled receptors (GPCR) *via* pertussis toxin sensitivity; specifically attributed to G_i protein coupling (Lavigne *et al.*, 2002). The human family was successfully cloned in the in 1990 (Boulay *et al.*, 1990) with three genes encoding FPR1, FPR2/ALX and FPR3 clustered on chromosome

19q13.3. The FPR has significant evolutionary divergence across mammalian species with differential gene expansion particularly notable in the mouse (Gao *et al.*, 1998). The murine Fpr gene family is located on chromosome 17 and consists of seven members. There are two direct orthologs between human and mouse with *FPR1* and *FPR2/ALX* represented by *fpr1* and *fpr2* respectively (Figure 1.4). Despite close homology between the human and murine receptors, sharing almost identical intracellular domains, murine Fpr1 has approximately a 100-fold reduction in affinity for the prototype formyl peptide fMLF (Gao *et al.*, 1993).

FPR1 and FPR2/ALX have been shown to have very similar cellular distribution with expression on PMN and mononuclear cells (both myeloid and lymphocytes). This profile is mirrored by the murine orthologs, suggesting they may share physiological roles across species (Fu *et al.*, 2006). Interestingly there is evidence suggest divergent evolution has occurred between *FPR1* and *FPR2/ALX*. To date seven common *FPR1* polymorphisms, two associated with juvenile periodontitis, have been identified whereas only one *FPR2/ALX* haplotype could be found (Sahagun-Ruiz *et al.*, 2001).

The third human receptor, FPR3, is the least characterised of the family and has no current ortholog in the mouse. This receptor is not expressed by neutrophils but is found on monocytes and DC. Interestingly FPR1 and FPR3 are expressed by immature DC, however only FPR3 is retained on mature DC (Devosse *et al.*, 2009). FPR3 is unusual as it is unable to respond to

formylated peptides and currently only has one high affinity endogenous ligand, a peptide termed F2L, derived from a heme-binding protein. In the human and mouse there is a degree of functional cross-over between FPR3, FPR2/ALX and Fpr2 respectively (Gao *et al.*, 2007).

The further five members of the mouse Fpr family are found in a third distinct cluster (Figure 1.4) and remain orphan receptors. Like FPR3 they do not respond to formylated peptide and have unusual expression patterns e.g fpr-rs3 on skeletal muscle, for putative chemotractant receptors. Their distinctive distribution may implicate tissue-specific roles or denote a degree of redundancy within the murine family (Gao *et al.*, 1998).

Notably FPRs are not restricted to binding formylated peptides but have been shown to be a highly promiscuous receptor family interacting with structural disperate ligands (Migeotte *et al.*, 2006).

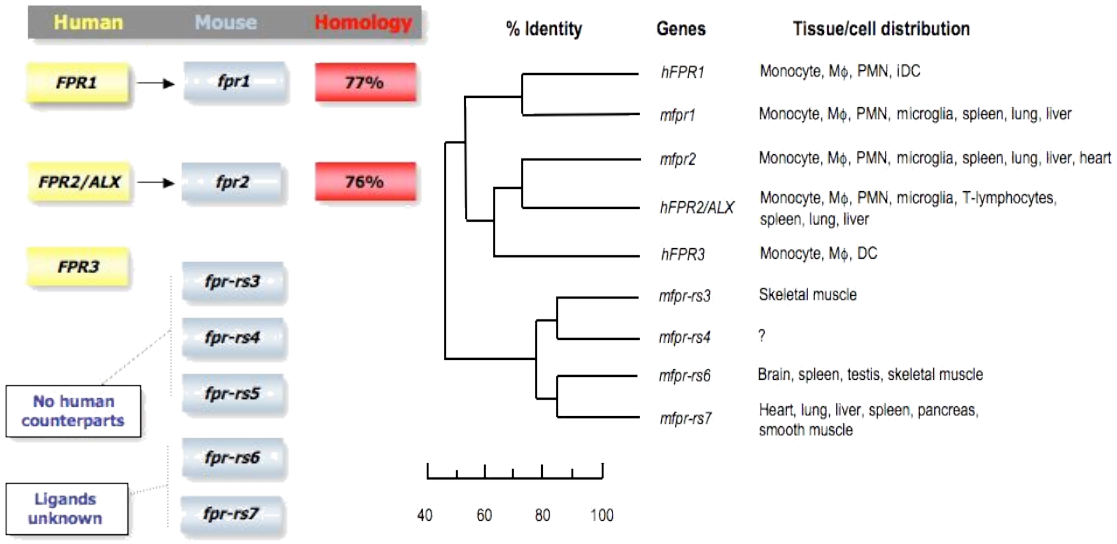


Figure 1.4 Schematic comparison representation of the FPR receptor family in the human and mouse. By sequence homology human and mouse families share two gene clusters. *hFPR1* and *mfpr1* share ~77% homology while *hFPR2/ALX* and *mfpr2* genes, ~76% homology, also share similarities with *hFPR3*. Structure homology can be compared by percentage within certain gene clusters. There are a number of disparities in the gene expansion between the two species with the further member of the murine *fpr* family. Adapted from (Ye *et al.*, 2009).

1.4.1 FPR1

FPR1, the first receptor of the family to be discovered, was identified for its ability to transduce the chemotactic effect of a synthetic *E.coli*-derived *N*-formyl-methionyl-leucyl-phenylalanine peptide (fMLF) (Schiffmann *et al.*, 1975). fMLF is a highly lipophilic compound with a flexible backbone which is important for establishing conformation to interact with FPRs (Dalpiaz *et al.*, 2003). Since this discovery fMLF has become the principal chemotractant agonist extensively characterised *in vitro*. Due to the popularity of fMLP its signalling cascade, via FPR1, is the best characterised among any neutrophil receptor, notably sharing many traits with both human family members and mouse orthologues.

Following ligation by their agonists, FPRs undergo conformation changes allowing functionally interactions with $G_i\alpha1$, $G_i\alpha2$ and $G_i\alpha3$ as well as putative association with G_0, G_z and $G\alpha_{16}$ (Migeotte *et al.*, 2006). Downstream of G-protein interaction there are a number of signalling pathways including calcium (Ca^{2+}) flux, phospholipase A, C and D (PLA, PLC and PLD respectively), phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (Selvatici *et al.*, 2006; Figure 1.5).

PLC is initial is an essential upstream mediator of both PI3K and MAPK pathways *via* protein kinase C (PKC). It has been shown that receptor desensitisation and internalisation is reliant on PKC following both homologous and heterologous activation of FPR or other GPCRs respectively (Le *et al.*, 2001b). PLA_2 is also well characterised for mediating PLC activation (Section 1.2.1.1) central to the biosynthesis of eicosanoids. The activation of PI3K following fMLF has been shown to selectively regulate oxidative burst and actin relocalisation, essential for cell polarisation, in neutrophils. The MAPK pathway, in particular ERK-1/2, selectively regulates chemokinesis as well as a multitude of signal transducers and activators of transcription (STAT) proteins and adaptor proteins (Wenzel-Seifert *et al.*, 1998).

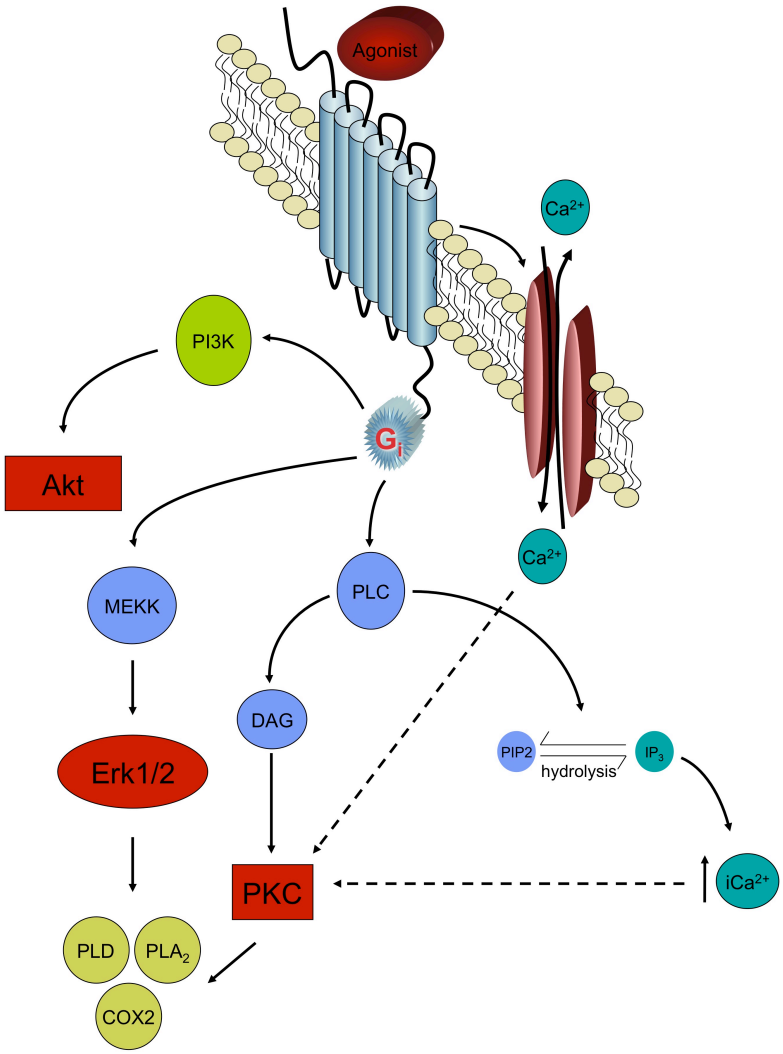


Figure.1.5. Schematic of the generic downstream signalling cascade of FPR family. FPR receptors are coupled to Gi-protein activating PI3K, MAPK pathways and transient Ca2+ flux to influence pro- and anti-inflammatory mediators.

Signalling *via* FPRs is regulated by two processes, receptor desensitisation and agonist-induced internalisation. Desensitisation is the result of uncoupling of G-protein from the receptor, and can occur either by direct ligation or following activation of a similar GPCRs. After initial activation of FPR1 with fMLF, the receptor rapidly reduces its responses to secondary stimulation with the same agonist; this is termed homologous desensitisation. FPR1 is also susceptible to heterologous desensitisation *via* ligation of a GPCR receptor,

C5aR or IL-8R, in a concentration-dependent manner (Ali *et al.*, 1999). These processes complement the classical idea of concentration-gradient dependent migration by peripheral cells to a site of inflammation.

The FPR family is known to internalise in a ligand-specific manner however the exact processes involved are unclear (Gilbert *et al.*, 2001). It may be reasonable to assume that conformational differences in ligand-FPR complexes are responsible for the distinct pharmacology attributed to each agonist.

Apart from the ability of FPR1 to mediate chemotaxis it was also noted that fMLF induced a rapid Ca^{2+} mobilisation (Andersson *et al.*, 1986). Although Ca^{2+} has been a robust functional response to fMLF, its functions are unclear. Ca^{2+} is certainly required for cytoskeleton reorganisation however phagocytosis and chemotaxis can occur in Ca^{2+}-depleted cells (Fu *et al.*, 2006). The necessity or otherwise of this secondary functional parameter, and the identification of the two further FPR family members, revealed that activation of a given signal transduction pathway was not only concentration dependent but also ligand specific.

FPR1 is most notably associated with host defence, with fMLF noted to elicit shape change, adhesion, phagocytosis, cytokine production, superoxide production and degranulation in both granulocytes and phagocytes (Selvatici *et al.*, 2006). The importance of FPR1 in preventing bacterial infection is particularly evident within human periodontitis. Moreover, the connection

between cancer progression and inflammation has become an emerging area and indeed FPR1 has been associated with mechanistic pathogenesis (Huang *et al.*, 2009). Furthermore human immunodeficiency virus-1 (HIV-1) peptide derivatives have been shown to activate FPR1 (Su *et al.*, 1999) (Table 1.1). This leads to two plausible conclusions that FPR1 plays an important role in immune surveillance by responding directly to foreign antigen or that HIV-1 has evolved to desensitise the important classical chemotactic and effector functions attributed to FPR1 (Le *et al.*, 2001b). If either hypothesis is correct there is considerable scope for the development of FPR1 specific therapeutics within this area of research.

To determine the potential roles of FPR1, pharmacological tools and $Fpr1^{-/-}$ transgenic mice have been developed. The replacement of the formyl group of fMLF with tertiary butyloxycarbonyl group (t-BOC) produced the first FPR1 antagonists (Freer *et al.*, 1980). However the most successful approach to specific inhibition of this multi-faceted receptor has been the development of an $Fpr1^{-/-}$ transgenic mice (Gao *et al.*, 1999; Section 1.6).

1.4.2 FPR2/ALX

Originally characterised as the low-affinity fMLF receptor (efficacy ~1000 fold lower than FPR1; Gao *et al.*, 1993), FPR2/ALX has a short but complicated history.

The human receptor was initially reclassified as the receptor for the lipid-derivative lipoxin A4 (LXA_4), termed ALX (Fiore *et al.*, 1994). However it soon became apparent that this was not the only competitive agonist capable of binding this receptor with reports describing HIV peptides and serum amyloid A (SAA; Su *et al.*, 1999), Annexin A1 (AnxA1; Perretti *et al.*, 2001b; Perretti *et al.*, 2002), as well as synthetic peptide (W-peptide; Le *et al.*, 1999) and chemical compounds (C43; Burli *et al.*, 2006). There are currently ~30 ligands that have been shown to bind the FPR family and although many are peptides, they are structurally unrelated. Most intriguingly, I believe, this is the first receptor family to be shown to interact with ligands spanning lipids, proteins and peptides (Table. 1.1). Furthermore although there is considerable promiscuity across the FPR receptors, ligands can transduce either pro- or anti-inflammatory actions *in vitro* and, in some cases, *in vivo* (shown in my thesis).

The paradigm surrounding FPR2/ALX pharmacology is further complicated by the differential expansion of the FPR family in the mouse. Indeed when the gene families were first assessed it was thought FPR2/ALX was represented by two related murine genes, noted as *fpr-rs1* and *fpr-rs2*. Notably, *fpr-rs1* showed closer sequence homology with ALX, but *fpr-rs2* was shown to represent more functional characteristics of the receptor (Gao *et al.*, 1998). My thesis, and work performed in other laboratories, has revealed that these genes are formed by alternate transcription and therefore can be considered cumulatively as *Fpr2*. This is reflected by the nomenclature used in this thesis,

which follows the very recent recommended guidelines from IUPHAR (Ye *et al.*, 2009).

The signal transduction of FPR2/ALX is therefore a difficult topic, however it is reasonable to assume it shares similar intracellular machinery with FPR1 as they have similar intracellular domains. A comprehensive study of the FPR1 and FPR2/ALX signalling was conducted by creating chimaeric receptors with segments of FPR2/ALX replaced by FPR1 revealed numerous specific binding sites. Notably fMLF recognised the same extracellular loops on both FPR1 and FPR2/ALX but with different affinity. Furthermore, this analysis it revealed that N-glycosylation is required for peptide binding (again not necessarily at the same domain) to FPR2/ALX but not for the lipid agonist LXA_4 (Le *et al.*, 2005).

FPR2/ALX, as with FPR1, is strongly associated with host defence. However the novel ability of FPR2/ALX to convey both pro- and anti-inflammatory signalling makes it an intriguing GPCR.

Interaction of FPR2/ALX with fMLF and the acute phase protein SAA leads to NF-κB activation and secretion of IL-8 by human neutrophils (He *et al.*, 2003). The reduced affinity for formylated peptides, such as fMLF, was initially thought to reflect receptor redundancy, whereby the desensitisation of one receptor, FPR1, may result in the prevalence of a second lower affinity

counterpart, FPR2/ALX (Hartt *et al.*, 1999b). Although this hypothesis may be true *in vitro* it is slightly simplistic when considering the role of heterologous densensitisation (Section 1.4.1) *in vivo*. Indeed FPR2/ALX is proposed to play an integrated role with FPR1 in attenuating CCR5 signalling when exposed to HIV-1 peptides.

SAA, its peptide-derivative amyloid-Aβ_{42} (Aβ_{42}) (Section 1.5.3.1) and Prion peptide PrP106-126 are potent amyloidogenic ligands for FPR2/ALX in the periphery and central nervous system (CNS) respectively. The formation of amyloid plaques in atherosclerotic legions (Wilson *et al.*, 2008) and Alzheimer's disease (Cui *et al.*, 2002b) are important pathological processes attributed to FPR2/ALX.

However unlike FPR1, FPR2/ALX can transduce anti-inflammatory responses including deactivation and detachment of leukocytes, apoptosis, phagocytosis and regulation of COX-2. The most striking attribute of this receptor pharmacology is mediated by endogenous ligands; glucocorticoid-induced protein annexin-A1 (AnxA1), lipid-derived lipoxin A_4 (LXA_4), neuroprotective protein humanin and heme-binding protein-deriving peptide F2L (Table 1.1).

The dual roles of FPR2/ALX make association with disease pathogenesis difficult to ascertain. FPR2/ALX expression is induced by both pro-inflammatory stimuli, e.g TNF-α (Cui *et al.*, 2002b) and pattern recognition

receptors (Chen *et al.*, 2009) as well as anti-inflammatory signalling, such as through the glucocorticoid receptor (Hashimoto *et al.*, 2007; Sawmynaden *et al.*, 2006). The strongest mechanistic links are with autoimmune diseases including airway allergy (Bonnans *et al.*, 2007) and synovial fibroblasts in arthritic joints (Fiore *et al.*, 2005). Clinical studies have noted the receptor is up-regulated on circulating mononuclear cells following acute ischemic stroke (Grond-Ginsbach *et al.*, 2008).

The therapeutic potential of this receptor has not gone unnoticed by academia or industry with a ever increasing number of synthetic agonists being developed. The first specific peptide described was Trp-Lys-Tyr-Val-$_D$-Met (also termed WKYMVm or W-peptide), a hexapeptide identified by high-throughput peptide library screening (Baek *et al.*, 1996). Despite potent activity *in vitro*, inducing Ca^{2+} flux, chemotaxis, superoxide production (Le *et al.*, 1999), W-peptide has shown little promise *in vivo* (Gavins *et al.*, 2005).

Although the majority of agonists identified are peptides, a number of non-peptidic compounds are also currently in development. Amgen were able to identify a number of pyrazolone compounds, in particular Compound 43 (C43), by high-throughput screening. C43 was shown to have potent anti-inflammatory pharmacology by inhibiting fMLF and IL-8 mediated chemotaxis *in* vitro, as well as a pronounced reduction oedema in *vivo* (Burli *et al.*, 2006). Amgen continue to pursue this avenue of research with studies assessing further modifications of a benzimidazole core to generate other small

molecules specific for Fpr2/ALX noting both pro- and anti-inflammatory responses *in vitro* (Frohn *et al.*, 2007).

As alluded to with FPR1, the use of current antagonists is controversial due to concentration dependent effects. As with synthetic agonist development, both peptide and non-peptide approaches are available. Ryu's group who developed W-peptide subsequently, used the same peptide library to generate the WRW4 peptide (Bae *et al.*, 2004). More recently, high-throughput screening of a series of quinazolinone derivative identified both agonistic, Quin-C1, and antagonistic, Quin-C7, molecules (Zhou *et al.*, 2007). The area of FPR2/ALX pharmacology has however struggled with the lack of specificity of many of the agonists and antagonists, for this reason we chose a transgenic route to determine specific Fpr2 pharmacology (Section 1.6).

Receptor	Natural Agonists	Synthetic Agonists	Antagonists	Promoter Activity
FPR	Formylated-mitochondrial peptides Capthepsin G AnxA1	fMLF (≥1nM) Ac2-26 Ac2-12 Ac9-25 T20/DP107 gG-2p20 AG-14 WKYMVM WKYMVm MMWLL	BOC derivatives Cyclosporin A Cyclosporin H Bile Acids Spinorphin LDLLDL CHIPS CDCA DCA	TNF-γ IL-10 TLR4
FPR2/ALX/ ALX	Formylated-peptides Serum amyloid A β-amyloid peptide 42 Urokinase-receptor-derived peptide CRAMP AnxA1 Humanin LXA_4 F2L NADH-dehydogenase subunit 1 PACAP27 Prion protein Hp (2-20) SHAAGtide sCKβ8-1	fMLF (≥10μM) HIV-derived peptides Ac2-26 Ac2-12 Anti-flammin-2 Compound 43 Formylated humanin Quin-C1 MMK-1 WKYMVM WKYMVm LL-37 Temporin D2D3 uPAR	BOC derivatives (≥10μM) WRW4 peptide FLIPr Quin-C7	GC TNF-γ TLR2 TLR4 NOD2
FPR3	Formylated-peptides F2L Humanin Hp (2-20)	Ac2-26 fMLF (>10μM) WKYMVM WKYMVm	WRW4 peptide	Currently unknown

Table 1.1 Non-exhaustive table of human formyl peptide receptor family ligands. Agonists are listed as pro-inflammatory,anti-inflammatory or currently unspecificed. There are numerous ligands, both agonists and antagonists that bind across two or more FPR receptors. Adapted from Migeotte *et al.*, 2006

1.5 Therapeutics and the FPR pathway

As demonstrated (Table 1.1) there is broad scope for development of therapeutically active synthetic compounds targeting both FPR1 and FPR2/ALX. However, it is important to first investigate and characterise the roles and regulation of both the receptor and its endogenous ligands during homeostasis, inflammation and following current therapeutic intervention.

Three of the endogenous agonists specific for FPR2/ALX are strongly linked with current, globally available treatments; glucocorticoid-regulated AnxA1, aspirin-triggered lipoxin A_4, statin-modulated/cholesterol-linked serum amyloid A.

1.5.1 Glucocorticoids

Ever since the first observation by Dr Philip Hench (subsequent Nobel laureate) that steroids extracted from adrenal gland cortex could exert potent anti-inflammatory effects (Hench *et al.*, 1949) glucocorticoids (GC) have become the front-line anti-inflammatory therapy. Over the last half century their ability to suppress the inflammatory response and indeed their specific modes of action have been under continued investigation. Therapeutic GC have shown widespread efficacy in a number of auto-immune diseases however clinicians have become acutely aware that persistent elevation of GC levels can cause a plethora of side effects with therapeutic regimes often based on assessing risk against benefit in individual patients. For this reason chronic GC treatment is often referred to as a ‘double-edged sword’.

Endogenous GC are essential to a variety of processes that underpin mammalian development, homeostasis, behaviour and host defence. Their actions, largely attributed to cortisol in humans and corticosterone in rodents, are tightly regulated by the hypothalamo-pituitary-adrenocortical (HPA) axis following well-documented circadian patterns (Dickmeis, 2009). During physical or emotional stress GC are rapidly secreted to prepare the body to react and adapt to a given situation. The body's ability to regulate GC secretion is particularly important in inflammatory micro-environments where GC act in a negative feedback loop to prevent over-activation of the immune system (Yeager *et al.*, 2004).

GC are highly lipophilic, readily crossing the plasma membrane to interact with their cytoplasmic receptors. There are two receptors responsible for mediating the actions of endogenous GC pharmacology termed the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). The MR shows a high affinity (Kd ~0.5-2nM) for cortisol, corticosterone and aldosterone and has a distinct expression profile within the gut, kidney and some areas of the CNS. GR however has a wide expression profile but has a lower affinity (Kd ~10-20nM) for GC, but it does not readily bind mineralocorticoids. Therefore in 'stress', when GC levels can exceed 300nM, GR is largely responsible for their local and systemic pharmacology (Buckingham, 2006). There are two known GR receptor isoforms, termed α and β, however GRβ is thought to be non-functional as it lacks the GR binding domain (Oakley *et al.,* 1996). The GR transduces its wide-ranging functions

by two prominent intra-cellular mechanisms; the genomic and non-genomic pathways.

The 'classical' genomic pathway is the result of GC binding cytoplasmic GR, leading to activation and dissociation of chaperone proteins and transcription factors normally associated with the receptor. Subsequent homodimerisation of receptor-ligand complex translocate to the nucleus. The GC-GR complex will bind specific DNA sequences, termed GC response elements (GREs), thus governing the transcription of target genes. Indeed so broad are the genomic actions of GC that they are thought to influence the transcription of approximately 10% of human genes (Buckingham, 2006). Notably this pathway inhibits the generation and release of inflammatory mediators (e.g. IL-1, TNF-α, $cPLA_2$) while inducing anti-inflammatory proteins such as IL-10, IL-1RA and IL-13. However these 'classical' responses are relatively slow (occurring within hours) because *de novo* synthesis is required.

Our research base is strongly focused on the ability of GC to induce rapid, non-genomic regulation of cellular processes through direct protein-protein interactions (Croxtall *et al.*, 2000). This machinery involves the release of pre-formed intracellular mediators to convey immediate anti-inflammatory actions. A clear example is the direct modulation of platelet function (which are non-nucleated) within 5 min of prednisolone treatment (Moraes *et al.*, 2005).

One established example of an anti-inflammatory GC-induced protein is AnxA1, which was first described in the late 1970's.

1.5.1.1 Annexin A1 (AnxA1)

Simultaneous investigation by three laboratories discovered that glucocorticoid-induced inhibition of PLA_2 was mediated by a 'second messenger' terming the protein renocortin, macrocortin and lipomodulin (Flower *et al.*, 1979). These proteins were subsequently shown to be indentical and renamed as lipocortin 1 (Pepinsky *et al.*, 1986; Wallner *et al.*, 1986) before genetic, proteomic and biochemical analysis categorised it as a member of a superfamily of related proteins termed annexins (Crumpton *et al.*, 1990; Pepinsky *et al.*, 1986; Wallner *et al.*, 1986).

The term annexin, from the Greek *annex* "hold together" describes their common characteristic of binding negatively charged phospholipids in a calcium dependent manner. The annexin superfamily has grown steadily in the 1990s with over 160 unique annexin proteins observed across 65 different species ranging from fungi and protists to plants and higher vertebrates. There are currently 13 member of the annexin family in mammalian biology, involved in a wide variety of physiological processes (Gerke *et al.*, 2002).

Annexins have common structural characteristics comprising of highly homologous core domain consisting of 4-8 repeats of a 70-75 amino acid sequence and an amino-acid tail (N-terminal) unique to each family member (Wells *et al.*, 2004). The formation of their three-dimensional structure has reveal that annexins form α-helical disks with slight curvature to form novel Ca^{2+} binding sites (Rosengarth *et al.*, 2003). Indeed AnxA1 consists of an N-

terminal of 40 residues, and 4-conserved core domain repeats. In the absence of Ca^{2+} the protein N-terminal binding site (yellow; Figure 1.6 B) is 'folded' within the protein structure. However upon Ca^{2+}-dependent membrane binding the protein conformation changes to allow association of AnxA1 binding sites with cell surface proteins (Figure 1.6 A). The N-terminal domain has phosphorylation sites for tyrosine and serine-kinases as well as glycosylation and transglutamination sites, with several proteolytic motifs spanning the N-terminal capable of significantly modifying its physical and biological properties. Interestingly tyrosine phosphorylated AnxA1 is more susceptible to proteolysis. The formation of small pharmacologically active peptides by synthetically targeting these different proteolytic sites has been exploited (Section 1.4.2). The first acetylated polypeptide described in the early 1990s, represented the entire N-terminus (residues 2-26, termed Ac2-26), and conserved the anti-migratory effects of the full-length protein (Cirino *et al.*, 1993). It is important to note that although Ac2-26 is functional it shows considerable reduction in potency with ~200 fold reduction by molar ratio compared to the full length parent protein (Perretti *et al.*, 1993b).

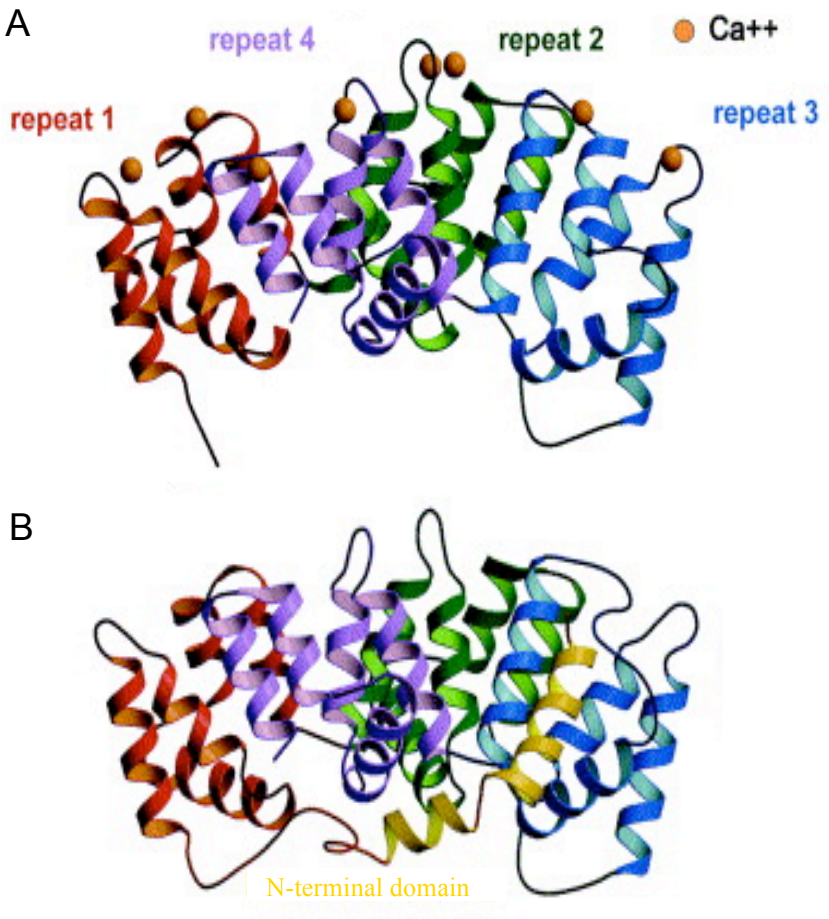


Figure 1.6. Ribbon diagram of full-length human annexin A1. AnxA1 protein structure consists of 4 conserved repeats and a N-terminal domain. (A) in the presence of Ca^{2+} (orange) the protein externalises the N-terminal domain allowing functional binding to occur. (B) If no Ca^{2+} is absent the N-terminal (yellow) is folded within repeat 3 to regulate its activity. Adapted from (Rosengarth *et al.*, 2003).

The AnxA1 promoter activity is strongly regulated by endogenous and exogenous GC (Wallner *et al.*, 1986) it is clear that a multitude of other transcription factors, including AP-1 and GATA, induced by inflammation play a significant regulatory role. The acute phase cytokine, IL-6 (Solito *et al.*, 1998), thought to play a key role in linking innate and adaptive immunity, is a notable addition to the regulation of AnxA1 and a strong tie to its involvement in acute inflammation.

AnxA1 is presented in high abundance in granulocytes and particularly neutrophils, where the protein is packaged within gelatinase granules (Perretti *et al.*, 2000). AnxA1 is also an abundant cytoplasmic protein in monocytes, Mϕ, lymphocytes, vascular endothelial cells and synoviocytes. Indeed its widespread distribution across immune cells and tissues suggest links with multiple functions and pathologies. Within the context of the spontaneously resolving model of inflammation discussed within this thesis I will focus on the biological roles of AnxA1 within the innate immune system.

AnxA1 constitutes between 2-4% of total cytosolic protein within human neutrophils (Ernst *et al.*, 1990) and is therefore integral to their overall functional biology. Notably 50-70% of total AnxA1 can be externalised onto the outer leaflet of neutrophil membrane upon adhesion to an endothelial cell monolayer (Perretti, 1997). The presence of AnxA1 on the neutrophil surface instigates deactivation pathways including, detachment from vascular endothelium, apoptosis, phagocytosis and ultimately improved resolution (Perretti *et al.*, 2009). Interestingly the surface of an activated neutrophil is a hostile environment for AnxA1, readily degraded by proteinase 3 (PR3) and elastase, limiting its activity (Vong *et al.*, 2007). These processes can therefore undermine the activity of AnxA1 during active inflammatory with leukocytes preventing binding of AnxA1 to the cell surface (Perretti *et al.*, 1993e).

Several studies have linked the inhibition of inflammatory mediators by GC with AnxA1-dependent pathways in both monocyte and Mϕ cells (Kamal *et al.*,

2005). Intriguingly the maturation of peripheral monocytes cells to tissue Mϕ increases GC-sensitivity and AnxA1 synthesis (Ambrose *et al.*, 1992). The role of membrane-bound AnxA1 in Mϕ has largely been studied in the context of PMN phagocytosis. Externalisation of AnxA1 plays an important role in the recognition of apoptotic bodies, binding PS to cross-link cell membranes and facilitate engulfment (Fan *et al.*, 2004).

In acute and chronic inflammatory pathologies within rodent and man, the anti-inflammatory actions of AnxA1 have been well established, by our laboratory and others. However AnxA1 has many other actions both intra- and extracellular in nature. The first study to associate AnxA1 peptides with a specific receptor is now a seminal work within the field with Gerke's group identifing FPR1 as a putative receptor (Walther *et al.*, 2000). Following this discovery it was soon revealed that AnxA1 was co-localised with FPR2/ALX on the surface of activated human neutrophils (Perretti *et al.*, 2002). Furthermore competitive binding studies using AnxA1 and its peptide derivatives together with LXA_4 (Hayhoe *et al.*, 2006) as well as functional activity measured by calcium flux using transfected cell lines (Babbin *et al.*, 2006) underlined the preference of full-length AnxA1 for FPR2/ALX over FPR1. To ascertain whether these binding properties were equally true in mice our group investigated AnxA1 and Ac2-26 in ischemic models of leukocyte recruitment in transgenic $Fpr1^{-/-}$ mice (Gavins *et al.*, 2005). It was apparent from this study that AnxA1 retained significant efficacy suggesting that although Fpr1 was capable of mediating some pharmacology it was not the prominent Fpr receptor in mouse. To address this question a further

transgenic strategy was implemented (Section 1.6) forming the basis of this thesis.

Despite the specific associations of AnxA1 with the FPR2/ALX receptor being relatively recent many of its signalling characteristics have already been identified. AnxA1 and Ac2-26 are both capable of inducing transient changes in intracellular Ca^{2+} and L-selectin shedding by neutrophils (Solito *et al.*, 2003). Both are predominantly associated with a MAPK signalling cascade inducing rapid phosphoryation of ERK1/2 in a dose dependent manner (Hayhoe *et al.*, 2006). AnxA1 has also been shown to induce phosphorylation of Akt during ligation to T lymphocytes (D'Acquisto *et al.*, 2007). Indeed it has been shown that AnxA1 augments T-cell receptor (TCR) signalling initiating an effector phenotype suggesting that distinct signalling pathways may be cell-specific.

1.5.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

The NSAIDs are among the most popular therapeutics used with over 50 NSAIDs on the global market since their discovery in 19th Century. Aspirin, acetylsalicylic acid, is arguably the best known of the NSAIDs, and therefore I will describe some of the generic characterises of this drug. Notably NSAIDs share three common actions, namely anti-inflammatory, analgesic and antipyretic effects.

Aspirin was introduced as an anti-inflammatory medication in 1899 by Bayer, however it was not until the 1970's that the mechanisms of action was elucidated by J R Vane's laboratory (Vane, 2000). The role of PG (Section 1.2.1.1) and thromboxane A_2 (TXA_2) production by the cyclooxygenase (COX) pathway is now well established within inflammation. The potent anti-inflammatory actions of aspirin are attributed to non-selective inhibition of the COX pathway, also resulting in gastrointestinal toxicity, a common trait of NSAIDs. It is now clear that there are three isoforms of COX enzyme, constitutively expressed COX-1, inducible COX-2, and centrally distributed COX-3, a splice variant of COX-1. The anti-inflammatory role of aspirin is mediated through inhibition of COX-2, but non-specific blockade also diminishes the gastroprotective and platelet aggregation functions of eicosinoids derived from COX-1. To address this issue of toxicity by blockade of COX-1, selective COX-2 inhibitors were developed, however controversially adverse cardiovascular events has lead to withdrawal of many of the COX-2 inhibitors from the global market (Rao *et al.*, 2008).

Interesting some NSAIDs, such as paracetamol, do not share the anti-inflammatory actions within the periphery are though to be centrally active, although the mechanisms are still unclear. NSAIDs are often classed as mild analgesics, are thought to be as effective in reducing inflammatory pain, particularly postoperative pain in comparison to opioid-based therapeutics. Their action is again reliant on inhibition of PG synthesis to reduce the hyperalgesic effects of PG, which lower the activation threshold of C fibers.

Finally NSAIDs act to reduce body temperature during fever. Elevated systemic PGE_2 triggers an increase in cAMP in the hypothalamus to elevate body temperature. Systemic fever is often the result of increased cytokine production (e.g IL-1), which in turn induces PG synthesis.

Aspirin's action is mediated by the irreversible blockade of COX-1 and COX2. Interestingly to complement inhibition of pro-inflammatory mediators, aspirin-acetylated COX-2 catalyses the production of anti-inflammatory 15-epimeric lipoxins (Fiorucci *et al.*, 2003).

1.5.2.1 Aspirin-triggered lipoxins (ATLs)

Lipoxins, a condensed term for lipoxygenase (LO) interaction products (Serhan *et al.*, 1984), are novel eicosanoids generated from arachidonic acid (AA). The most notable of the native lipoxins are LXA_4, LXB_4 and their epitopes, which consist of a unique trihydroxytetraene structure. There are two endogenous pathways of biosynthesis via 5-, 12- and 15-LO as well as aspirin-acetylated COX-2 (Figure 1.7).

The "classic" pathways involves two-stage metabolism of AA following the interaction of leukocytes with platelets or epithelial cells. Leukocytes express 5-LO capable of converting AA to an epoxide, leukotriene A_4 (LTA_4). Although platelets are unable to produce lipoxins alone, they express 12-LO, which can metabolise LTA_4 to either LXA_4 or its structural isomer LXB_4. A second pathway involves 15-LO within epithelial cells which oxygenates AA to 15*S*-

hydroxyleicosatetraenoic acid (15*S*-H(p)ETE), this metabolite is readily taken up by leukocytes and converted by 5-LO to active lipoxins (Chiang *et al.*, 2006). Thirdly, as alluded to in the previous section aspirin acetylates COX-2 resulting in the production of 15-epi-lipoxins by the metabolism of 15*R*-HETE by 5-LO. Despite both ATLs being produced as stereo-isomers they retain many of the characteristic of the endogenous epitopes inducing vasorelaxation, prostacyclin synthesis and nictric oxide synthesis (Morris *et al.*, 2006). Recent studies have also noted that GC can promote 5-LO activity in circulating leukocytes suggesting an intriguing common anti-inflammatory pathway shared by both GC and aspirin (Hashimoto *et al.*, 2007).

As previously mentioned, LXA_4 was the first endogenous FPR2/ALX ligand to be identified (Section 1.4.2), and has sometimes been refered to as the lipoxin A_4 receptor (ALX; (Fiore *et al.*, 1994). Superficially the actions of LXA_4 have been shown to parallel the anti-inflammatory properties of AnxA1, mediating reduced PMN activity (Pouliot *et al.*, 2000), promoting detachment of adherent leukocytes from mesenteric microcirculation (Gavins *et al.*, 2003) pro-apoptotic and pro-phagocytic (Mitchell *et al.*, 2002). The most notable divergence is observed within the specific pharmacology of LXA_4. Firstly LXA_4 interacts with a distinct binding domain different to that of peptide ligands (Chiang *et al.*, 2006; Le *et al.*, 2005), furthermore unlike other ligands it has been shown to impair MAPK signalling (El Kebir *et al.*, 2009). Secondly there is some controversy over the ability of LXA_4 to induce Ca^{2+} mobilisation, with Ca^{2+} flux shown in a monocytic cell line (Romano *et al.*, 1996) but not apparent in neutrophils. Finally LXA_4 acts as a potent chemoattractant for

human monocytes (Maddox *et al.*, 1996). Importantly FPR2/ALX is not alone in mediating the actions of $LXA_{4,}$, which also binds the nuclear aryl hydrocarbon receptor (AhR) (Schaldach *et al.*, 1999). However the role of this receptor pathway has currently not been delineated.

Lipoxins are unstable, rapidly inactivated by dehydrogenation, therefore their evanescent nature make exogenous lipoxins a complicated therapeutic concept.

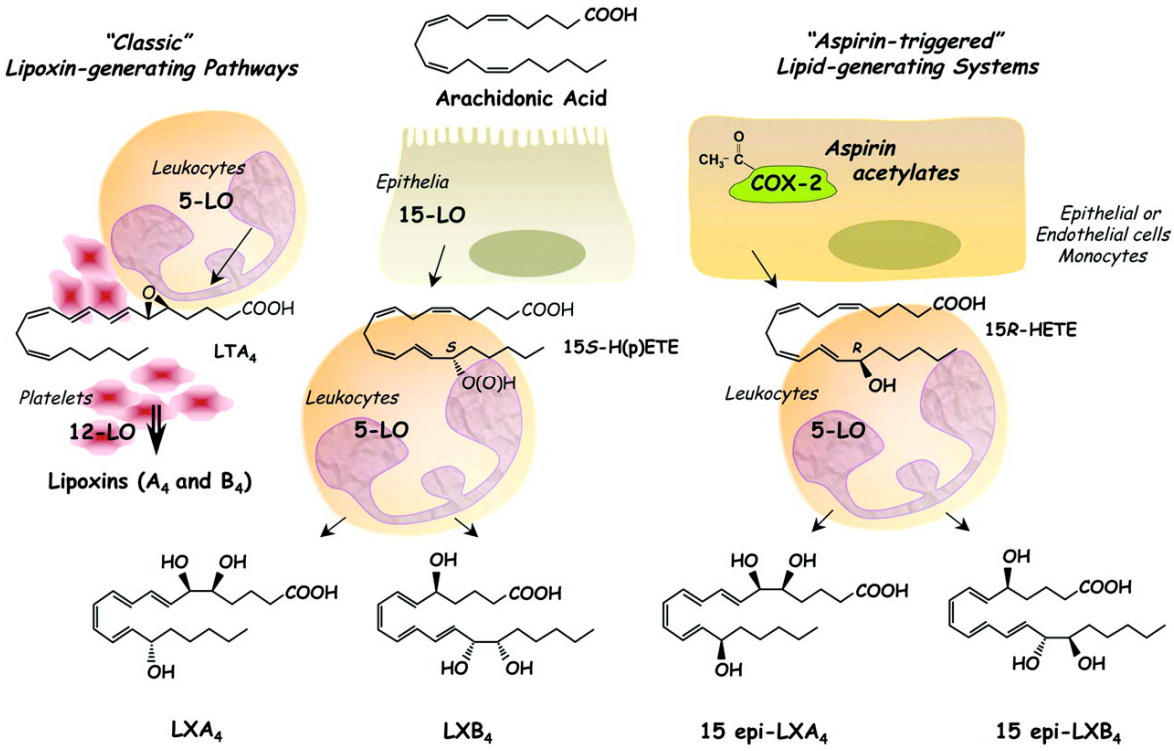


Figure 1.7. Schematic of lipoxin generation and structures. In humans lipoxins can be induced using lipoxygenase (LO) activity, either in a classical or inducible fashion such as up-regulation and acetylation of COX2 by aspirin. (Chiang *et al.*, 2006)

1.5.3 Statins

Statins, first identified as inhibitors of cholesterol biosynthesis in the mid-1970's (Endo *et al.*, 1976), are currently the gold–standard class of therapeutics for combating dyslipidemia. They mediate their effects by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses the conversion of HMG-CoA to mevalonic acid, subsequently modulating low density lipoprotein (LDL) levels to regulate cholesterol deposition.

LDL is one of many lipoproteins which sequesters and transports cholesterol from the liver to muscle and adipose tissue in the periphery. Elevated cholesterogenesis leads to increased circulating LDL-cholesterol complexes strongly associated with cardiovascular risk factors. Furthermore this reduces formation of high-density lipoprotein (HDL)-cholesterol complexes responsible for mobilising peripheral cholesterol back to the liver for clearance. Statins redress this balance by reducing cholesterol biosynthesis and upregulating LDL receptor gene expression to enhance clearance of LDL-cholesterol {Dergunov, 2008 #425}. In 1987 lovastatin became the first statin approved by the food and drug administration (FDA) in the USA it rapidly became a worldwide therapeutic due to prominent efficacy and tolerance (Endo, 1992).

Dyslipidemia can be either primary, via dietary or genetic factors, or secondary, the result of systemic disease pathogenesis such as auto-immune diseases, alcholism, renal and liver failure to name but a few. The complex

pathogensis of this disease suggests a strong role of inflammation, particularly in cardiovascular disease where cholesterol plays a prominent role in plaque formation and ischemic events. Indeed during the pathogenesis of acute tissue injury local cell death generates large quantities of cell membrane fractions rich in cholesterol (Tam *et al.*, 2005).

Statins themselves have been increasingly linked with direct anti-inflammatory actions particularly with respect to atheromatous disease (Rossen, 1997). Of the multiple diagnostic tools currently applied to clinical risk factors acute phase proteins, particularly C-reactive protein (CRP), fibrinogen and serum amyloids, are the most common systemic inflammatory markers. Interestingly although CRP is well characterised as a marker of inflammation it has not been functionally linked to disease pathology, this is in contrast to serum amyloid A (SAA) which is a potent pro-atherogen (Wilson *et al.*, 2008). The deleterious role of SAA and its peptide $A\beta_{42}$ have long been associated with Alzheimer's disease (AD) however its pro-inflammatory pharmacology, and specificity for FPR2/ALX, is a recent discovery (Su *et al.*, 1999). The link between cholesterol biosyntheisis, inflammation and serum amyloid A has only come to light in the last couple of years with lovastatin shown to significantly the reduce SAA activity, but not CRP, and therefore inhibit amyloidosis and plaque formation in atherosclerosis (van der Hilst *et al.*, 2008).

1.5.3.1 Serum amyloid A and amyloidosis

Serum amyloid A is a highly inducible component of the acute phase response particularly prevalent in chronic diseases, notably it is currently used as a disease marker for clinical diagnosis and prognosis in Crohn's disease, atherosclerosis and rheumatoid arthritis (Cunnane *et al.*, 2000). In common with other acute phase mediators SAA is secreted in large quantities from the liver during inflammation, with serum levels increasing up to 1000-fold within hours of an inflammatory stimulus (Kushner, 1982). Notably SAA is unique among the acute phase proteins as it is also generated at local sites of inflammatory insult (Vreugdenhil *et al.*, 1999).

There are currently three members of the human SAA gene family located on chromosome 11 (11p.15.1), comprising *SAA1, SAA2* and *SAA4*, as well as the pseudogene *SAA3*. SAA1 and SAA2 have a major part in the acute phase response, capable of amyloid formation, whereas SAA4 is constitutively expressed in a variety of tissues (Sellar *et al.*, 1994); all three are amphipathic proteins. Gene transcription of both acute phase proteins is markedly up-regulated by pro-inflammatory mediators including IL-1, IL-6 and TNF-α (Westermark *et al.*, 2009), a response that can be significantly enhanced in the presence of GC (Jensen *et al.*, 1998). The multiple actions of SAA1 and SAA2 are further complicated by the identification of numerous isoforms (Raynes *et al.*, 1991) further more their exact structure has remained elusive as they are insoluble at neutral pH (Munishkina *et al.*, 2007).

SAA is primarily associated with the amyloidosis, involving the deposition of amyloid peptides to form fibrils particularly within mononuclear cells (Munishkina *et al.*, 2007). The mechanisms that underlie the formation of amyloid plaques are currently poorly understood. During non-inflamed conditions SAA is predominantly (~90%) bound to HDL (Benditt *et al.*, 1977) however it dissociates at high concentration. The SAA/HDL complex has a significant influence on lecithin cholesterol acyltransferase (LCAT) activity to induce reverse cholesterol transport from the site of inflammation utilising high affinity macrophage scavenger receptors (Lindhorst *et al.*, 1997). These observations have lead to interest in delineating the link between chronic degenerative diseases, such as Alzheimer's or RA, and secondary amyloidosis in the formation of atherosclerotic plaques and vice versa (Schulz *et al.*, 2007). Sequestrating acute phase SAA by elevating HDL, notably in the case of statins, maybe a useful anti-inflammatory strategy.

In vitro studies have shown that unbound SAA can increase the production of pro-inflammatory cytokines such as TNFα, IL-8, IL-1β (He *et al.*, 2003) and G-CSF (He *et al.*, 2008). SAA and its metabolite amyloid β_{42} are potent chemotractants *in vitro* using transfected HEK293/ALX (Le *et al.*, 2001a), human PMN (Liang *et al.*, 2000) and murine microglial cells (Cui *et al.*, 2002b). Functional roles of SAA *In vivo* are less well characterised, despite its elevated levels it retains its chemokinetic effects, as well as binding and opsonization of gram-negative bacteria, by recognition of outer membrane protein A (OmpA) (Shah *et al.*, 2006). This could represent a pathogen-

associated molecule pattern (PAMP) recognition pathway that strengthens the parallels between the FPR family and TLRs.

To further complicate its roles within inflammation SAA has been associated with multiple receptors including FPR2/ALX (Liang *et al.*, 2000), CD36/LIMPII Analogous-1 (CLA-1; Baranova *et al.*, 2005), TLR2 (Cheng *et al.*, 2008) and TLR4 (Sandri *et al.*, 2008). Each receptor has been shown to mediate distinct pharmacological actions of SAA however the inter-relationship between these responses are undetermined in a physiological environment.

SAA signalling attributed to FPR2/ALX was first noted to mediate a chemotactic response in human phagocytes (Su 1999). Unlike the other two endogenous ligands, SAA also induces respiratory burst (Bjorkman *et al.*, 2008), inflammatory cytokine production (He *et al.*, 2003) and is a pro-survival factor (Christenson *et al.*, 2008). The association with Alzhemier's disease is also prominent with parallel functions of SAA derivative βA_{42} peptide on microglial acting through FPR2/ALX (Cui *et al.*, 2002b) (Figure 1.8).

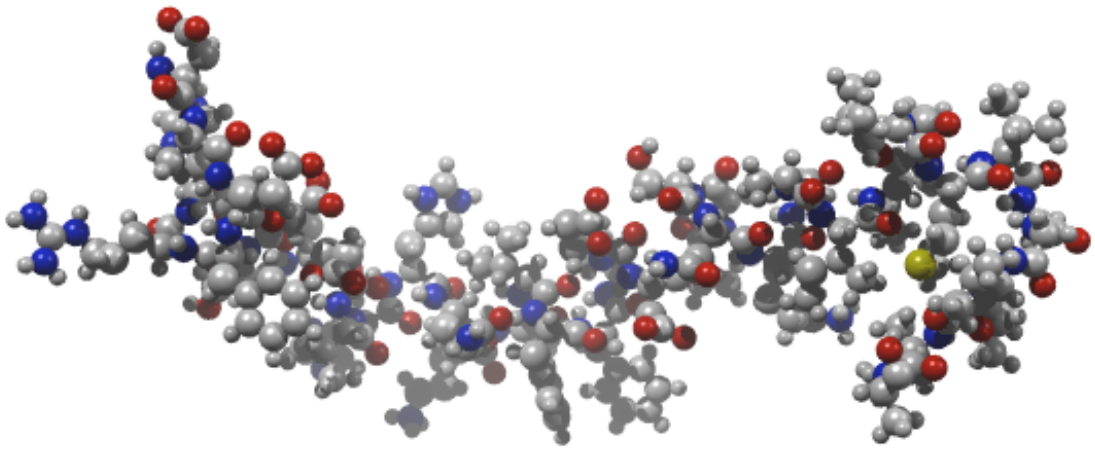


Figure 1.8 3-D Structure of beta-amyloid 1-42. The starting point (N-terminal) is at the left. Change in conformation from α-helical to β form of the peptide is thought to be key in conveying its neurotoxic properties (Tomaselli *et al.*, 2006).

CLA-1 (human) or scavenger receptor B-1 (rodent) play an important role in lipid metabolism, and is widely expressed in liver, adrenal gland, monocytes and macrophages (Rigotti *et al.*, 1995). Both receptors show high affinity for HDL and are strongly expressed on mononuclear cells, hepatocytes, adrenal glands and atherosclerotic legions. These receptors recognise the presence of common amphipathic helices apparent on many apolipoproteins and also found on unbound SAA, to induce MAPK signalling (Baranova *et al.*, 2005).

Within the last two years research by two groups have suggested SAA can mediate some of its pro-inflammatory pharmacology via either TLR2 or TLR4. The use of anti-TLR2 Ab and *tlr2*$^{-/-}$ mice have shown reduced ability of SAA to induce cytokine production, MAPK activation and NF-κB activity *in vitro* (Cheng *et al.*, 2008). Furthermore *in vivo tlr2*$^{-/-}$ mice were shown to have a

significantly reduced neutrophil infiltration and G-CSF production when stimulated with exogenous SAA (He *et al.*, 2008). A slightly less convincing study using *tlr4*$^{-/-}$ mice suggested a TLR4 dependent pathway induced NO release by macrophages following SAA stimulation (Sandri *et al.*, 2008).

Taken together, it can be summarised that SAA plays a wide variety of roles via multiple receptors to convey its role in cholesterol transportation (Banka *et al.*, 1995), bacterial recognition (potential via protein-protein interaction and receptor signalling) and cytokine-like pro-inflammatory signalling via FPR2/ALX.

1.6 Transgenic animals

The accessibility of transgenic technology has revolutionised the entire field of pharmacology providing invaluable insight and almost boundless detail by exploiting gene manipulation on a physiological scale. Our laboratory is an eager exponent of this technique both importing and generating transgenic colonies to investigate multiple biological functions.

There have been a number of transgenic mice directly relevant the determination of AnxA1 biology. The first was the development of the Fpr1$^{-/-}$ mouse (Gao *et al.*, 1999), which was subsequently identified as the putative AnxA1 receptor. The efficacy of exogenous AnxA1 was significantly reduced in Fpr1$^{-/-}$ mice but not completely abolished (Perretti *et al.*, 2001b). In parallel with these studies we successfully generated a novel AnxA1$^{-/-}$ mouse colony,

uncovering a distinctive phenotype with AnxA1$^{-/-}$ mice exhibiting exaggerated inflammatory response and resistance to the effects of GC, suggesting AnxA1 is an important 'secondary messenger' (Roviezzo *et al.*, 2002). This transgenic has been an invaluable to tool revealing a multitude of cellular functions orchestrated via AnxA1 (D'Acquisto *et al.*, 2008). This novel transgenic was imbued with the reporter technology, a powerful technique to specifically label the silenced gene and therefore monitor specific promoter activity (Maggi *et al.*, 2005). Robert Hannon incorporated β-galactosidase (*LacZ*) to monitor gene expression in a wide variety of tissues (Hannon *et al.*, 2003). The implication of that Fpr1 was not the specific AnxA1 receptor in human and mouse assays lead to Robert Hannon to embark on a second transgenic assessed in this thesis, the Fpr2$^{-/-}$ colony. To distinguish promoter activity a second, green fluorescent protein (GFP), reporter strategy was chosen in line with previous studies (Chiocchetti *et al.*, 1997).

The Fpr2$^{-/-}$ colony is unique, however other attempts of over-expressing human FPR2/ALX or silencing Fpr2 in mice have been reported. Serhan's group selectively expressed human FPR2/ALX in a myeloid-specific manner. This study revealed that FPR2/ALX could orchestrate neutrophil recruitment and diminish eicosanoid production following inflammatory stimuli (Devchand *et al.*, 2003). Unconventionally, the Fpr2 gene has been reported to be silenced in a study conducted with the FPR3 agonist F2L (Gao *et al.*, 2007). The paper however did not contain details or validation of the molecular approach used and no such study has since been published.

The culmination of these experiments will hopefully provide key input in delineating the roles of both agonists and receptors in GPCR pharmacology. Intriguingly the most powerful tool maybe a combination of two or more transgenic animals.

1.7 Scope of the thesis

This PhD project has endeavoured to characterise some of the complex pharmacological roles of murine Fpr2. Over the last decade an intriguing novel field of 'ligand-biased' pharmacology has emerged around the FPR family, which recognise structurally disperate ligands spanning protein, peptides, lipids and synthetic molecules. This project utilised both known human pharmacological tools and a novel transgenic mouse nullified for Fpr2. This approach was derived to both validate the anti-inflammatory roles previously attributed to Fpr2 *in vitro* and *in vivo* as well as to make distinct comparisons between the functional similarities of murine Fpr2 its human orthologue FPR2/ALX.

1.7.1 Aims

1. Confirm the generation of a functional transgenic $Fpr2^{-/-}$ mouse colony.
2. Validate and assess the distribution of *fpr2* promoter activity by monitoring a target/reporter construct.
3. Elucidate phenotypic difference between wild type and $Fpr2^{-/-}$ mice within naïve and inflammatory environments *in vivo*.
4. Investigate the role of Fpr2 pathways in mediating the pharmacology of putative human agonists in murine cells *ex vivo* and *in vivo.*
5. Validate the potential of endogenous Fpr2 ligands for therapeutic intervention during a variety of inflammatory models.

1.7.2 Hypothesis

My hypothesis is that the human FPR2/ALX is the receptor that transduces the biological effects of ANXA1. The murine Fpr2 receptor is a direct orthologue of the human FPR2/ALX receptor allowing comparable analysis of human ligands under physiological conditions. The experiments described in this thesis are designed to test this hypothesis.

Chapter 2.

Materials and Methods

In Vitro Protocols

2.1 Chemicals and reagents

LPS serotype *E.coli* 0111:B4, fMLF, Zymosan A, dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, Poole, UK, Dorset, UK); recombinant human apo-SAA, murine IL-1β (PeproTech, Rocky Hill, NJ), Lipoxin A_4 (LXA_4) (Calbiochem, San Diego, CA), W-Peptide and peptide Ac2-26 (acetyl-AMVSEFLKQAWIENEEQEYVVQTVK; Mr 3050) were synthesised by Cambridge Bioscience (Cambridge, UK); F1, B11 and GB4 primers (Thermo Electron, Waltham, MA); rat IgG2a isotype control PE, anti-mouse Ly6G (GR-1) PE, anti-mouse CD2 (LFA-2) PE, anti-mouse F4/80 PE-Cy5 (eBioscience, San Diego, CA); phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) antibody, ezrin/radixin/moesin antibody (Cell Signalling Technology, Danvers, MA).

Human recombinant annexin A1 (hrAnxA1) was produced by purifying GST-tagged protein by sepharose column purification using GSTrap (GE Healthcare, Little Chalfont, UK, Little Chalfont, UK).

Compound 43 (C43) was a generous gift from Amgen (Thousands Oaks, CA).

2.2 Generation of murine Fpr2$^{-/-}$ colony

The transgenic Fpr2$^{-/-}$ animals were generated by Dr. Robert Hannon in a similar manner to the previously generated AnxA1 null mice (Hannon *et al.*, 2003) using homologous recombination in embryonic stem (ES) cells with a dual-purpose targeting/reporter vector. Genomic clones containing Fpr sequences were isolated from a bacteriophage lambda library (129/SvJ; Stratagene, La Jolla, CA) by plaque hybridisation. Inserts from positive plaques were subcloned into pZero (Invitrogen, San Diego, CA), end-sequenced and then aligned with the FP locus on Chromosome 17. A pgk-neo cassette was inserted into one of these clones (p2.1) just downstream of the ATG start codon for Fpr2, using the technique of site-specific recombination in bacteria. The sequences of the primers used to achieve this step are forward 5' tcagaaggagccaaatatctgagaaatggttgtttttgaaaactttcaggtgcagacaaaATGgctagccc ttctgcttaatttgtgcctgg and reverse 5' tgctgtgaaagaaaagtcagccaatgctagattcagataccagatagtggtgacagtgtgtggcgtagagg atctgctcatgtttgac. Using the plasmid pPGK-neo-FRT as a template for PCR, these primers amplify a fragment of 2.2 kb containing the pgk-neo cassette in reverse orientation, flanked by 63 bp arms showing homology to Fpr2. This fragment was electroporated along with plasmid p2.1 into E.coli strain HS996 using the RED-ET subcloning kit (Gene Bridges, Dresden, DE). The novel Nhe I site (gctagc) located immediately after the ATG start codon in the forward primer was used for the subsequent in-frame insertion of GFP

(Qbiogene, California, USA) and also to facilitate Southern blot screening. All steps were confirmed by sequencing.

The targeting vector was linearised by digestion with SnaBI and electroporated into ES cells (strain 129SvEv). Neomycin resistant colonies were picked and screened for correct insertion by Southern blotting using probes located beyond both the 5' and 3' ends of the vector arms, and also a probe for GFP. Clones showing homologous recombination into the Fpr2 locus were expanded, karyotyped by G-banding, and then injected into the blastocysts of C57/Bl6 females (Caliper Life Sciences, Cambridge, MS).

Male chimaeras showing greater than 95% agouti coat colour were paired with C57Bl6 females. F1 offspring were screened by PCR of tail clip DNA for germline transmission of the targeted allele using the Extract-N-Amp system (Sigma-Aldrich, Poole, UK). The primers used for genotyping are F1 (tgagtgtcatgtcagaaggagcc), B11 (cggaatccagctacccaaatc) and GB4 (ataaccttcgggcatggcactc). The F1/B11 pair produces a band of 233 bp from the wild-type allele, whereas F1 and GB4 produce a band of 351bp if the targeted allele is present. Cycling conditions were 92°C for 30s/54°C for 15 s/ 72°C for 15 s x 33 cycles. Heterozygotes were mated together to produce F2 homozygotes. Genotyping was performed by PCR and confirmed by Southern blotting.

2.3 Detection of 'in frame' GFP construct within target/report vector

Flow cytometry analysis was used to assess the fluorescent properties of the GFP insertion within $Fpr2^{-/-}$ animals. The GFP construct, introduced in section 2.2, was specifically targeted 'in frame' within the promoter region of fpr2 (Figure 2.1 A). This strategy was developed to assess constitutive and induced promoter activity by FACS (Section. 2.19). This methodology was capable of confirming the genotype further validating successful germline transmission.

The consistent but subtle modulation of this fluorescent signal was further amplified by using GFP specific antibodies. Detection of conjugated GFP was only apparent in $Fpr2^{-/-}$ cells following permeabilisation of the cell membrane with saponin to allow intracellular staining (Figure 2.1 B).

Cells were resuspended in round-bottom 96-well plate with 100μl PBS containing 0.2% bovine serum albumin (BSA) and 1.3mM $CaCl_2$ (PBC). Cells were washed (400*g*, 30s) before non-specific binding was blocked with 16mg/ml human immunoglobulin G (IgG) for 5 min at 4°C. Cells were washed prior to fixation with 4% paraformyladehyde (PFA) (10 min; 4°C). The GFP construct was not expressed on the cell surface, therefore PBC containing 0.1% saponin was used to permeabilise the cell membrane and allow intracellular-GFP to be targeted with a goat-anti-GFP antibody (1:200 dilution; 30 min at 4°C; Serotec, Oxford, UK). Anti-GFP binding was subsequently

detected with a conjugated chicken-anti-goat Alexa 488 (1:200; 20 min at 4°C; eBioscience, San Diego, CA). Cells were washed with PBC/0.1% saponin between each staining step following fixation. Finally cells were washed twice in PBC/0.1% saponin before being resuspended in 200μl PBC for analysis.

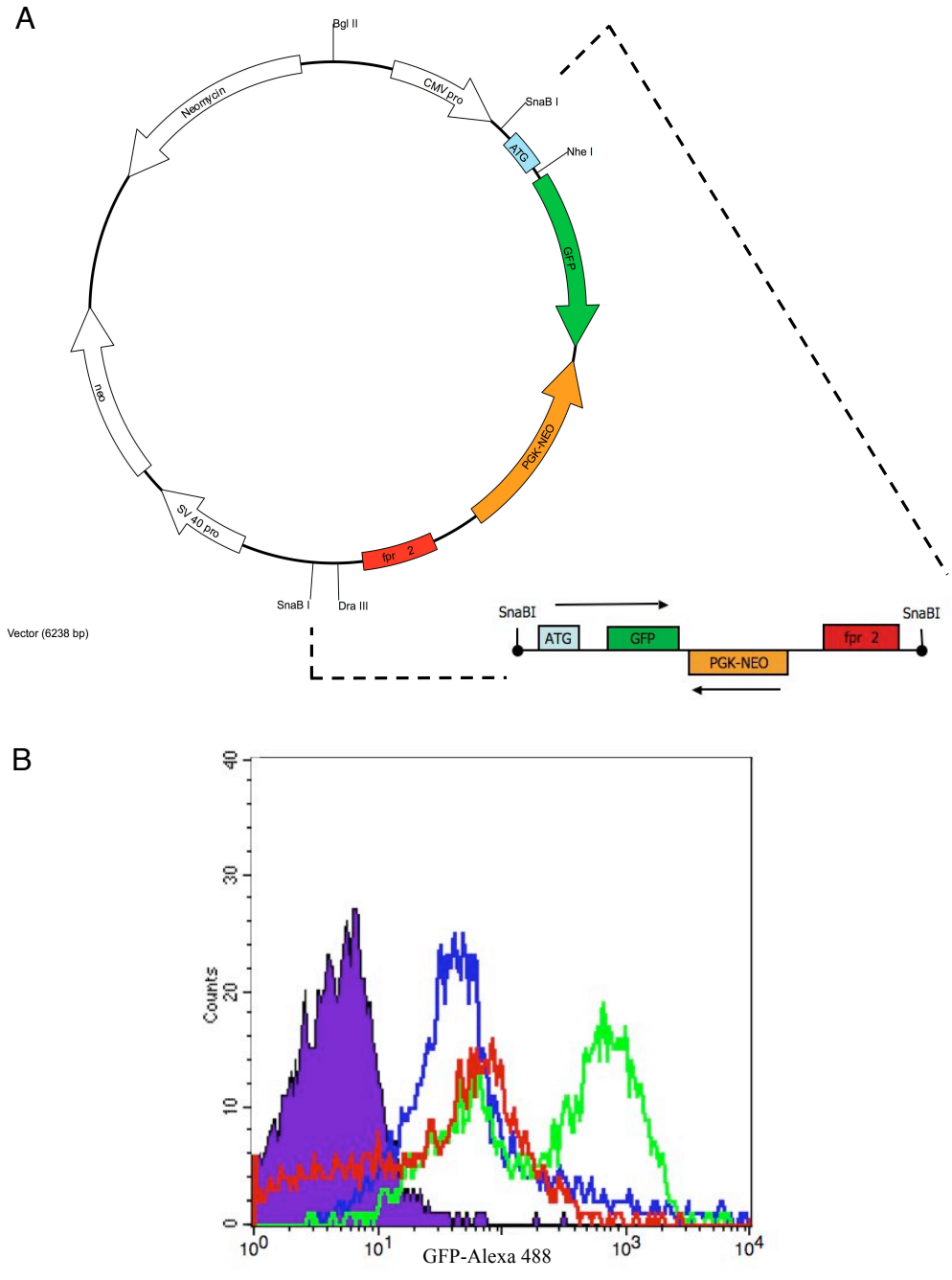


Figure 2.1. Schematic of GFP insertion strategy. (A) The development of a dual-purpose targeting vector by inserting a gene silencing sequence into a pQBI 25 vector. The vector was linearised by digestion with SnBI so it could be inserted into mouse embryonic stem cells for cloning. (B) GFP signal was enhanced by using a conjugated anti-GFP antibody. The histogram is representative of 3 experiments comparing non-specific binding of the anti-GFP antibody with specific intracellular staining in $Fpr2^{-/-}$ cells (Green), intracellular staining with an Isotype control (Purple), WT (intracellular; Blue) and $Fpr2^{-/-}$ (cell surface; Red).

2.4 Genotyping: Polymerase-chain reaction (PCR)

F1 offspring were screened by PCR of tail clip DNA for germline transmission of the targeted allele using the Extract-N-Amp system (Sigma-Aldrich, Poole, UK). To prepare genomic DNA Extraction Solution was mixed in a ratio of 4:1 with Tissue Preparation Solution and a 100µl was added to each tail clip. Samples were incubated at 37^0C for 15 min, 94^0C for 3 min and finally on ice for 3 min.

The primers used for genotyping were F1 (tgagtgtcatgtcagaaggagcc), B11 (cggaatccagctacccaaatc) and GB4 (ataaccttcgggcatggcactc) were stored at 100 µM (-20^0C), these primers were diluted for a 10µM working concentration. A master mix was made up containing 10µl Reddy mix™ (Sigma-Aldrich, Poole, UK), 3µl of F1/B11 primer pair, 3µl of F1/GB4 primer pair. 4µl of each sample was added to 16 µl of the master mix a placed in a Primus 96 plus™ thermocycler (Aviso, Salisbury, UK); cycling conditions were, 92^{o}C for 30s (denaturation); 54^{o}C for 15s (annealing); 72^{o}C for 15 s (extension), x 33 cycles.

High melting point agarose (Invitrogen, San Diego, CA, San Diego, CA) was integrated into boiling TAE buffer (40mM Tris base and 1mM EDTA; Sigma-Aldrich, Poole, UK). As the solution cooled, ethidium bromide (Invitrogen, San Diego, CA) was added (2µl per 100ml agrose gel). 10µl of each sample was separated by electrophoresis and calibrated with molecular weight markers (New England Biolabs, Ipswich, MA). DNA was visualised by exposure to ultra

violet light. The F1/B11 pair produced a band of 233 bp for the wild-type allele, whereas F1/GB4 produce a band of 351 bp when the targeted allele is present. Heterozygotes were mated together to produce F2 homozygotes.

2.5 Reverse-transcprition polymerase-chain reaction (RT-PCR)

Quantitative real-time PCR assays were performed as described previously in Sawmynaden et al. 2006. Inflammatory cell pellets were washed and resuspended in Tri-reagent (Ambion, Austin, TX), following manufacturers instructions. Total RNA was used to synthesis cDNA with dNTP mix RetroScript, Oligo (dT) primer and SuperScript III reverse transcriptase (Invitrogen, San Diego, CA). *fpr1/anxa1* Quantitech Primers (Qiagen, West Sussex, UK) in Power SYBR green PCR mix (Applied Biosystems, Foster City, CA) were detected by an ABI prism 7900 real time PCR system (Applied Biosystems, Foster City, CA). Expression was normalised compared to corresponding GAPDH mRNA expression.

2.6 Human recombinant Annexin-A1

BL21 competent cells were maintained in aliquots at –80^{o}C, upon thawing cells were transfected with GST-tagged (GE Healthcare, Little Chalfont, UK) human recombinant Annexin 1 (hrAnxA1) construct with an ampicillin (AMP) resistant region. Viable cells were allowed to grow on agar/ampicillin plates over night to select positively transfected colonies. Three or four discreet surviving colonies were selected and grown in 5-10ml Lennox L broth base $(LB)_{AMP}$ broth (Invitrogen, San Diego, CA) for 2 h at 37^{o}C in a shaker before 500ml $(LB)_{AMP}$ broth was added for 4-6 h in an incubator. To induce hrAnxA1 fusion protein expression Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, Poole, UK) was added (final concentration 0.1mM) to the flasks and incubated on a shaker for 12-16 h at room temperature. The bacterial broth was centrifuged (1500*g*, 20 min, 4^{o}C). The supernatant was discarded and the cell pellet resuspended in ice-cold lysis buffer. To ensure cells were fully lysed the cell suspensions were sonicated on ice. Finally a detergent, 1% IGEPAL (Sigma-Aldrich, Poole, UK), was added to solublise any remaining cytoplasmic membrane. The samples were mixed thoroughly before being centrifuged (1500*g*, 20 min, 4^{o}C). The supernatants were collected for Sepharose column purification using GSTrap (GE Healthcare, Little Chalfont, UK).

2.7 Primary macrophages (Mϕ)

Bone marrow derived cells were used interchangeably with polyacrylamide gel-elicited peritoneal cells with no difference in results. Both preparations were therefore termed primary macrophages Mϕ.

2.7.1 Bone marrow macrophages

WT and Fpr2$^{-/-}$ mice (4–6 wk old) were killed by exposure to carbon dioxide (CO_2). The hind legs were exposed and cleaned with 70% ethanol prior to the extraction of the femur and tibia in a sterile flow cabinet. The bones were washed and cleaned, to remove all attached muscle, before removing the epiphyses. Bone marrow was flushed with 5ml Dulbecco's Modified Eagle Medium with GlutaMAX (37^{o}C; DMEM; Invitrogen, San Diego, CA) through a 25-gauge needle before mechanically disruption of marrow plugs and centrifugation (400*g*; 10 min). Cells were then resuspended, 2x10^{6} cells/ml in DMEM supplemented with L-glutamine, penicillin-streptomycin, 20% FCS, and 30% L929 conditioned medium, and maintained in 100mm x 20mm cell culture dishes (Corning, Schiphol-Rijk, NL) at 37°C.

L929 fibroblasts were cultured as a source of the Macrophage-Colony Stimulating Factor (M-CSF) required to direction bone marrow pre-cursor cells towards a mature macrophage phenotype. L929 fibroblasts were resurrected from FCS-rich, 10% DMSO, aliquots stored in liquid nitrogen. The aliquot was gently warmed and washed twice in 20ml DMEM supplemented with L-

glutamine, penicillin-streptomycin, 10% FCS, to remove DMSO. Finally cells were seeded into T175 cm^2 flasks (Corning, Schiphol-Rijk, NL) containing 50ml media. Once the cells became confluent fresh media (25ml) was added to the flask and fibroblasts were incubated for 2 d releasing M-CSF. The L929 condition media was gently removed and filtered through 0.22μm sterile syringe filters (Corning, Schiphol-Rijk, NL) ready for use or stored at -20^oC.

Fresh culture medium was added on day 3. The differentiation of Mϕ was confirmed by morphology using FACS at day 5 (Figure 2.2).

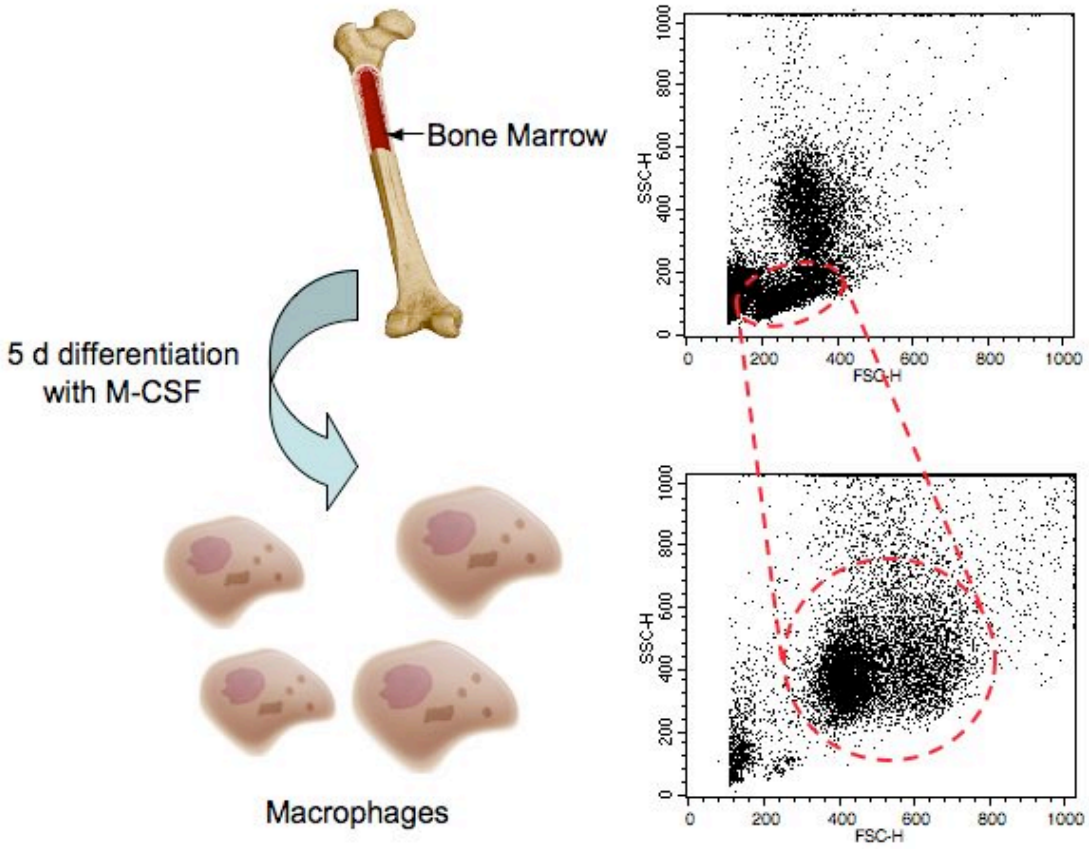


Figure 2.2. Schematic of macrophage differentiation from precursor bone marrow cells. Cells are eluted from the tibia and femur of 4-6 week old mice. Supernatant harvested from L929 fibroblast cells supplies a source of M-CSF enabling bone marrow cells to be differentiated over a 5 day period. A change in cell morphology, assessed by FACS, is notable between day 0 (top) and day 5 (bottom).

2.7.2 Polyacrylamide gel-elicited macrophages

45μm-90μm polyacrylamide gel beads can be used to elicit mature macrophages following injection into the peritoneal cavity. The Bio-Gel beads are too large to be ingested by resident macrophages potentiating the migration and maturation of monocytes within the peritoneum. This produces a homogeneous population of mature macrophages that peaks at 4 d post injection (Davies *et al.*, 2005).

Sterile beads were washed with distilled water by centrifugation at 400*g* for 5 min. Gel beads were reconstituted to a suspension of 2% w/v sterile 0.9% saline. 1ml of the 2% w/v Bio-Gel (Bio-Rad, Hemel Hempstead, UK) was injected i.p. and macrophages were recovered by peritoneal lavage (3ml PBS, 25u heparin, 0.3mM EDTA) 4 d post injection. Cell suspensions were passed through 40μm cell strainers (BD Bioscience, Oxford, UK) before being washed and seeded.

Cells were counted by Neubauer haemocytometer (Section 2.18) and resuspended at $2x10^6$ cells/ml in serum-free DMEM (Invitrogen, San Diego, CA), supplemented with 50μg/ml gentamicin (Sigma-Aldrich, Poole, UK). Macrophages were transferred to 6-well cell culture plates (Corning, Schiphol-Rijk, NL) $2x10^6$ cells/well and allowed adhere for 1 h. Non-adherent cells were removed by washing twice with sterile PBS and fresh media is re-applied.

2.8 Chemotaxis

The commercially available Neuroprobe ChemoTxplate™ 96-well plate (Receptor Technologies Ltd, Leamington Spa, UK) with polycarbonate membrane filters and 5μm membrane pores was utilised as described before (Lim *et al.*, 2000). Mϕ were obtained as above, and resuspended at $4x10^6$ c/ml in Roswell Park Memorial Institute (RPMI; Lonza, Slough, UK) medium containing 0.1% BSA. The chemotaxis assay was performed using known FPR family agonists (fMLF, Ac2-26, SAA, AnxA1) added to the bottom wells as chemotactic stimuli (27μl), the filter was placed on top and 25μl of the Mϕ cell suspension placed above the membrane. Plates were incubated for 180 min in a humidified incubator at 37°C with 5% CO_2.

Cells remaining on top of the filter were removed and the surface was washed. The plate was centrifuged (312*g*, 1 min), the filter removed and cell pellet re-suspended. An aliquot (20μl) was removed and mixed with 30 μl of AlamarBlue (pre-diluted 1:10 in PBS; Serotec, Oxford, UK) in a 96-well plate and incubated (37°C, 5% CO_2) for 4 h (Figure 2.3). A standard curve was constructed using known cell concentrations between 0 - $4x10^6$ Mϕ, 20μl with AlamarBlue. Plates were read at 530-560nm excitation wavelength and 590nm emission wavelength for fluorescence values. Unknown values were converted using the standard curve constructed with known Mϕ numbers.

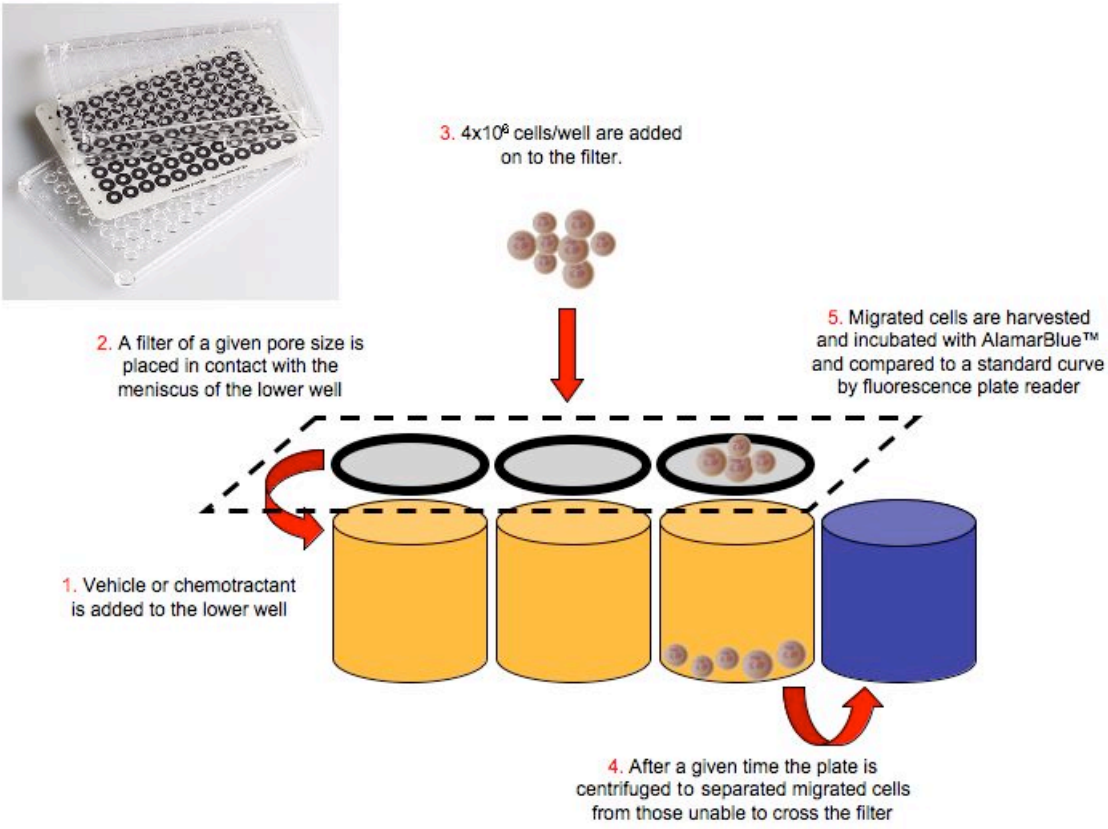


Figure 2.3. Schematic of 96-well plate chemotaxis assay using Neuroprobe chemoTxplate™ 96-well plate (Receptor Technologies Ltd).

2.9 In vitro PMN phagocytosis

Human PMNs were isolated from peripheral venous blood drawn from healthy volunteers, after informed written consent, as previously described (Godson *et al.*, 2000). Briefly, mononuclear cells were separated by centrifugation on Ficoll-Paque (Sigma-Aldrich, Poole, UK) and PMN plated at 2 x 10^6 cells/ml in RPMI 1640 (Lonza, Slough, UK) supplemented with 10% autologous serum, 2mmol/L glutamine, 100U/ml penicillin, and 100µg/ml streptomycin (Invitrogen, San Diego, CA).

Mϕ were treated with the appropriate stimuli as indicated for 15 min at 37°C. The treated cells were washed with RPMI 1640 before co-incubation with apoptotic PMNs (4 x 10^6 PMNs/well) at 37°C for 30 min. Mϕ were exposed to human apoptotic PMNs at 37°C for 30 min. Non-ingested cells were removed by three washes with cold PBS. Phagocytosis was assayed by myeloperoxidase staining (Section. 2.23) of cocultures fixed with 2.5% glutaraldehyde. For each experiment, the number of Mϕ containing one or more PMN in at least five fields (minimum of 400 cells) was expressed as a percentage of the total number of Mϕ and an average between duplicate wells was calculated.

This work was undertaken at both the William Harvey Institute and the Conway Institute, UCD, Dublin where I spent a week learning the technique with Paola Maderna.

2.10 Western blotting by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Both cell pellet-derived and supernatant proteins were analysed using the SDS-PAGE technique. Proteins are negatively charged and denatured before being subjecte to polyacrylamide gel electrophoresis, which separates protein primarily by size with the smallest protein moving the furthest down the gel.

The SDS acts to disrupt to hydrogen bonds and confers a uniform negative charge across the protein. This negates the charge across the proteins in each sample and therefore allows proteins to be separated according to size. DTT is a reducing agent that cleaves disulphide bonds to completely unfold the protein structure. Bromophenol blue is a dye used to identify the protein front as it moves through the polyacrylamide gel.

The density of the polyacrylamide gel can be altered to facilitate the separation of the protein of interest. For the purposes of this my thesis I used 10% polyacrylamide gels to monitor proteins ranging from 17-75kDa.

The gels were cast using a Mighty Small casting cassette (Hoefer Scientific Instruments, Holliston, MA) containing a plastic separator sheet, a ceramic end plate with two 1.5 mm spacers on either side, and a glass plate front. The space between the ceramic end plate and glass front provides the space for the gel to set. Protogel Resolving Buffer (National Diagnostics, Atlanta, GA)

and distilled water mixture is polymerised using ammonium persulfate (APS; Sigma-Aldrich, Poole, UK) and N,N,N',N'-Tetramethyethylene (TEMED; Sigma-Aldrich, Poole, UK) as catalysts, added immediately prior to pouring the gel into the cassette. A gap ~ 2 cm from the top allows space for the stacking gel, and any air bubbles are removed by adding a small amount of iso-propanol, which acts as a surfactant. The resolving gel is given 15 -20 min to polymerise, any excess water/iso-propanol is drained, before the stacking gel (National Diagnostics, Atlanta, GA) is mixed and added on top. To form wells within the stacking gel 10-well combs were placed at the top of the cassette and again the gel was left to set.

Samples were separated for 60-90 min by electrophoresis at 110V and calibrated using a High Range Molecular Rainbow™ (GE Healthcare, Little Chalfont, UK) molecular weight markers. The separate proteins were subsequently transferred from the polyacrylamide gel to an Immobilon-P™ polyvinylidene difluoride (PVDF; Millipore, Watford, UK) membrane at 4^0C for 75 min using 100V (Figure 2.4).

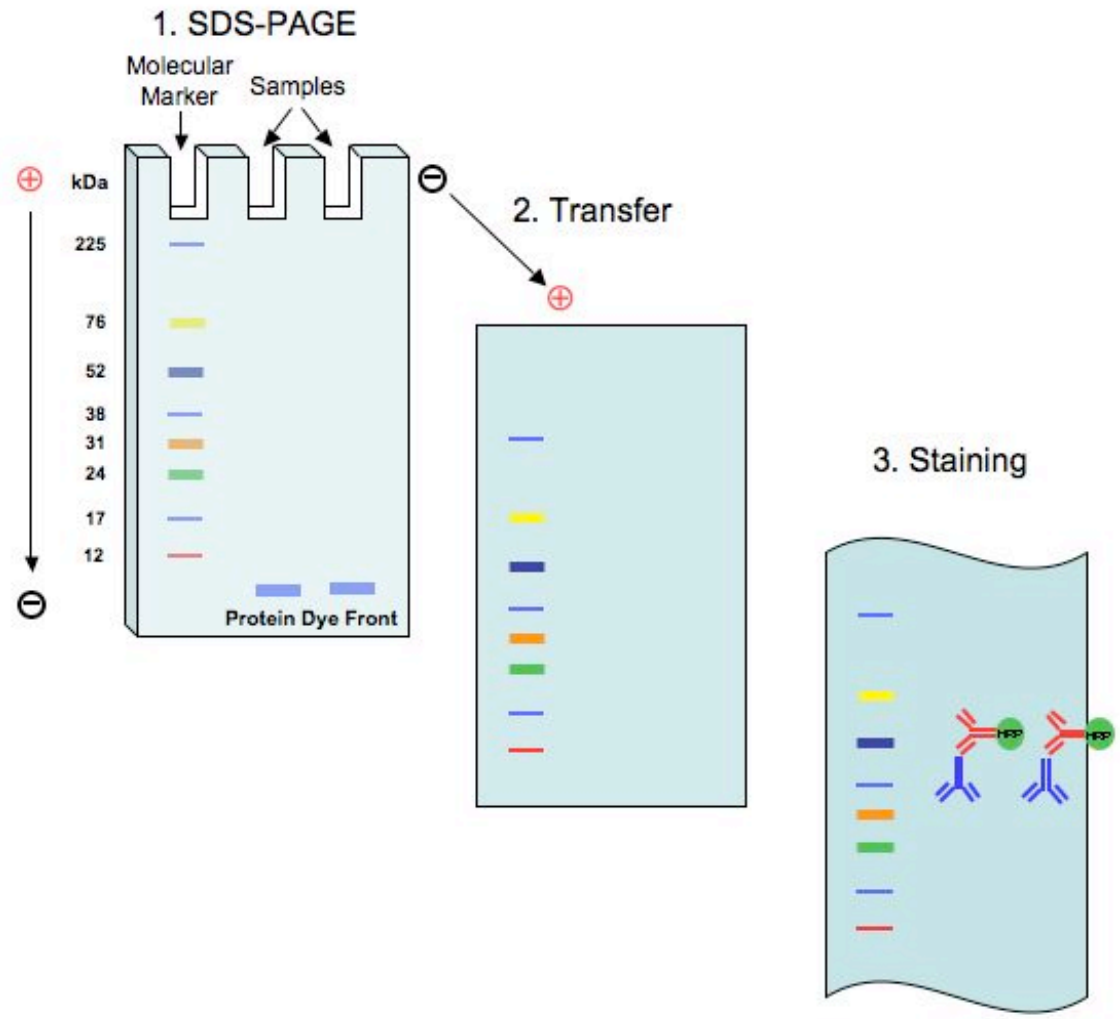


Figure 2.4. A Schematic of the three step technique to probe for specific protein by immunoblot. Negatively charged proteins are separated by electrophoresis through a polyacrylamide gel. These samples are then transferred by to a PVDF membrane before being probed with specific primary antibodies (blue). Secondary HRP-conjugated antibodies (red) can be used to visualise protein by luminescence, which can be detected on photographic film. A molecular weight marker is used to calculate protein siz**e.**

2.11 Protein Assay

Protein content was determined using BCA protein assay (Pierce, Rockford, IL) and calculated by comparison with known concentrations of BSA. This is a colorimetric assay utilising the ability of protein to reduce Cu^{3+} to Cu^{2+}. Bicinchoninic acid reacts with Cu^{2+} to produce a purple colour complex, which is generated in a robust linear relationship to protein concentration.

10 μl of sample or standard was added to a 96-well plate in duplicate. 200 μl of working reagent was added and incubated for 30 min at 37^0C. Absorbance was measured at 570 nm using a Multiskan plate reader (Labsystems, Waltham, MA). Optical density was compared to a known standard curve (0.1-1mg/ml) to calculate unknown protein concentrations.

2.12 Sample preparation for phosphorylated proteins

Purified primary macrophages were plated at on 6-well plates at $1x10^6$ cells/well. Cells were treated with a variety of ALX ligands for 10 min and incubated at 37^oC. Cells were immediately lysed following treatment with boiling hot 20mM Tris-HCL (pH 6.8) and then placed on ice. Cell lysates were mechanically dissociated by passage five times through a 21-gage needle. Homogenates were centrifuged at 4500*g* for 2 min to remove cell debris; supernatants were then transferred to fresh tubes. Lysates were finally denatured with using a 6 x volume of sample buffer (12% sodium dodecylsulphate (SDS), 600mM dithiothreitol (DTT) and 0.6% bromophenol blue in 120 mM Tris-HCL (pH 6.8) and 60% glycerol; Sigma-Aldrich, Poole, UK).

Following SDS-PAGE the PVDF membranes were blocked with 3 % BSA in Tris-Tween Buffered Saline (TTBS) for 2 h. Either rabbit anti-phospho-p44/42 MAP Kinase (P-ERK) or Rabbit Anti-Phospho-Ezrin/Radixin/Moesin antibodies (Cell Signalling) was diluted 1:1000 in 0.3% BSA/TTBS and incubated with the membrane overnight. Membranes were washed 3 times for 15 min with TTBS before a secondary HRP-conjugated anti-rabbit antibody (Dako, Cambridge, UK) was added 1:10000 dilution and incubated for 1 h. Membranes were washed 3 times before target protein luminescence was identified using Enhanced chemiluminescence (ECL) to amplify the HRP

signal. Luminescence was detected by a 10 min exposure on Hyperfilm (GE Healthcare, Little Chalfont, UK).

Each western blot was repeated at least 3 times with and results were collated using ImageJ™ to determine opitical density of each band. Total MAP Kinase (Cell Signalling Technology, Danvers, MA) was used as a measure of total protein to calculate a ratio of phosphorylation and therefore correct for loading errors.

2.13 Radio-ligand binding

Radio-ligand binding assays were conducted as described previously in Hayhoe *et al.* (2006). Briefly, primary macrophages (Mϕ) were resuspended at $10x10^6$ cells/ml in PBS containing Ca^{2+} and Mg^{2+} and placed on ice. The tracer ($Iodine^{125}$ W-peptide) custom made by Phoenix Pharmaceuticals (Burlingame, CA) was prepared following the manufacturer's instructions. An aliquot was resuspended on the day of the experiment in 1 ml of distilled water. The concentration was calculated using the specific activity (1507.17 Ci/mmol), quantity provided (10μCi) and the relative molecular mass of the peptide (856.11) and was determined to be of 7 ng/ml (82pM).

Unlabelled W-peptide was also prepared by resuspension in distilled water to a final concentration of 500μM. Subsequently a 1μM working stock was prepared, this was then employed to determine the extent of Non Specific

Binding (NSB) by the radio labeled tracer. Together with total binding (TB) of the tracer; specific binding can be estimated by TB – NSB.

The reaction mixture was then incubated for 1 h on ice after which it was transferred on to a vacuum filtration unit equipped with 25mm GF/C filter membranes on to which any cells and bound tracer would be retained. The filters were then washed 3 times using 4ml aliquots of 10mM ice cold Tris-HCL, to remove any unbound tracer. Following the wash step the filter paper was transferred into recipient tubes and the amount of bound tracer was determined using a gamma−counter.

In Vivo Protocols

2.14 Animals

Fpr2$^{-/-}$ mice and wild type (WT) littermate controls were bred in-house. All animals were fed on a standard chow pellet diet with free access to water and maintained on a 12 h light–dark cycle. Animal work was performed in accordance with the U.K. Home Office regulations Animals (Scientific Procedures) Act 1986.

2.15 Zymosan-induced peritonitis

Zymosan A (Sigma-Aldrich, Poole, UK) is an extract prepared from yeast (*Saccharomyces cerevisae*) cell wall capable of triggering 'classical' inflammatory machinery. Experimental peritonitis is widely used as an inflammatory model for drug screening with cell infiltration and inflammatory components widely characterised (Rao *et al.*, 1994). More recently, this self-resolving model of inflammation has been used to study the mechanisms and molecules that contribute for the resolution of inflammation (Serhan, 2007).

2.15.1 Acute Peritonitis

The Zymosan-induced peritonitis model was has previously been applied to both FPR null (Perretti *et al.*, 2001b) and AnxA1 null (Chatterjee *et al.*, 2005) mice to pre- assess the pharmacology of AnxA1 and therefore was the initial model assess in my thesis.

Peritonitis was induced in 6-8 week old mice by injecting 1mg Zymosan A in 0.5ml phosphate-buffered saline (PBS) intraperitonally (i.p.). 4 h post injection WT and $Fpr2^{-/-}$ mice were killed and peritoneal cavities were lavaged with 3ml PBS, 25u heparin, 0.3mM EDTA. Cell suspensions were stained with Turk's Solution (1:10 dilution; Section 2.18) and total, PMN and PBMC cell counts were assessed by light microscopy. Specific cell populations were identified and quantified by FACS analysis (Section. 2.19).

2.15.2 Spontaneously Resolving Peritonitis

The effects of endogenous and exogenous AnxA1 was first characterised in a resolving model of Zymosan-induced peritonitis (Getting *et al.*, 1997) which revealed a role for AnxA1 in both acute phase inflammation and active resolution throughout the time course. The AnxA1 null mouse was also assessed across a 96 h time course to investigate the AnxA1 gene expression throughout this resolving model (Damazo *et al.*, 2006).

To assess the profile of Fpr2 endogenous ligands peritonitis was again induced with 1mg Zymosan A in 0.5ml PBS (i.p) across a range of time-points spanning 4-120 h. Cells were counted and leukocytes identified and quantified by FACS (Section 2.18 and 2.19).

Cell pellets and cell-free exudates were separated by centrifugation (4500*g,* 5 min) and stored at -80^{o}C for gene (Section. 2.5) and protein (Section. 2.20–2.24) based analysis, respectively.

2.16 IL-1β-induced air pouch

The air-pouch was originally developed in rat as an *in vivo* model representative of an inflamed synovium (Edwards *et al.*, 1981). Subcutaneous injection of air (day 0 and day 3) leads to the formation of a *lining tissue*, a very thin layer of 2-3 cells where a combination of macrophage- and fibroblast-like resident cells coexist. These features offer both a simplistic and robust microenvironment that allows great flexibility for experimental design.

The role of IL-1β in leukocyte emigration was first noted for its induction in an endotoxin-induced air pouch (Ribeiro *et al.*, 1991) but not fully characterised as a dependent stimuli until the early 90's (Perretti *et al.*, 1993c).

The air pouch procedure was carried out as described previously (Perretti *et al.*, 1994). WT and $Fpr2^{-/-}$ mice (6-8 weeks old) were injected with 2.5ml sterile air intra-dermally (i.d.) in the dorsal inter-scapula region with a 23-gauge needle on day 0 and again on day 3 (Figure 2.5).

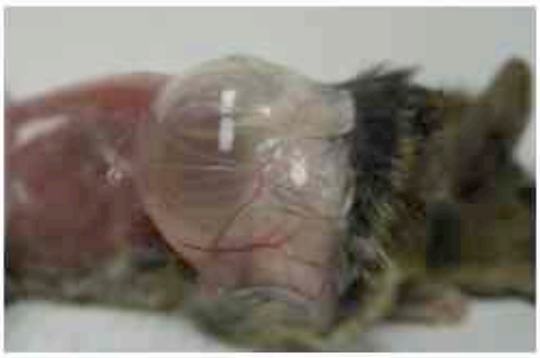
Animals were prophylactically treated with either FPR family agonists or saline (control) intra-venous (i.v.) on day 6 or 7. To induce cell infiltration air pouches were injected with 0.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, Poole, UK) containing 20ng IL-1β per mouse for 4 h.

The cell infiltrate was subsequently harvested by gentle lavage of the air pouch using PBS supplemented with 0.3mM EDTA and 25u heparin. As

described with the peritoneal lavage (Section. 2.15) cell suspensions were differentially counted by Turk's staining and prepared for FACS analysis.

Day 0 and 3 inject 2.5 ml of air by intra-dermal (i.d) injection into the dorsal inter-scapula region

By day 6 the air pouch lining shows features similar to synovium. These features can be reinforced with repeated injections of air and extended time courses of up to 30 days



Characteristic macroscopic view of a 6 d air pouch membrane displaying the developed microvasculature

Figure 2.5. Schematic air pouch formation and picture of membrane structure. Sampaio, Dufton and Perretti 2009.

2.17 K/BxN Arthritis

The K/BxN T cell receptor (TCR) transgenic mouse line is susceptible to a spontaneous joint disorder bearing many of the pathogenic hallmarks of human RA (Matsumoto *et al.*, 1999). K/BxN serum, containing atherogenic immunoglobulins, produces disease pathogenesis when injected into healthy animals. The transfer of induced arthritis model has become a popular technique for investigating the effector phase of inflammatory arthritis.

Mice received 150μl of pooled sera (i.p.) from K/BxN arthritic mice at a single time-point (day 0). The development of disease was monitored by assessing the clinical index: one point was given for each tarsal or wrist joint which presented with erythema plus swelling; A maximum of 22 points could be scored per animal.

Cumulative disease incidence was determined by the number of mice that presented a minimum of two paws with a clinical score (≥ 3) and was quantified as a percentage of the total group.

2.18 Differential cell counting with Turk's solution

To determine the number of cells within a given population the samples were diluted in Turk's solution. 10μl of the cell suspension is counted by a Neubauer haemocytometer. The cells can be identified and characterised as either PMN or PBMC by their nuclear morphology, stained violet by the highly permeable Turk's solution (Figure 2.6).

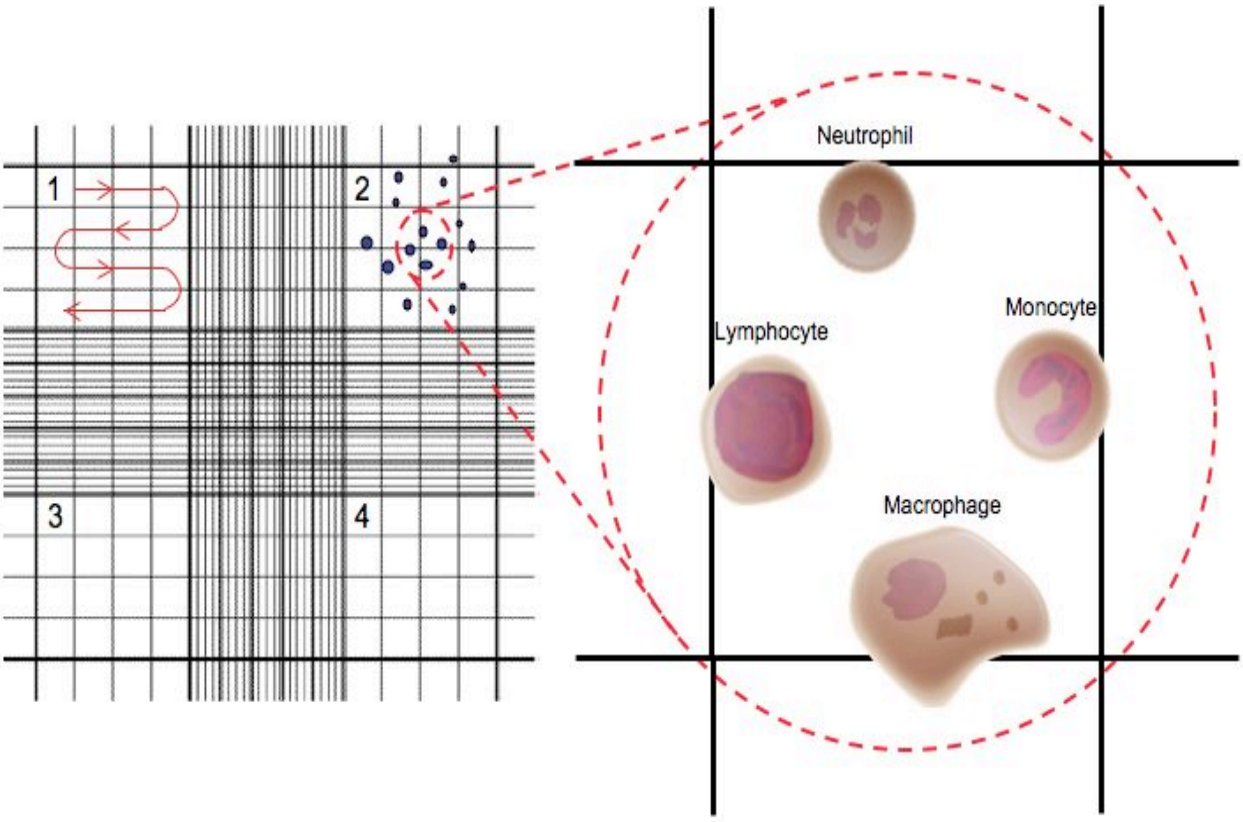


Figure 2.6. Illustration of a Neubauer haemocytometer and differential cell counts by nuclear morphology. Cells are counted in the four numbered sections (1-4) and so a more accurate average can be obtained. Turk's solution allows the identification on PMN (e.g. neutrophils) and PBMC (e.g Lymphocyte, monocyte and macrophages) and therefore counted differentially.

2.19 Measurement of specific cell populations within inflammatory infiltrate by flow cytometry (FACS)

Cell infiltrates were analysed using a 96-well plate staining protocol previously described (Yona *et al.*, 2004). Briefly, cell pellets were resuspended in PBS containing 0.2% bovine serum albumin (BSA) 1.3mM $CaCl_2$ (PBC). Cells were washed (400*g*, 30s) before non-specific binding was blocked with 16mg/ml human immunoglobulin G (IgG). Cells were subsequently stained with specific conjugated antibodies, anti-mouse Ly6G (GR-1; Granulocytes) PE, anti-mouse CD2 (LFA-2; Lymphocytes) PE, anti-mouse F4/80 ($F4/80^{low}$ monocytes; $F4/80^{high}$ macrophages) PE-Cy5 (eBioscience, San Diego, CA), (45 min, 4^{o}C) or corresponding isotype control (final concentrations 5µg/ml). Cells were washed (400*g*, 30s) three times before being resuspended and run on a FACScalibur analyser (Becton Dickinson, Oxford, UK).

For quantitative analysis a given leukocyte population could be identified by various parameters including Forward Scatter (FSC; size), Side Scatter (SSC; internal complexity) and specificity of a given conjugated-antibody. For example Figure 2.7 depicts $GR\text{-}1^{+}$ cells gated predominantly for strong PE fluorescence in the FL-2 channel. The proportion of these $GR\text{-}1^{+}$ events could be compared with the total events (10000) to determine the percentage $GR\text{-}1^{+}$ population (Figure 2.7). Together with the total cell count a specific cell infiltrate per cavity or pouch can be approximated.

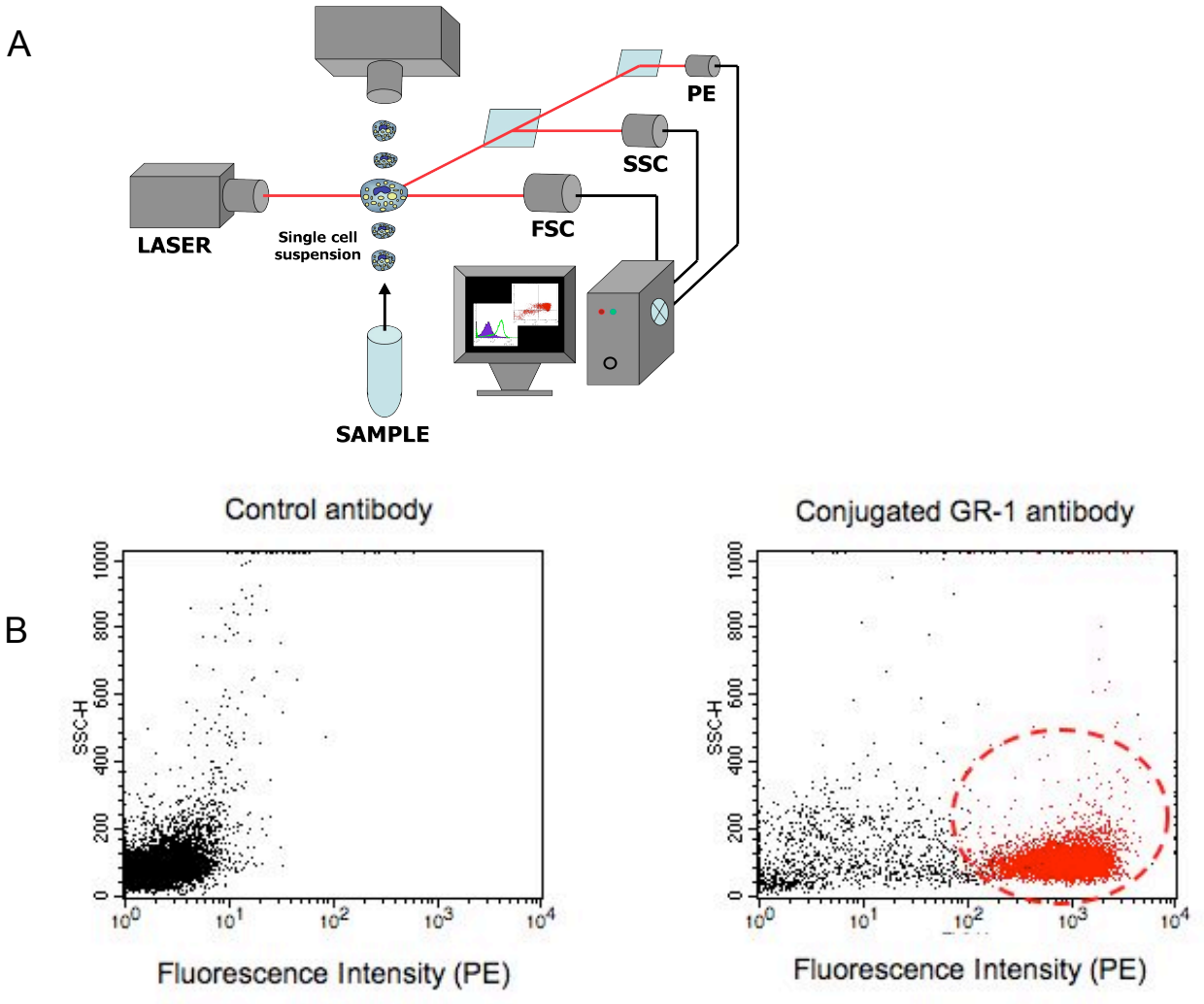


Figure 2.7. Identification of specific leukocyte subsets by FACS. (A) Depicts a schematic of a FACS assessing three parameters following excitation with an Argon 488 laser. (B) An example of conjugated anti-mouse GR-1 antibody used to identify a specific, GR-$1^{+\ high}$ population of granulocytes within an cells harvested from a 4h IL-1β-induced air pouch. A conjugated anti-rat IgG2 antibody is used as a negative control to identify non-specific antibody binding by measurement of fluorescence. Neutrophils are characterised by high GR-1 binding (red). A percentage of the total population can be derived and compared with total cell counts to derive total GR-$1^{+\ high}$ cells within a given cell suspension.

Peripheral blood was prepared as described previously (Chatterjee *et al.*, 2005). Briefly, blood was collected by cardiac puncture from both WT and $Fpr2^{-/-}$ mice under anaesthesia (3% halothane). I routinely collected 0.5-1ml of blood by cardiac puncture of 22-25g mice. Cells were washed (4ml PBS, 400*g*, 5 min), blocked and stained, as above, in 15ml tubes. Following staining the cell pellets were washed twice and lysed using Whole Blood Immuno-Lyse™ (Coulter, Luton, UK). Cells were washed twice and resuspended and data acquired on the FACScalibur analyser.

For each sample a minimum of 10,000 events were acquired for analysis by CellQuestTM software (Becton Dickinson, Oxford, UK) on a Power Macintosh G3 computer.

Optical Density endpoint and ELISA Assays

All assay endpoints were assessed using a Multiskan plate reader (Labsystems).

2.20 Mouse TNF-α/IL-6 ELISA

TNF-α and IL-6 production was measured by murine Ready-Set-Go!^{TM} ELISA kit according to manufacturers instructions (eBioscience, San Diego, CA). Briefly, samples were allowed to defrost on ice prior to plating on to the pre-coated NUNC Maxisorp 96 well ELISA plates with comparable standard. Plates were assessed after avidin-HRP conjugation and visualised with 3,3',5,5'-tetramethylbenzidine (TMB) (R&D Systems, Abingdon, UK). Optical density was assessed at 450nm.

2.21 Lipoxin A_4 Extraction and ELISA

Samples were initially purified to minimise background following an extraction method derived from Romano *et al.* (2002) using C_{18} Sep-Pak colums (Waters Corporation, Milford, MA). Lavage fluid from zymosan peritonitis samples were centrifuged at 400g for 5 min and supernatant was removed and stored at -20^{o}C. 100μl of each sample was mixed with 800μl 20% methanol v/v in distilled water (pH 3.5) immediately prior to extraction.

The C_{18} Sep-Pak columns were preconditioned with 4ml methanol followed by 4ml distilled water. 1ml of sample was placed on top of each column which was situated on a manifold, Vac Elute SPS 24 (Varian, Palo Alto, CA),

connected to a vacuum pump generating ~ 5 in Hg suction. Columns are washed with 4ml water followed by 4ml hexane to remove any non-polar molecules. Lipoxin A_4 could then be eluted with 2ml ethyl ethanoate, a highly polar solution. Samples were dried within a vacuum centrifuge for 2 h and residues were reconstituted with extraction buffer provided within the Lipoxin A_4 ELISA kit (Neogen, Lexington, KY).

Subsequently 50μl of the extracted samples were applied to a pre-coated 96-well plate and incubated with an enzyme conjugate for 1 h at room temperature. The plate was washed by submersion in wash buffer three times and a 150μl of secondary HRP-substrate was applied for 15-20 min. The reaction wash was stopped with 1M HCl and read at 450nm, values were derived by comparison to a standard curve ranging from 0.02-2ng/ml.

2.22 Keratinocyte-derived Cytokine (KC) ELISA

KC production was measured by a Mouse CXCL1/KC DuoSet ELISA kit according to manufacturers instructions (R&D Systems). Briefly, NUNC Maxisorp 96-well ELISA plates were pre-coated overnight with a capture antibody, diluted to 2μg/ml in PBS. The plates were washed and blocked with 300μl of PBS containing 1% BSA for 1 h at room temperature. The plate was repeatedly washed and 100μl of a 1:10 dilution of each sample or known dilution of standards added for 2 h at room temperature. The plate was washed again and 50μl HRP-conjugated detection antibody (200ng/ml) was added for 1 h. Streptavidin-HRP was added for 20 min followed by TMB to

visualise protein, the reaction was stopped with 1M HCl. Optical density was assessed at 450nm.

2.23 Mouse Serum Amyloid A (SAA) ELISA

Levels of SAA within exudate samples were measured using a mouse SAA ELISA kit (Immunological Consultants Laboratory, Newburg, OR). The assay was performed in a pre-coated 96-well plate. Samples were diluted 1:200 for air pouch exudates and 1:400 for Zymosan-induced peritonitis exudates before 100μl of each sample was added to each well. Samples were incubated at room temperature for 1 h and then washed by submersion in wash buffer provided. A secondary HRP-conjugated substrate was added for 30 min and again unbound substrate was removed by washing. Finally TMB solution was used to bind the HRP-conjugated protein, this reaction was stopped with 1M HCL and plates were read at 450nm.

2.24 Myeloperoxidase (MPO) Assay

MPO enzyme activity was assayed by measuring hydrogen peroxide (H_2O_2) (Sigma-Aldrich, Poole, UK) dependent oxidation of TMB as previously described (Cuzzocrea *et al.*, 1997).

20μl exudate samples or known concentrations of MPO were mixed with 160μl of TMB and 20μl H_2O_2 (30% w/w) in a 96-well plate. Contents were

incubated for 5 min at room temperature before optical density was read at 620nm.

2.25 Statistics

Data are expressed as mean ± SEM of *n* experiments performed in duplicate, triplicate or quadruplicate where stated. All data were initially assessed for normal distribution GraphPad Prism 4.0 software (GraphPad, San Diego, CA) prior to further statistical analysis.

Student's *t*-test was used to compare two groups with parametric data distribution. Mann-Whitney *U*-test was used for non-parametric data (ERK phosphorylation). Comparison of clinical scores and paw volumes between groups was made using ANOVA. All analysis was performed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). In all cases, a *P* value <0.05 was taken as significant.

Chapter 3.

Results

In Vitro Characterisation of Fpr2 signalling in primary cells

3.1 Confirmation of generation of Fpr2$^{-/-}$ *transgenic colony*

The targeting vector underwent homologous recombination in 8 out of 96 ES cell clones (Figure 3.1 A). The alignment of the 14.12 kb lambda insert p2.1 is shown, along with the locations of the Nhe I restriction sites used for Southern blot screening. Germline transmissions was primarily confirmed by Southern blot screening (Figure 3.1 B top) of tail clip DNA, the enzyme Nhe I. Probe 5b generates bands of 21.9 kb for WT and 15.7 kb and Fpr2$^{-/-}$ targeted alleles, respectively.

A multiplex PCR was subsequently optimised to target the in-frame green fluorescent protein (GFP) target/reporter construct as a method of discriminating between WT and Fpr2$^{-/-}$ alleles. The f1/b11 primer pair produces a band of 233 bp using WT DNA, whereas the f1/Gb4 pair gives a band of 351 bp if the targeted allele is present (Figure 3.1 B bottom).

To ensure the specificity of the transgenic strategy Multiplex PCR was used to compare the expression of *fpr1* and *fpr2* in WT (+/+) and Fpr2$^{-/-}$ (-/-) mice. Primers were compared to the internal control (IC) house-keeping gene (18s) (Figure 3.1 C).

The transgenic strategy and confirmation of germline transmission was performed by Dr Robert Hannon. I oversaw the maintenance, including genotyping, breeding and subsequent backcrossing of the $Fpr2^{-/-}$ colony and littermate controls during my PhD.

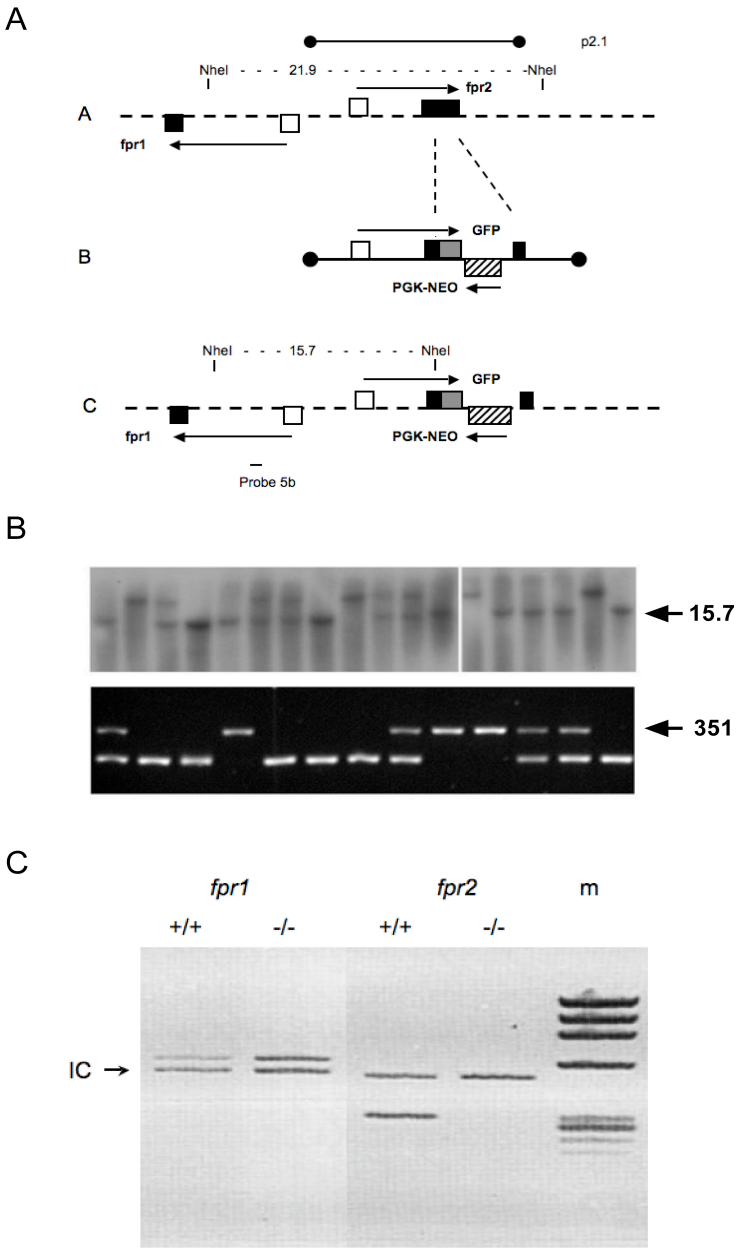


Figure 3.1. Confirmation of the generation of the $Fpr2^{-/-}$ mouse (A) Schematic representation of a region of ~ 30 kb of mouse genomic DNA spanning the *fpr1* and *fpr2* genes. (B) top panel; Southern blot screening using a Nhe I. Probe 5b producing bands of 21.9 kb (WT) and 15.7 kb ($Fpr2^{-/-}$). (B) bottom panel; PCR was used to genotype heterozygote offspring, f1/b11 primer pair produced a band of 233 bp (WT), whereas the f1/Gb4 pair gives a band of 351 bp if the targeted allele is present. (C) PCR was used to compare *fpr* gene family expression in WT (+/+) and $Fpr2^{-/-}$ (-/-) animals. The arrow denotes a housekeeping gene (18S) to confirm the comparable presence of cDNA.

3.2 Induction of fpr2 promoter activity

The transgenic strategy utilised an insertion of a GFP target/reporter construct located in frame of the promoter region. Expression of GFP could therefore be monitored as a measure of promoter activity.

3.2.1 fpr2 promoter activity during macrophage (Mϕ) differentiation

Mϕ were chosen for their previously established phenotype in AnxA1$^{-/-}$ mice (Yona *et al.*, 2005) within our laboratory, complementing data pertaining to Mϕ expression of Fpr2 by another group (Hartt *et al.*, 1999b). The presence of *fpr2* was confirmed in my thesis with the initial observation of *fpr2* promoter activity in a variety of leukocyte populations by FACS *ex vivo* (Figure 3.12 B).

Promoter activity, assessed as GFP Median Fluorescence Intensity (MFI), was monitored by FACS over 5 d bone marrow progenitor cell maturation to Mϕ by incubation with M-CSF. Bone marrow cells increased promoter activity throughout the Mϕ differentiation time course (Figure 3.2 A), expressed as a percentage increase in GFP MFI compared naïve bone marrow cells. Promoter activity was significantly (P<0.01) induced at 3 d and remained at a consistent plateau until 5 d.

To compensate for the unavailability of antibodies targeting Fpr2 in WT cells, activity of WT and Fpr2$^{-/-}$ Mϕ were compared by monitoring production of a

potent inflammatory cytokine, TNF-α. Inflammatory cytokines are known to play a key role in the regulation of the FPR family (Mandal *et al.*, 2005) and (Cui *et al.*, 2002b). TNF-α release into the supernatant was significantly (*P*<0.001; 4-5d) increased during Mϕ maturation, measured by ELISA and revealed a close correlation with the modulation of GFP expression in $Fpr2^{-/-}$ macrophages (Figure 3.2 B). There were no notable (NS) differences between the genotypes suggesting similar Mϕ activity within each culture.

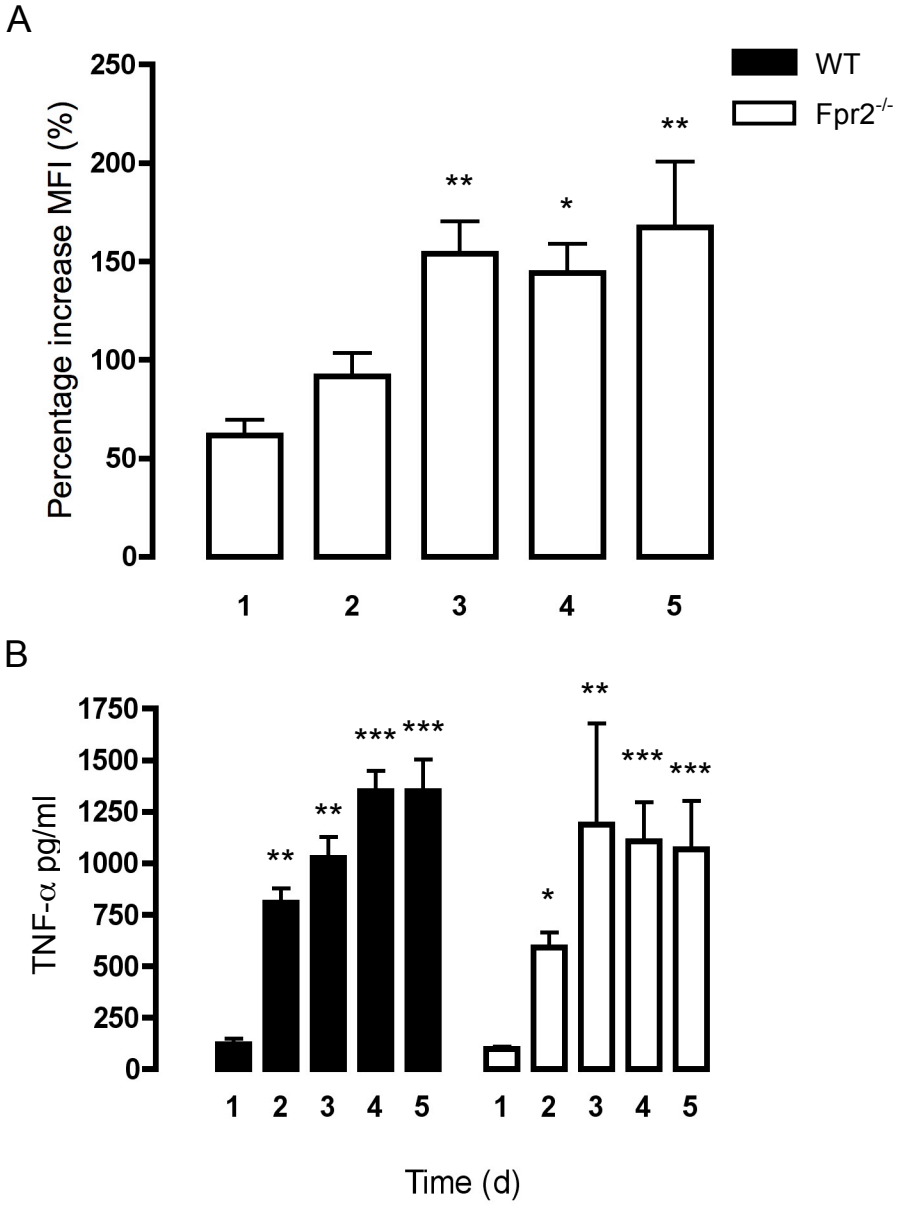


Figure 3.2. *fpr2* promoter activity during bone marrow Mϕ differentiation. Mϕ are differentiated from bone marrow progenitor cells by 5 d culture in media enriched with L929 condition media (M-CSF). (A) *fpr2* promoter activity is expressed as a percentage increase in the median fluorescence intensity (MFI) of GFP fluorescence compared to 0 d Fpr2$^{-/-}$ cells. (B) TNF-α secretion into the culture supernatant was assessed by ELISA across the time course. All data are mean ± SEM of n=3. **P*<0.05, ***P*<0.01, ****P*<0.001 compared to 0 d by one-way ANOVA and Dunnett's post-hoc test compared to day 0 control.

3.2.2 Modulation of fpr2 promoter activity in macrophages (Mϕ) with pro- and anti-inflammatory stimuli

Differentiated Fpr2$^{-/-}$ Mϕ were stimulated with pro-inflammatory or anti-inflammatory treatments *in vitro* to modulate promoter activity. Lipopolysaccharide (LPS; 100ng/ml) significantly (P<0.05) increased GFP expression at 8 h post treatment compared to 5 d. However, prednisolone alone had the opposite effect on promoter activity in GFP expression noted at both 4 and 24 h had levels below day 5 Mϕ (Figure 3.3 A).

TNF-α release by Mϕ was again monitored in Fpr2$^{-/-}$ cells and revealed an acute secretion profile peaking at 4 h (P<0.01) following stimulation with 100ng/ml LPS, however prednisolone reduced TNF-α production at both 4 and 24 h (Figure 3.3 B). It is interesting to note that TNF-α secretion profile of LPS treated Mϕ pre-emptively match the peak of promoter activity. This would suggest *de novo* synthesis is important when monitoring promoter activity via the GFP reporter construct. Equally prednisolone reduced TNF-α secretion below levels measured on day 5 Mϕ paralleling the sub-basal promoter activity observed (Figure 3.3 A).

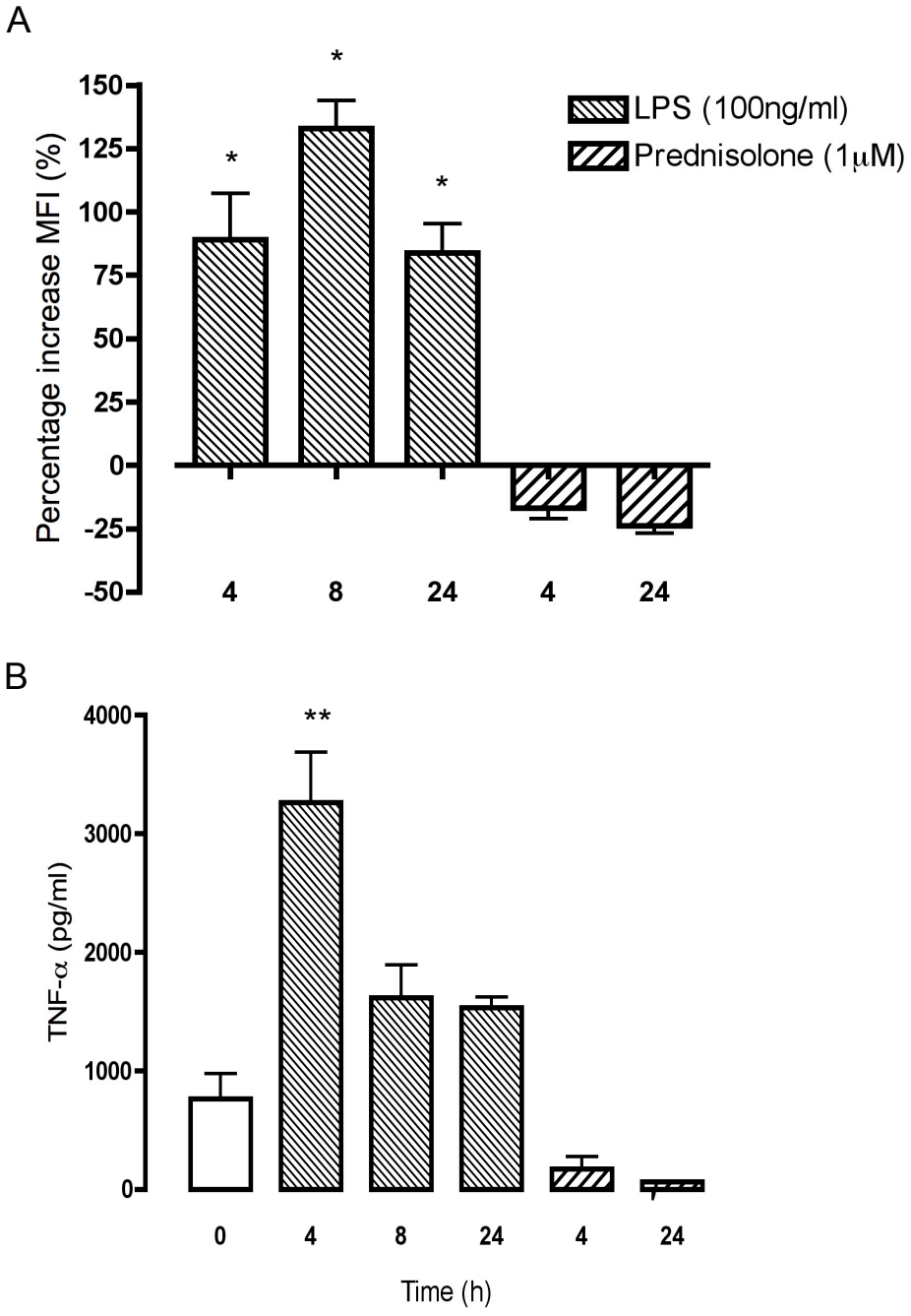


Figure 3.3. *In vitro* modulation of *fpr2* promoter activity in Fpr2$^{-/-}$ Mϕ. BM differentiated Mϕ were stimulated with either 100ng/ml LPS or 1μM prednisolone over a 24 h time course. (A) Percentage increase in GFP MFI was calculated compared to untreated control MFI value from 5 d Mϕ represented as 0 h treatment. (B) The TNF-α secretion from Mϕ into the culture supernatant was measured by ELISA. All data is mean ± SEM of n=3. **P*<0.05, ***P<0.01* compared to 0 h by one-way ANOVA and Dunnett's post-hoc test.

3.3 Radio-ligand binding on primary macrophages (Mϕ)

To validate our transgenic strategy employed to generate the Fpr2$^{-/-}$ mouse it was important to confirm absence of a surface receptor by assessing the binding capability of a specific Fpr2 ligand. ^{125}Iodine-labelled W-peptide (tracer), a synthetic hexapeptide, was used as a known high-affinity ligand for murine Fpr2 (Le *et al.*, 1999). Specific binding was assessed in WT Mϕ by the addition of increasing amounts of tracer (0.1 - 820 pmoles) in the presence of a constant concentration of cold peptide (10μM; Figure 3.4 A). Specific binding (pmoles/mg) was used to generate a Scatchard plot (see Figure 3.4 A insert) revealing an apparent high and low-affinity binding site for W-peptide in line with a human receptor binding study (Strouse *et al.*, 2009). The high-affinity had a Kd of ~44 pM and the B_{max} ~12 pmol. There was no specific binding of the ^{125}Iodine-labelled W-peptide to Fpr2$^{-/-}$ Mϕ.

A displacement assay confirmed the deletion of the receptor in the Fpr2$^{-/-}$ Mϕ as the tracer was unable to bind the cell surface. WT Mϕ showed a concentration-dependent displacement of tracer by increasing concentrations of cold peptide (30 – 3000nM; Figure 3.4 B).

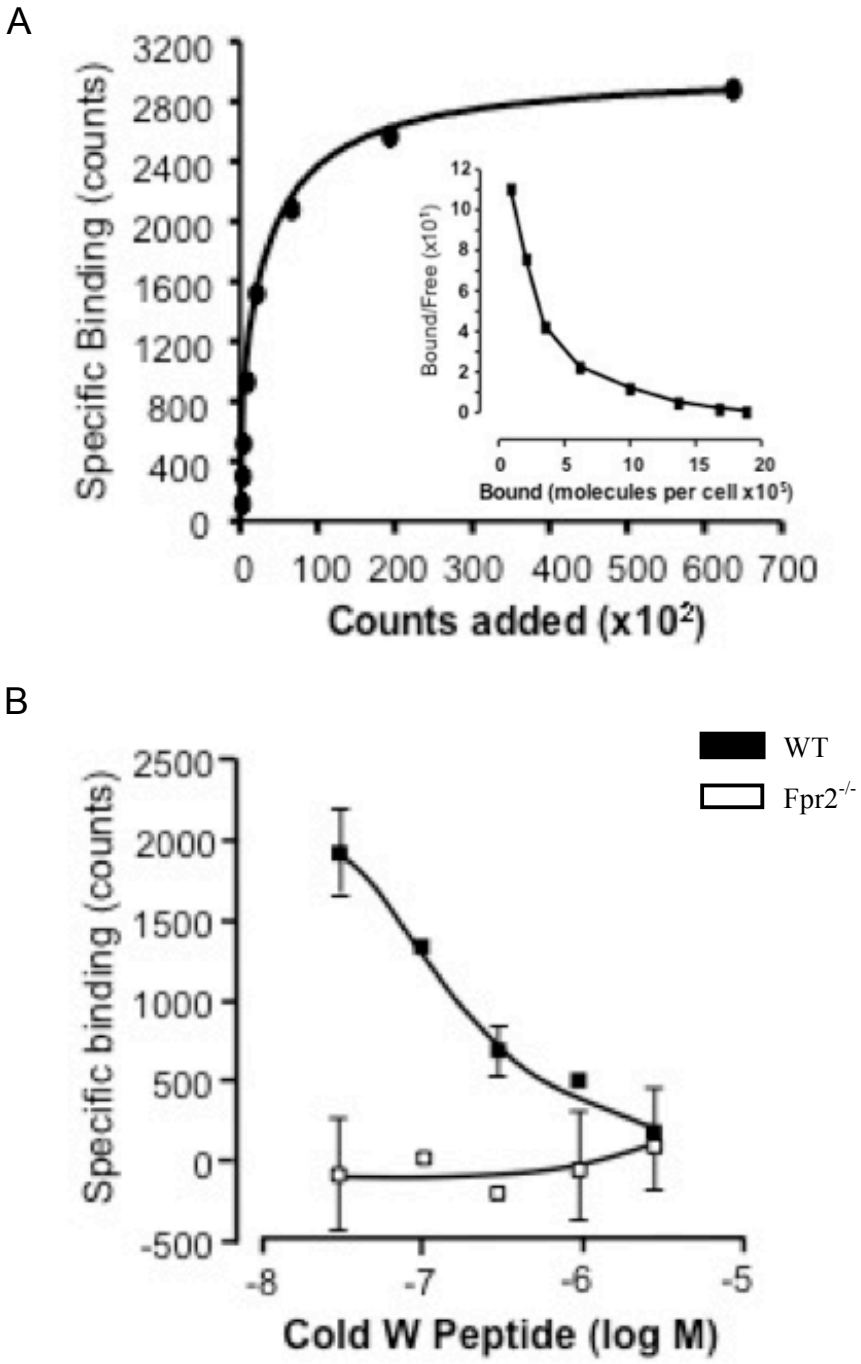


Figure 3.4. Radio-ligand binding assay. (A) Specific binding of ^{125}I-labelled W-peptide is represented as number of molecules bound compared to the gamma-counts. This data allowed the calculation of a Scatchard plot inset. (B) Fpr2 specific binding to WT and Fpr2$^{-/-}$ Mϕ was assessed by measuring the competitive displacement of ^{125}I-W-peptide trace by cold peptide. All data are mean ± SEM of n=3.

3.4 ERK phosphorylation signalling cascade in ALX ligand stimulated primary macrophages

To test the specificity of Fpr2 for functional ligation of reported ligands we chose to monitor ERK phosphorylation as a readout for receptor activation. This system has been reported to be robust in several studies both in-house (Hayhoe *et al.*, 2006) and elsewhere (He *et al.,* 2003). Phosphorylation was monitored at a selected time-point, that is 10 min post addition of each ligand.

W peptide, which shows high specificity for Fpr2, confirmed in radio-ligand binding assays (Section 3.3), produced a rapid phosphorylation of ERK in WT Mϕ across a concentration range (Figure 3.5 A). The inability of W peptide to transduce a functional response in the absence of Fpr2 would suggest a specificity for this receptor, at least within the given dose range applied.

C43, a non-peptidic molecule developed using a cell-based assay high-throughput screen of a compound library by Amgen (Burli *et al.*, 2006), also produced a notable concentration-dependent response in WT Mϕ (Figure 3.5 B). Previously no signalling cascade has been attributed to this molecule. The marked reduction ($P<0.01$) in ERK phosphorylation, suggests a significant role for the MAPK pathway in C43 pharmacology via Fpr2.

I then tested the AnxA1 N-terminal peptide, Ac2-26. This peptide, shown to activate all FPRs under *in vitro* experimental settings (Ernst *et al.*, 2004),

produced a reproducible concentration-dependent response in WT Mϕ that was significantly (P<0.01) reduced in Fpr2$^{-/-}$ Mϕ (Figure 3.5 C).

Finally we tested the response of SAA, an acute phase protein, that has been associated with activation of the ERK pathway following ligation of CLA-1 (Baranova *et al.*, 2005), TLR-2 (Cheng *et al.*, 2008), TLR-4 (Sandri *et al.*, 2008) and, most pertinently for my study, human FPR2/ALX (He *et al.*, 2003). The response of WT Mϕ revealed a concentration-dependent pattern of ERK phosphorylation that was retained in the Fpr2$^{-/-}$ Mϕ (Figure 3.5 D).

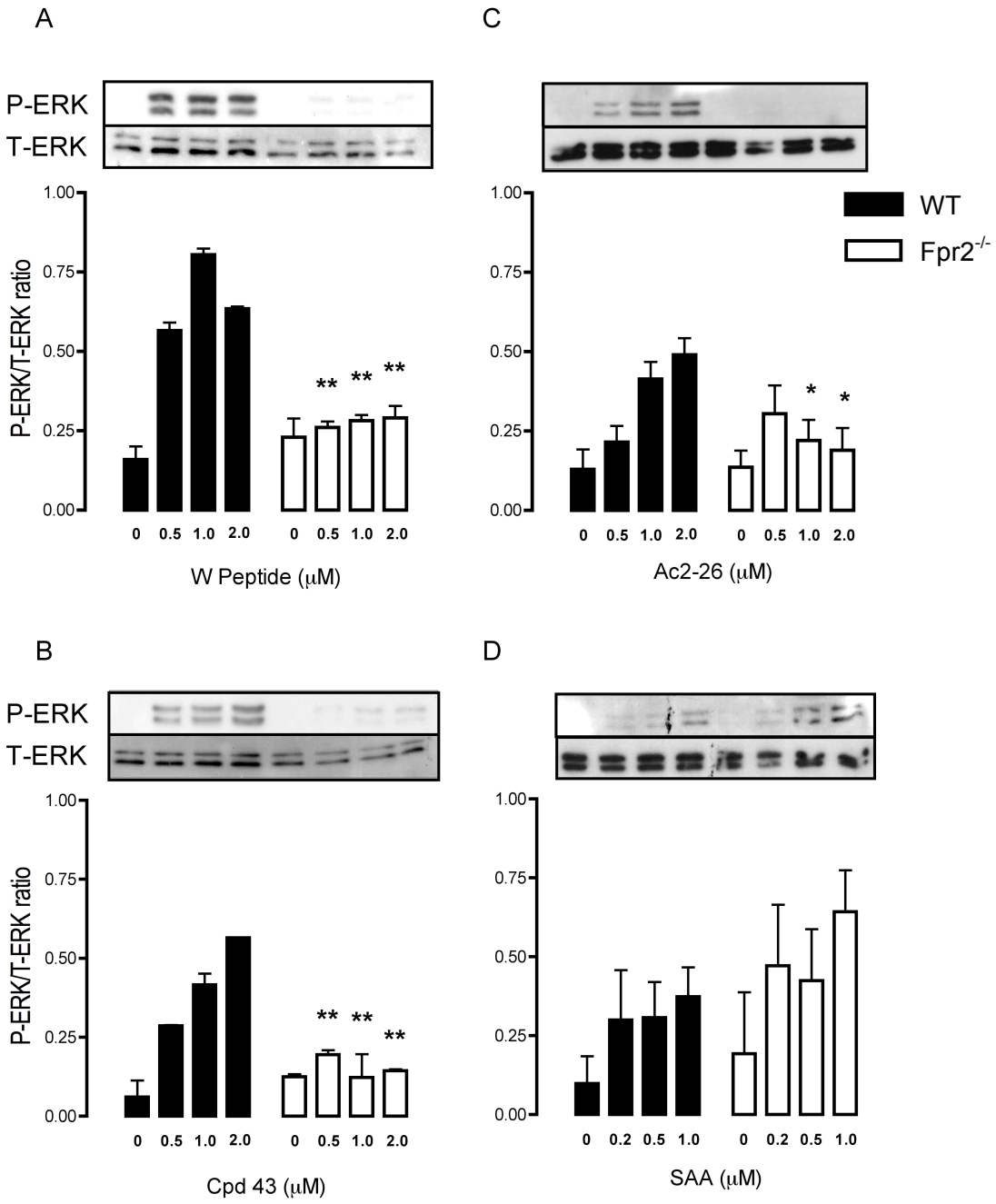


Figure 3.5. Intracellular signalling induced by Fpr2 ligation. Phosphorylation of ERK was monitored by western blotting, with Mϕ exposed to a dose range of W peptide, C43, peptide Ac2-26, and SAA (panel A to D, respectively) for 10 min at 37°C. Representative blots are shown with respective bar histograms showing cumulative data. Closed bars denote WT Mϕ and open bars Fpr2$^{-/-}$ Mϕ. Data, expressed as ratio P-ERK/total ERK, are mean ± SEM of three experiments. *P<0.05, **P<0.01 compared to respective WT Mϕ by Mann-Whitney U-test.

3.5 In vitro primary macrophage transmigration assay

The FPR family of receptors was initially characterised by their chemotactic properties, indeed Fpr2 was originally termed the low-affinity fMLF receptor (Ye *et al.*, 1992). To investigate the functional role played by Fpr2, the ability of FPR family ligands to transduce the chemokinetic effects was assessed in WT and Fpr2$^{-/-}$ Mϕ.

WT and Fpr2$^{-/-}$ Mϕ cells were capable of responding to 1μM concentrations of fMLF with similar efficacy. Notably, as the concentration range increased, the ability of the Fpr2$^{-/-}$ Mϕ to migrate was significantly attenuated compared to the response of WT Mϕ (Figure 3.6 A). This is in line with previous studies noting that HEK-293 cells transfected with mouse Fpr2 were responsive at high concentrations of fMLF (Hartt *et al.*, 1999b).

SAA has also been characterised for FPR2/ALX specificity to transduce a chemotactic response in HEK-293 transfected cells (Liang *et al.*, 2000). I was able to confirm this observation with a significant (P<0.05-P<0.01) reduction in Fpr2$^{-/-}$ Mϕ to transmigrate in response to a concentration range capable of mobilising WT Mϕ (Figure 3.6 B).

AnxA1 and its functionally active peptide derivative, Ac2-26, produced a mild concentration-dependent chemotactic response in both WT and Fpr2$^{-/-}$. Interestingly AnxA1 was capable of significantly (P<0.01) inducing a chemotactic response at 1μM, absent at the same concentration of Ac2-26.

Notably there was no specificity shown for Fpr2 with both WT and Fpr2$^{-/-}$ Mϕ revealing identical profiles (Figure 3.6 C and D).

From the chemotactic profiles of the four FPR ligands, Ac2-26 was noted to be the least chemokinetic. Indeed Ac2-26 was first characterised as a potent anti-migratory agent in vivo (Perretti et al., 1993b) with the distinct effects on both neutrophil and monocyte recruitment in a model of peritonitis (Getting et al., 1997). The notable ability of Ac2-26 to phophorylate ERK1/2 in Mϕ via Fpr2 (Figure 3.5 C) made it an ideal candidate to investigate the anti-migratory signalling attributed to Fpr2.

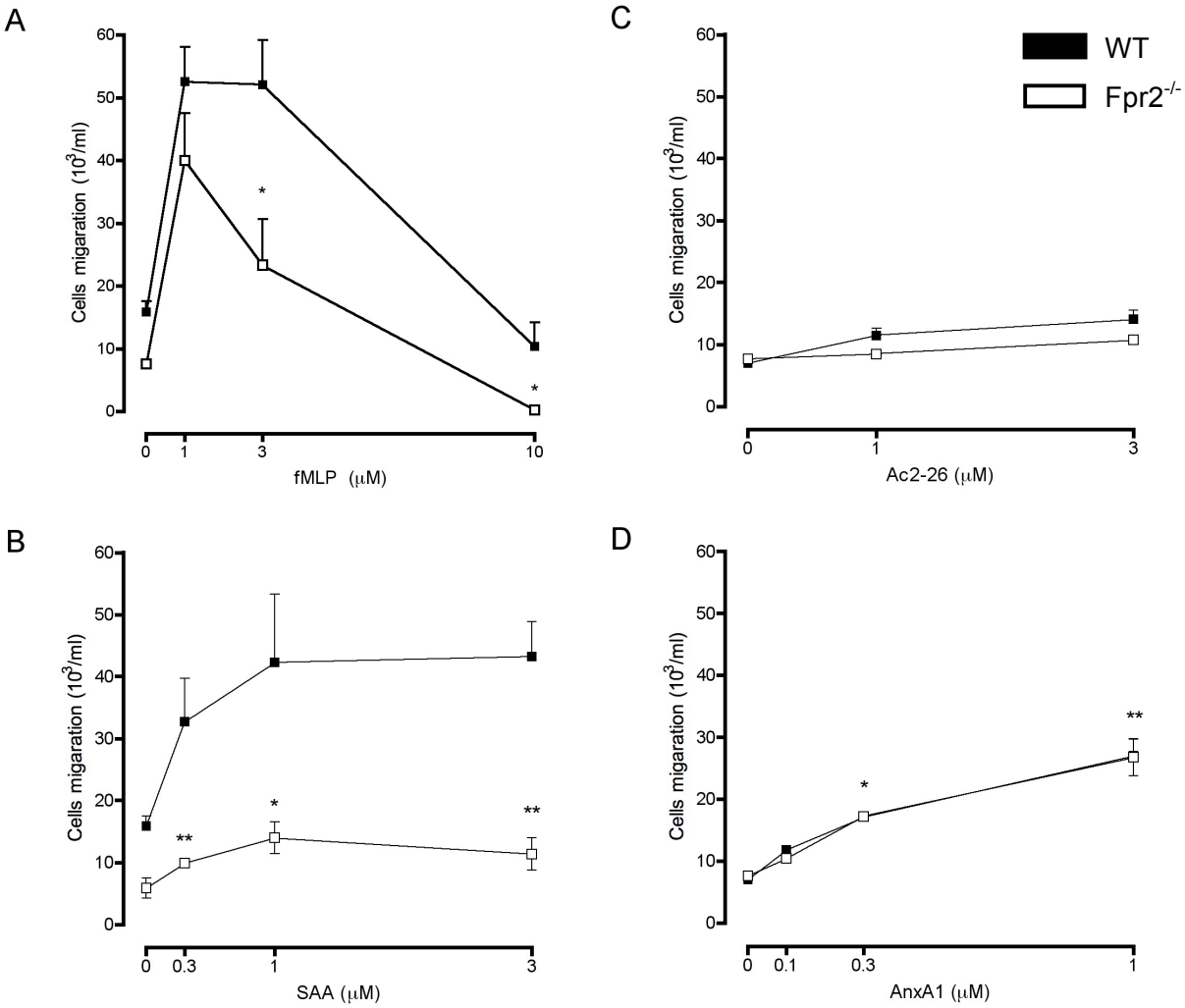


Figure 3.6. *In vitro* Mϕ locomotion in response to Fpr family ligands. Chemotactic response of WT (black) and Fpr2$^{-/-}$ (white) Mϕ towards different concentrations of (A) fMLF, (B) SAA and Ac2-26 or (C) AnxA1 was determined using Alamar blue staining. Mϕ chemotaxis occurred for 90 min at 37°C across 5μm pore membranes. Data are M ± SEM of 3 experiments in quadruplicate, with different Mϕ cultures; **P*<0.05, ***P*<0.01 compared to WT Mφ (one-way ANOVA and Student's t-test).

To test my hypothesis WT Mϕ, pre-treated with 1μM Ac2-26 10 min prior to migration to SAA (1μM, 90 min) was shown to have striking selectivity for Fpr2 mediated chemotaxis. When Mϕ were pre-treated with media, SAA was capable of significantly (*P*<0.05) inducing a migratory response compared to vehicle alone, confirming our previous data (Figure 3.6 B). This response was significantly (*P*<0.01) inhibited following pre-treatment of Mϕ with Ac2-26 (Figure 3.7). This result strongly suggests that both SAA and Ac2-26 mediate their converse effects on Mϕ migration via the Fpr2 receptor.

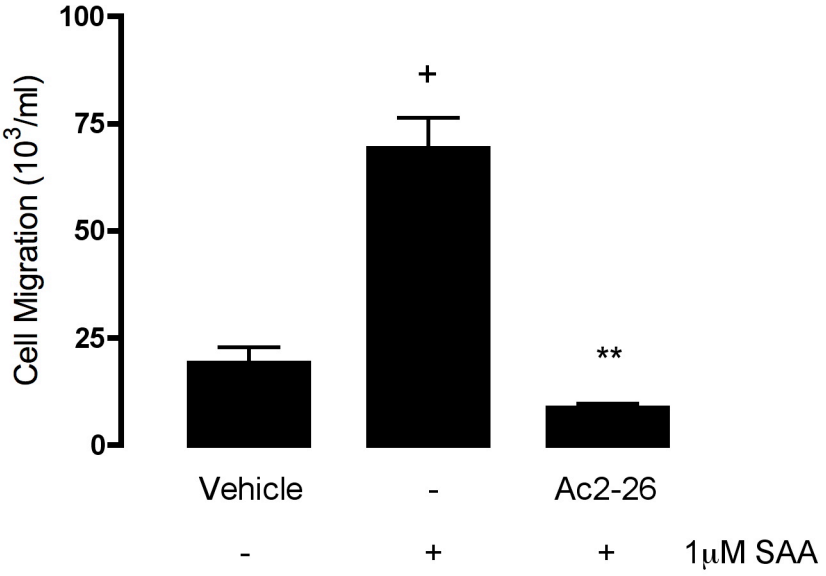


Figure 3.7. Inhibtion of SAA-mediated Mϕ chemotaxis by Ac2-26. Mϕ were incubated with vehicle or peptide Ac2-26 (1μM) 10 min prior to addition to the top well of the chemotaxis chamber, using 1μM SAA as a chemotactic stimulus. Vehicle alone had no effect on SAA-induced chemotaxis. However pre-incubation with Ac2-26 blocked chemotaxis. All data is mean ± SEM of n=4 analysis by Student's t-test +*P*<0.05 compared to vehicle alone; ***P*<0.01 compared to vehicle + SAA.

3.6 Phagocytosis of apoptotic neutrophils by primary macrophages (Mϕ)

An important property of the anti-inflammatory and pro-resolving profile of AnxA1, Ac2-26 and LXA_4 pharmacology is their ability to induce phagocytosis of apoptotic PMN by Mϕ. I therefore investigated the role of Fpr2 in the pro-phagocytic effect of Ac2-26 and LXA_4 by measuring the capacity of Mϕ to phagocytose human apoptotic neutrophils. Mϕ were isolated from both WT and $Fpr2^{-/-}$ animals and pre-treated with either Ac2-26 and LXA_4, used at previously published concentrations (Maderna *et al.*, 2005) shown to increase phagocytic capacity of WT Mϕ.

Ac2-26 significantly ($P<0.05$) increased ingestion of apoptotic neutrophils in a concentration dependent manner in WT, notably $Fpr2^{-/-}$ Mϕ did not increase their phagocytic capacity compared to vehicle treated cells (Figure 3.8). The treatment of WT Mϕ with 30μM Ac2-26 led to a significant ($P<0.05$) increase as calculated with respect to vehicle and $Fpr2^{-/-}$ Mϕ.

LXA_4 was observed to be a potent pro-phagocytic agent ($P<0.01$) with increased efficacy in WT Mϕ (>10,000 fold) compared to Ac2-26. The inability of LXA_4 to mediate pro-phagocytic signalling in $Fpr2^{-/-}$ Mϕ (Figure 3.8) would suggest that Ac2-26 and LXA_4 share common pharmacology through their interaction with Fpr2, in respect to this experimental model.

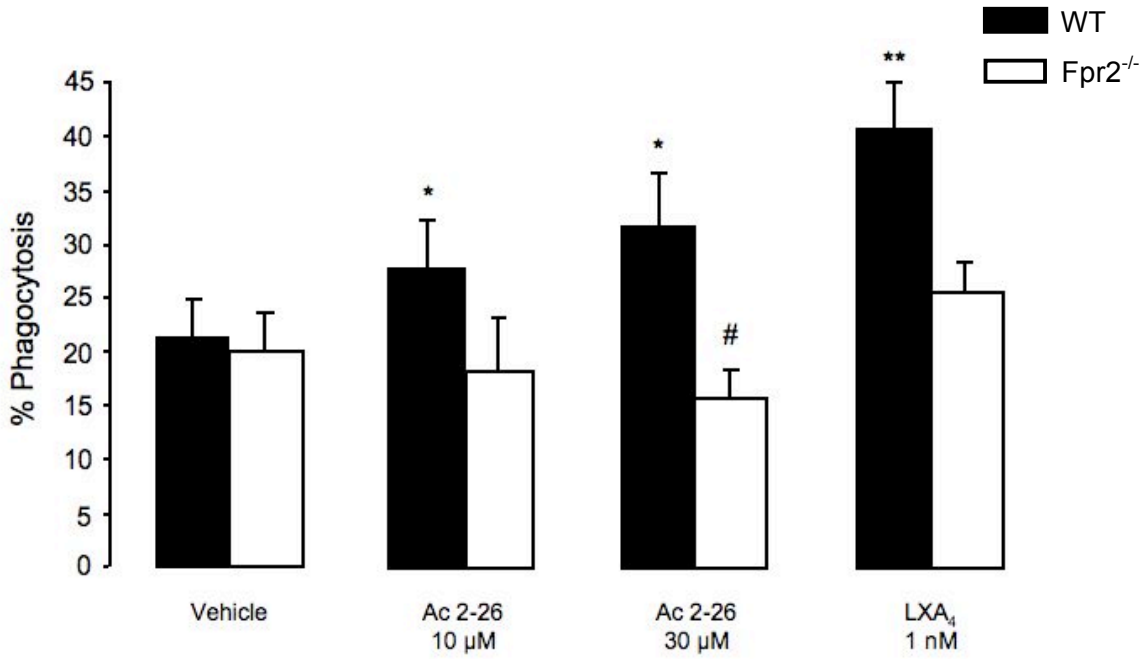


Figure 3.8. Fpr2-mediated Mϕ phagocytosis of human apoptotic neutrophils. Mϕ were treated with vehicle, Ac2-26 or LXA_4 15 min prior to the addition of human apoptotic neutrophils for 30 min. Ingested neutrophils were identified by MPO staining permeabilised Mϕ**.** Five fields (~150 Mϕ**/**field**)** of view were counted by light microscopy and an average percentage ascertained. All data is mean ± SEM of n=4-7 analysis by Student's t-test ***P*<0.01, **P*<0.05 compared to WT vehicle; #*P*<0.05 compared to WT 30μM Ac2-26 treated.

In Vivo characterisation of Fpr2 physiology

3.7 Comparison of phenotype

To ensure that Fpr2 deletion had no detrimental effects during the developmental stage of life a number of WT and Fpr2$^{-/-}$ litters were observed and documented until ~6 weeks of age.

Data pertaining to litter size and Mendelian ratios was collected from 16 litters comprising approximately 100 animals. The insertion of the transgene did not provoke any abnormality in breeding or variation to expected Mendelian ratio (Table. 3.1). The Fpr2$^{-/-}$ mice were viable, fertile and showed no obvious developmental or behavioural abnormalities.

Phenotypic Data	WT littermates	Fpr2$^{-/-}$	*P*
Litter size	7.15 ± 0.56	8.682 ± 0.58	NS
Male Births	3.62 ± 0.51	4.18 ± 0.36	NS
Female Births	3.54 ± 0.47	4.50 ± 0.45	NS
M/F ratio	1.02	0.93	NS
Weight (g) (4-6 weeks)	21.78 ± 0.51	23.22 ± 0.62	NS

Table 3.1. Phenotypic data of Fpr2$^{-/-}$ and WT littermates. Data was obtained from 16 litters, n>100 animals in WT or Fpr2$^{-/-}$ for the litter size and Mendelian analysis. The weight of animals was assessed in 26 male mice of same age. All data is mean ± SEM.

3.8 In Vivo characterisation of naïve cell populations

To understand the role of $Fpr2^{-/-}$ in inflammatory physiology it is important to clarify if resident cell populations would vary between genotypes. Two distinct cell populations, peripheral blood and peritoneal cavity, were assessed by flow cytometry to investigate cell activation and proportions of prominent phenotypic markers.

The total cell populations were characterised using Turk's stain to differentiate between leukocytes sub-populations, polymorphonuclear (PMN) and peripheral blood mononuclear cells (PBMC). Peripheral blood was seen to consist of approximately ~20% PMN of the total population (NS, ~$2.5x10^6$ cells/ml; Figure 3.9 A). The naïve peritoneal cavity consisted predominantly of PMBCs (Figure 3.10 A)

To confirm these observations three conjugated antibodies were used to characterised to each cell population. GR-1 is an antigen (Ag) marker of myeloid lineage, retained on circulating granulocytes and monocytes, particularly prominent on mature neutrophils. Monocytes and Mϕ were identified by F4/80 Ag marker with higher expression attributed to mature Mϕ phenotype (Austyn *et al.*, 1981). Both markers were used routinely throughout *in vivo* experiments discussed in my thesis. Finally CD2 (also termed LFA-2) molecule expression is common to all mouse lymphocytes. I was therefore able to assess, for each marker, the extent of Ag expression at the single cell level.

Strong GR-1$^+$ staining, measured by MFI units, in peripheral blood suggests a mature granulocyte component, as compared to more naïve phenotypes of F4/80$^+$ cells, probably denoting monocytes, or CD2$^+$ lymphocytes (Table 3.2). Resident cell derived from the peritoneum contained few granulocytes with markedly (P<0.0001; ~23 fold) lower expression profile of GR-1. Contrary to peripheral blood there was a strong increase in the expression of F4/80 (P<0.01; 20 fold), in line with the differentiation of monocytes to tissue macrophages. Finally CD2$^+$ cells showed almost twice (P<0.05) the expression of their circulating counterparts.

There were no genotypic noted (Table 3.2) in the this broad comparison of immune cells, however this should not deter further examination of subtle differences that may yet become apparent.

Blood	GR-1	F4/80	CD2
WT	1551 ± 55.5	15.7 ± 1.7	173 ± 16.2
$Fpr2^{-/-}$	1386 ± 85.7	17.8± 1.9	138± 3.2
Peritoneal Lavage			
WT	57± 6.5 ***	314± 17.8 **	255± 3.2
$Fpr2^{-/-}$	77± 22.9 ***	325± 20.4 **	246± 4.1 *

Table 3.2. Median Fluorescence Intensity (MFI) of constituent cells within peripheral blood and peritoneum. Mixed cell populations were obtained from naïve WT and $Fpr2^{-/-}$ mice by cardiac puncture and peritoneal lavage. Cell populations were stained for GR-1, granulocytes, F4/80, myeloid cells, and CD2, lymphocytes. All data is mean ± SEM of n=4-6 mice analysed by Student's t-test ***P<0.0001, **P<0.01, *P<0.05 compared to respective blood groups.

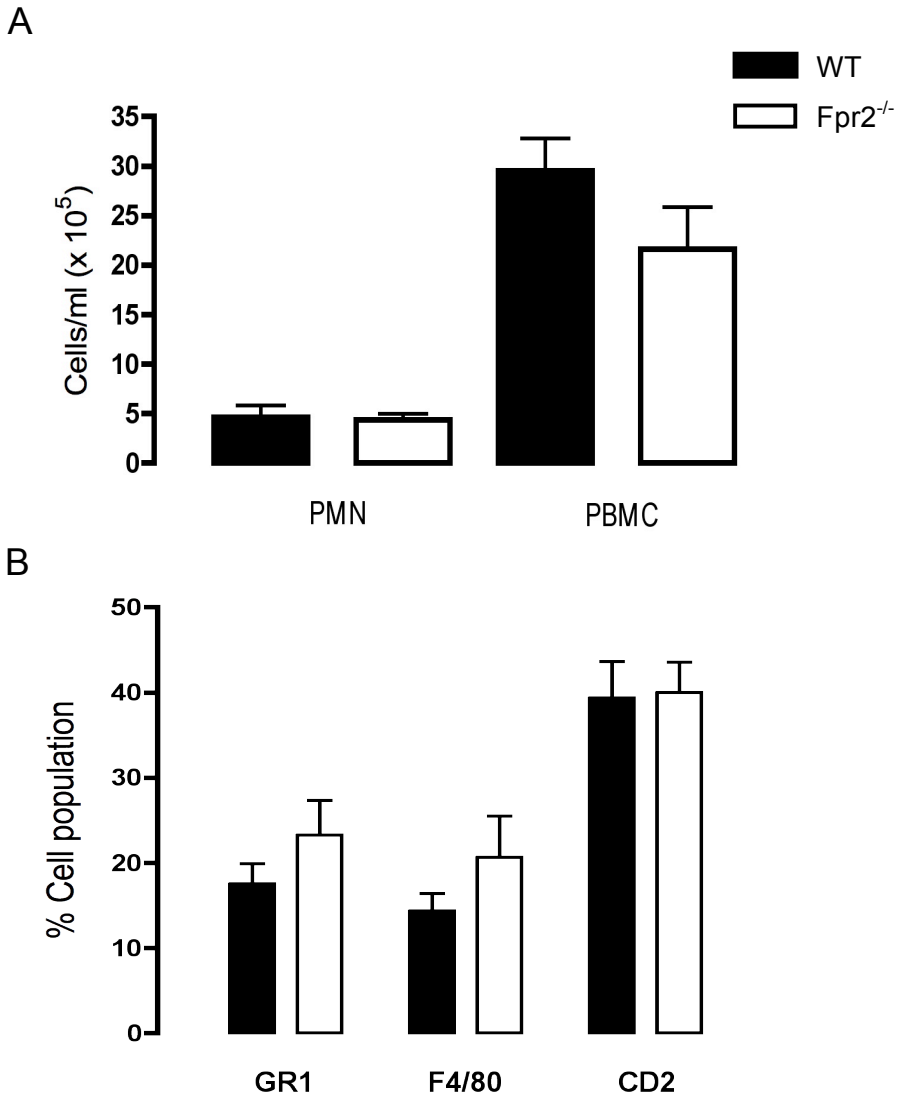


Figure 3.9. Characterisation of naïve peripheral blood cell populations by (A) differential cell counts using Turk's staining, (B) FACS was used to identify specific cell populations using GR-1 (PMN), F4/80 (monocytes/macrophages) and CD2 (lymphocytes). All data is mean ± SEM n=7.

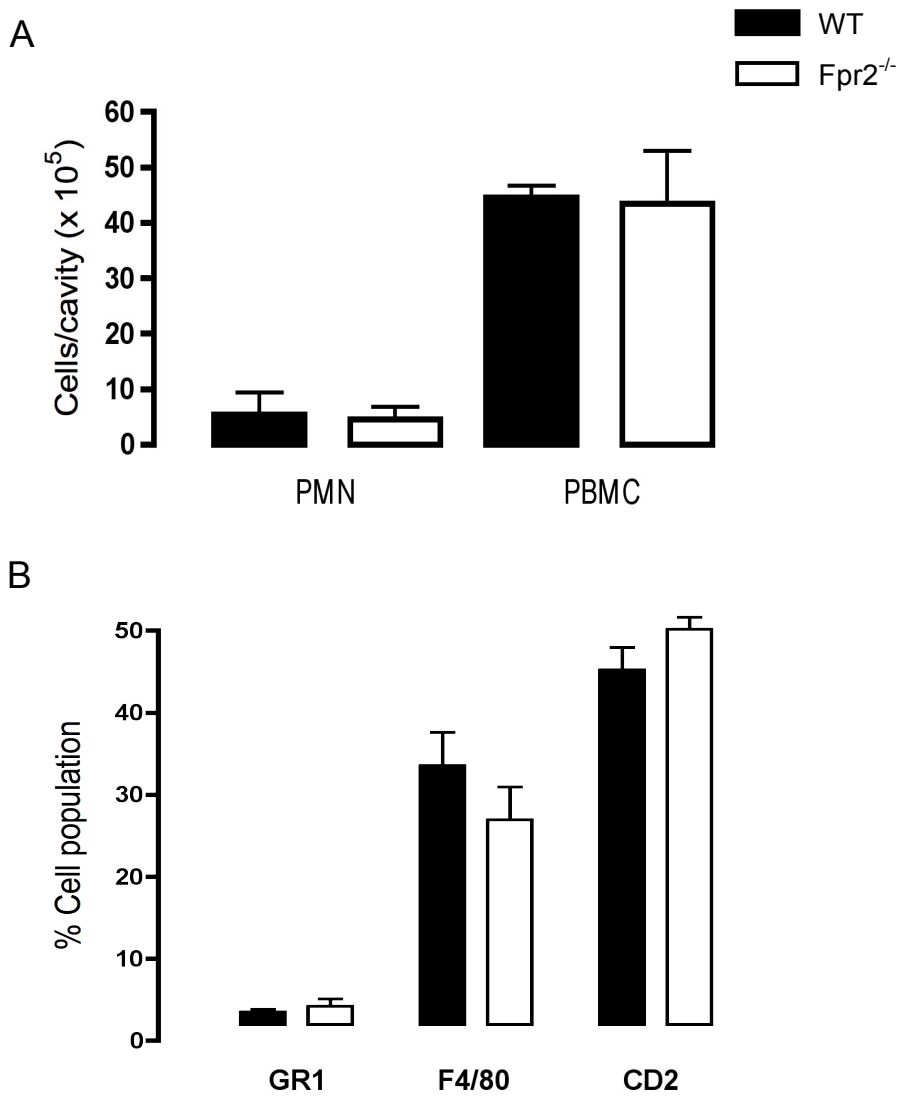


Figure 3.10. Characterisation of resident cell populations from peritoneal cavity (A) differential cell counts using Turk's staining, (B) FACS to identify specific cell populations using GR-1 (PMN), F4/80 (monocytes/macrophages) and CD2 (lymphocytes). All data is mean ± SEM n=6.

3.9 Gene transcription in naïve and acute inflammatory peritoneum

Reverse transcription PCR (RT-PCR) was undertaken to assess whether there were subsequent transcriptional discrepancies with *anxa1* (Figure 3.11 A) or *fpr1* (Figure 3.11 B) within the $Fpr2^{-/-}$ colony.

Peritoneal cells were harvested from naïve and 4 h zymosan (1mg; i.p) treated WT and $Fpr2^{-/-}$ animals. Gene transcription was investigated by quantitative PCR incorporating SYBRgreen (Ambion).

No notable differences in the expression of either *anxa1* or *fpr1* mRNA were observed between naïve or inflamed peritoneal cells for either genotype (Figure 3.11). These data do not reveal the presence of compensatory gene expression as both regulation of an endogenous ligand, AnxA1, and that of the close relative, *fpr1*, originally the putative receptor for AnxA1, remain unchanged in the absence of Fpr2.

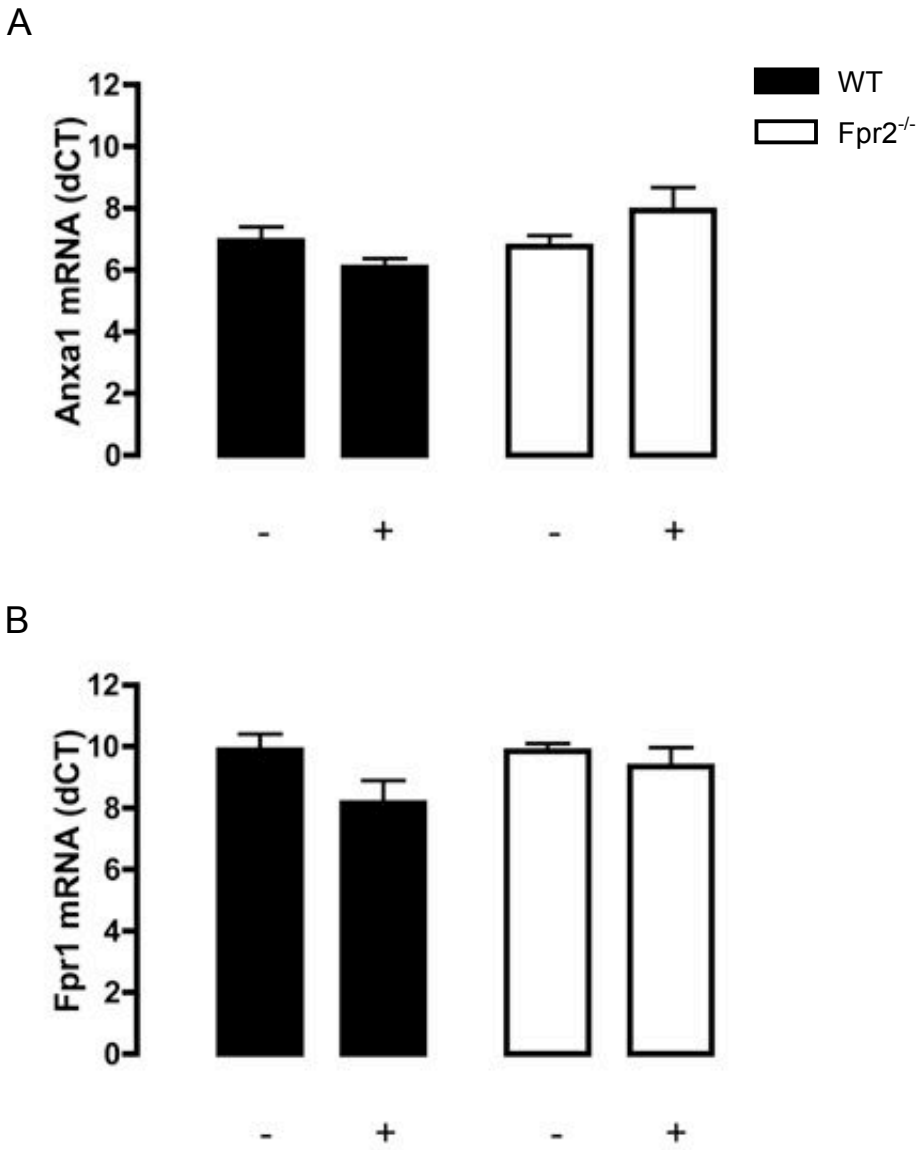


Figure 3.11. Reverse transcriptase PCR. The mRNA expression profile of the progenitor receptor *fpr1* and the Fpr2 ligand, *anxa1*, were compared to a housekeeping gene (GAPDH) in both naïve peritoneal cell (-) and cells harvested 4 h post zymosan peritonitis (+).All data is mean ± SEM n=6.

3.10 Fpr2 promoter activity in vivo

The incorporation of an in-frame GFP target/reporter construct into the promoter region of $Fpr2^{-/-}$ not only offered a novel method identifying genotype by FACS but also allowed promoter activity to be monitored in live cells *in vitro* (Section 3.2), this technique was therefore broadened to monitor a variety of *in vivo* cell populations.

3.10.1 Fpr2 promoter activity in naïve and inflammatory environments

Promoter activity, observed as GFP fluorescence (MFI units), was observed by FACS analysis in both naïve cell populations, peripheral blood (Figure 3.12 A) and bone marrow-derived Mϕ (Figure 3.12 B) used as a positive control. Furthermore I also measured inflammatory cell infiltrates from zymosan-induced peritonitis (Figure 3.12 C) and IL-1β-induced air pouch (Figure 3.12 D), indicating constitutive promoter activity of *fpr2*.

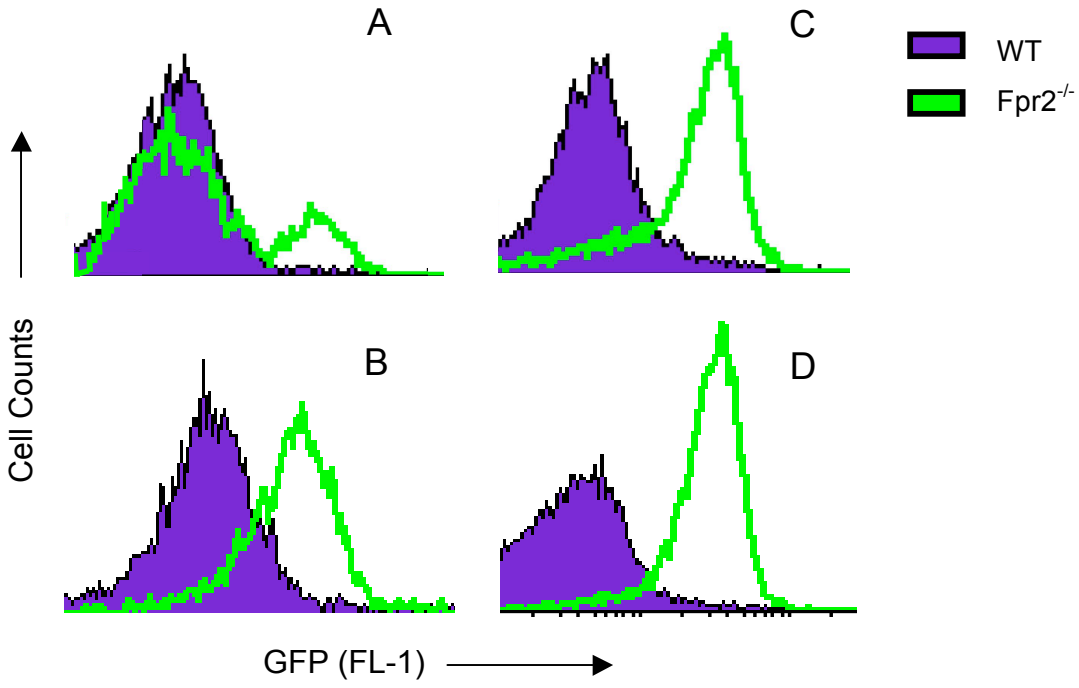


Figure 3.12. Histograms representing the promoter activity within *in vivo* cell populations in Fpr2$^{-/-}$ mice (green) compared to WT (purple) leukocytes for (A) peripheral blood (B) bone marrow-derived Mϕ (C) peritoneal cells 4 h post zymosan (i.p) (D) air pouch cells 4 h post IL-1β (s.c). Histograms are representative of 5 WT and Fpr2$^{-/-}$ mice.

3.10.2 Cellular distribution of Fpr2 promoter activity during acute inflammation

To assess the role of Fpr2 in an acute model of inflammation a variety of cell types were assessed for promoter activity following zymosan-induced peritonitis. To observe any subtle changes in GFP expression, leukocytes were permeabilised facilitating intracellular staining for GFP prior to FACS analysis.

Promoter activity was observed in both PBS- and zymosan-treated animals in peripheral blood, bone marrow and peritoneal cells. Specific leukocyte populations were determined by cell surface antigen staining and compared to total cell counts (Figure 3.13 A) to gauge proportionate cell numbers.

As later described (Section 3.13), acute zymosan-induced peritonitis is a granulocyte driven pathology with a large proportion of infiltrating cells expressing the granulocyte marker GR-1$^+$ (P<0.01; Figure 3.13 A and B). Interestingly GFP, hence *fpr2* promoter activity, was proportional increased (P<0.05) in the peritoneum in parallel to granulocyte migration. These results complement the data from the cellular compartments observed in previous histogram data (Figure 3.12).

Strong GR-1 staining is also seen within bone marrow cells denoting an immature population of myeloid cells with expression of GR-1 lost by circulating monocyte cells. Monocytes and Mϕ were identified using F4/80

staining, with a significant (P<0.05) reduction in F4/80^{high} Mϕ cells 4 h post zymosan injection (Figure 3.13 C). There was no apparent modulation of promoter activity in the mononuclear population between PBS- or zymosan-treated groups (Table 3.3).

Treatment	Peritoneal lavage		Bone marrow		Blood	
	Granulocyte	**Mononuclear**	**Granulocyte**	**Mononuclear**	**Granulocyte**	**Mononuclear**
PBS	49.6±3.1	32.6±2.2	36.3±2.6	35.3±0.3	61.6±1.6	40.3±1.8
Zymosan	53.6±1.2	34.0±2.6	33.6±0.6	33.6±1.2	78.0±2.3	33.0±1.5

Table 3.3. Median fluorescent intensity (MFI units) representing the *fpr2* promoter activity within *in vivo* cell populations in Fpr2$^{-/-}$ mice. Mice were either treated with 1mg zymosan or sterile PBS (i.p) for 4 h. Peritoneal lavage, bone marrow and peripheral blood were assessed for GFP expression by specific staining using α-GFP antibody. Granulocyte and mononuclear populations were distinguished by morphology. All data is mean ± SEM of n=3.

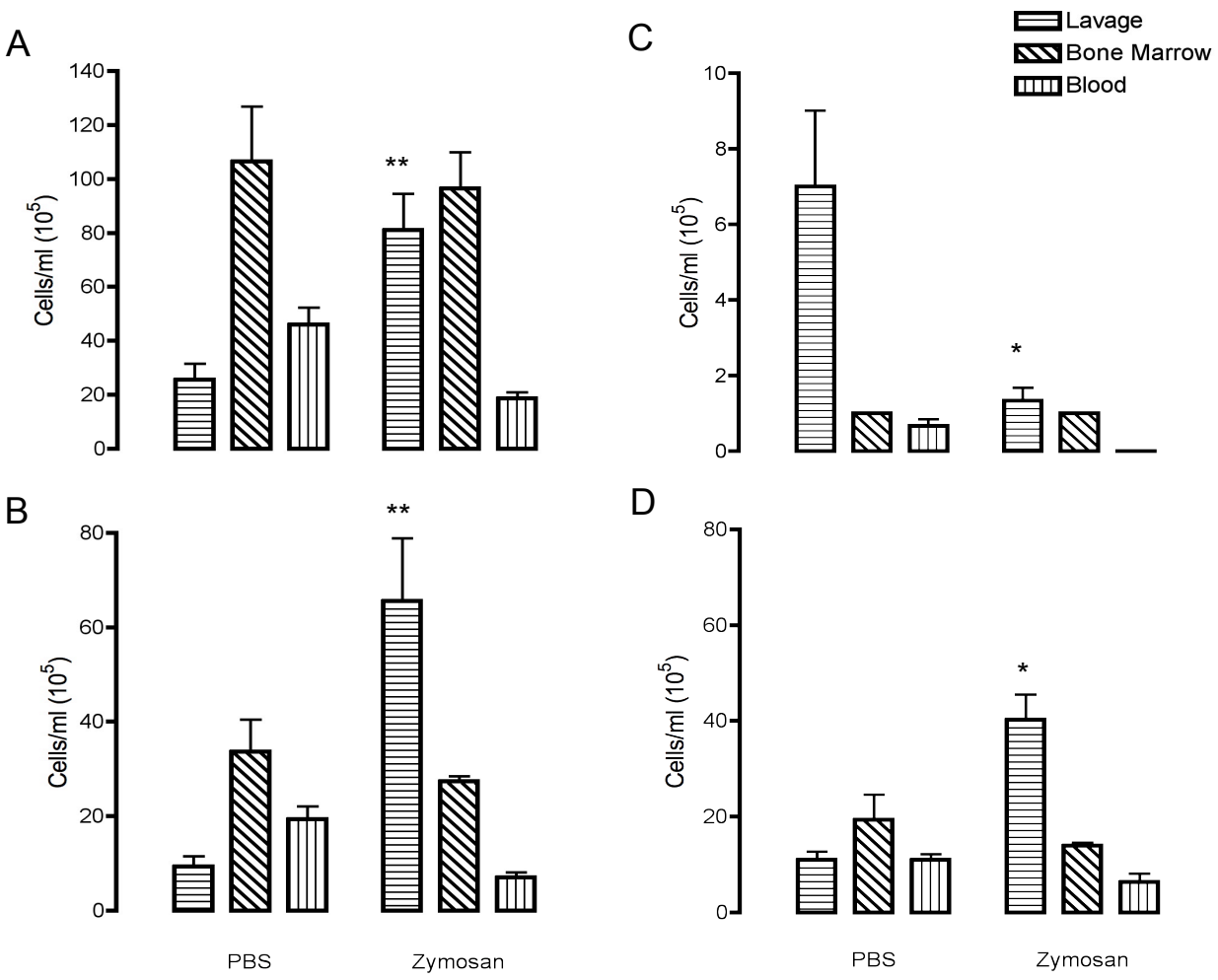


Figure 3.13. Phenotypic distribution of *fpr2* promoter activity during zymosan-induced peritonitis model. Peritoneal, bone marrow, and peripheral blood cells were assessed for promoter activity by FACS compared to WT controls. Leukocyte phenotype was assessed by (A) total cell counts, (B) granulocyte staining (GR-1^{+}), (C) F4/80 staining (D) GFP staining. All data is mean ± SEM of n=3 analysis by Student's t-test $^{**}P<0.01$, $^{*}P<0.05$ compared to PBS treated controls by Student's t-test.

3.11 Functional role of Fpr2 in IL-1β-induced acute air pouch

To investigate the functional consequences of $Fpr2^{-/-}$ deletion on the pharmacology of anti-inflammatory compounds I employed an acute, FPR-independent, leukocyte transmigration model *in vivo*.

An IL-1β-induced air pouch is a well-characterised model of acute granulocyte cell infiltrate (>80%) 4 h post induction, previously applied to both $AnxA1^{-/-}$ (Chatterjee *et al.*, 2005) and $Fpr1^{-/-}$ (Perretti *et al.*, 2001b) mice. WT and $Fpr2^{-/-}$ animals exhibited similar responses towards IL-1β in saline treated positive controls, with ~2-7x10^6 $GR\text{-}1^+$ c/ml harvested per pouch (Table 3.4).

When assessing multiple experiments it is important to qualify the variation between data sets. To ascertain whether data obtained from individual experiments could be compared using the cell counts. I assessed variation in vehicle treated IL-1-induced air pouches, using the control $GR1^+$ values of three experiments used to test 1μg/mouse AnxA1 (Table 3.4).

Although there was no variation (NS) between genotypes within a given experiment, I noted significant (P<0.05) variation across experiments when analysing each control group by one-way ANOVA (Table 3.4). I subsequently normalised data by calculating the percentage response compared to the control values obtained in each experiment.

Genotype	Experiment 1 (n=4)	Experiment 2 (n=4)	Experiment 3 (n=3)
WT	72±20.1	23.48±3.3 ∗	37±1.0
$Fpr2^{-/-}$	62±11.7	22.99±0.8 +	26±4.2 +

Table 3.4. Variation of GR-1^+ cell infiltrate to IL-1β-induce air pouch. The control response of WT and $Fpr2^{-/-}$ mice to mobilise GR-1^+ cells (x10^5/air pouch) towards IL-1β (20ng; 4 h time point) was compared in three separate experiments. All data is mean ± SEM n=3-4. **P*<0.05, compared to WT experiment 1, **P*<0.05, compared to $Fpr2^{-/-}$ experiment 1, by one-way ANOVA and Bonferroni multiple comparison test.

3.11.1 Anti-migratory action of AnxA1/Fpr2 interaction

Prophylactic treatment with hrAnxA1 (i.v), 10 min prior to 20ng IL-1β per pouch, was used to assess the concentration dependent pharmacology of AnxA1 biology.

Accumulation of GR-1$^+$ cells was significantly inhibited (0.5μg; P<0.05 – 10μg; P<0.01) across the dose range in WT mice, with a maximal response (88.5% inhibition) observed at 10μg hrAnxA1 (Figure 3.14).

There was a highly significantly (***P<0.0001) reduction of the effect of hrAnxA1 in Fpr2$^{-/-}$ mice by comparison of fit when dose-response curves were assessed using non-linear regression using the F test and global fit (Figure 3.14). Despite this, high doses of hrAnxA1, 3μg and 10μg still significantly (P<0.01) impaired GR-1$^+$ infiltrate by ~50% in both groups.

This may suggest the involvement of other receptors to mediate high dose AnxA1 pharmacology. Fpr1 was initially thought to be a putative receptor for mediating AnxA1 actions (Walther *et al.*, 2000) and would therefore be a candidate to convey this residual anti-migratory response in the absence of Fpr2.

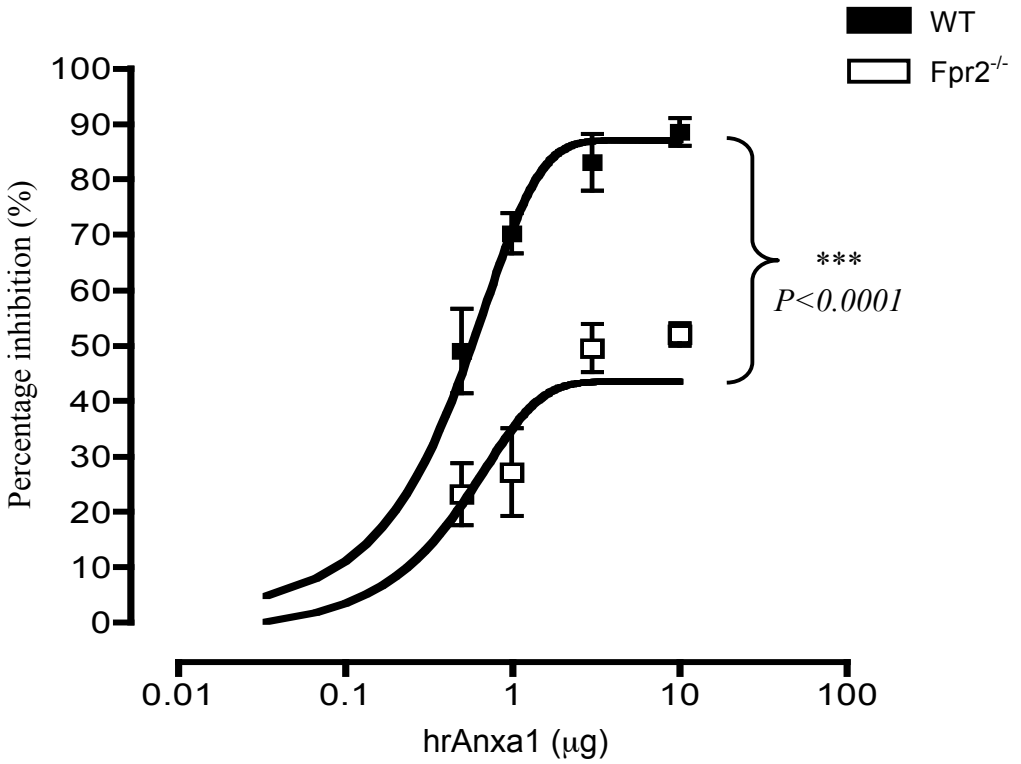


Figure 3.14. hrAnxA1 dose response curve of WT and Fpr2$^{-/-}$ PMN (GR-1+) cell infiltrate towards an IL-1β-induced air pouch. The percentage inhibitory action of prophylactic hrAnxA1 was assessed in WT (black) and *Fpr2*$^{-/-}$ (white) mice by comparison with positive control, saline treated, mice. Saline and dose range of hrAnxA1 were administrated (i.v) 10min prior to IL-1β (s.c). All data is mean ± SEM n≥4 WT and Fpr2$^{-/-}$ responses were significant across the dose range summarised by *P*<0.0001 comparison of fit following non-linear regression using F test and global fit.

3.11.2 Prostaglandin E_2 (PGE_2) release into air pouch exudates following hrAnxA1 treatment

The release of the pro-inflammatory mediator PGE_2 was assessed in the air-pouch exudates following 4 h IL-1β-induced leukocyte migration using an ELISA. Local production of PGE_2 showed no significant change in either genotype (Figure 3.15).

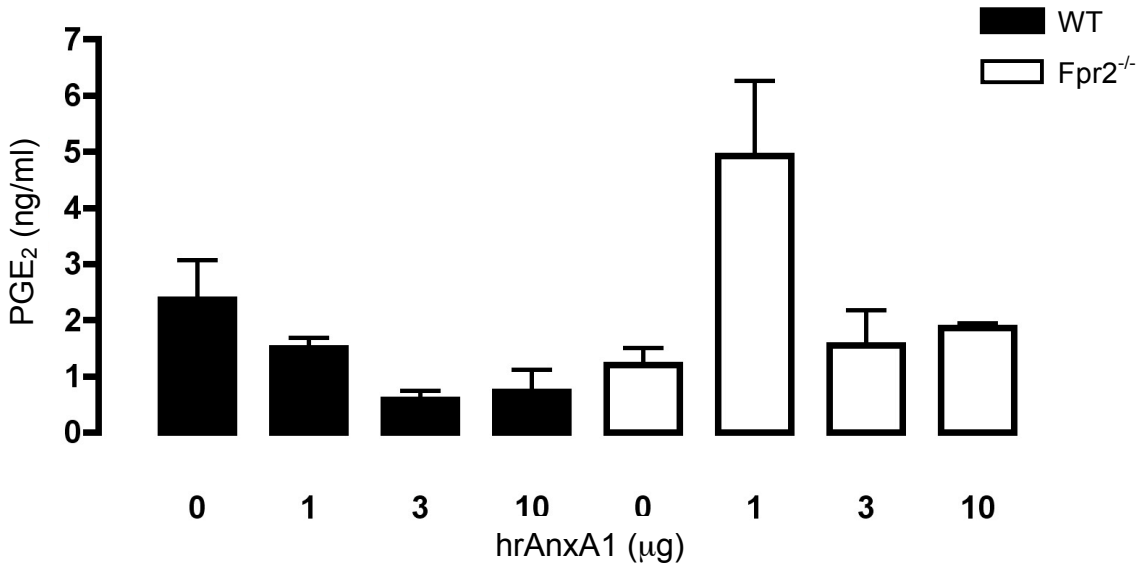


Figure 3.15. PGE_2 release into IL-1β-induced air pouch exudates. PGE_2 levels were measured by ELISA in WT (black) and *Fpr2*$^{-/-}$ (white) mice treated with a given dose of hrAnxA1 (i.v), 10 min prior to IL-1β (s.c). All data is mean ± SEM n=3-4.

3.11.3 Cytokine production in air pouch exudates following hrAnxA1 treatment

The production of chemotactic cytokines, keratinocyte-derived cytokine (KC; murine Gro-α) and Monocyte Chemoattractant Protein-1 (MCP-1) were measured by ELISA in inflammatory exudates from IL-1β-induced air-pouch.

Exudate levels of KC were significantly increased in both WT and Fpr2$^{-/-}$ following 1μg AnxA1 i.v. (P<0.01 and P<0.05 respectively; Figure 3.16 A). This trend was reduced at the highest dose of hrAnxA1 administered (10μg/animal) in both genotypes, however counter-intuitively KC levels were significantly more pronounced in the WT across the dose range (Figure 3.16 A).

There was only moderate fluctuation of MCP-1 production within the IL-1β-induced air pouch with no significant changes observed across the dose range or between genotypes (Figure 3.16 B).

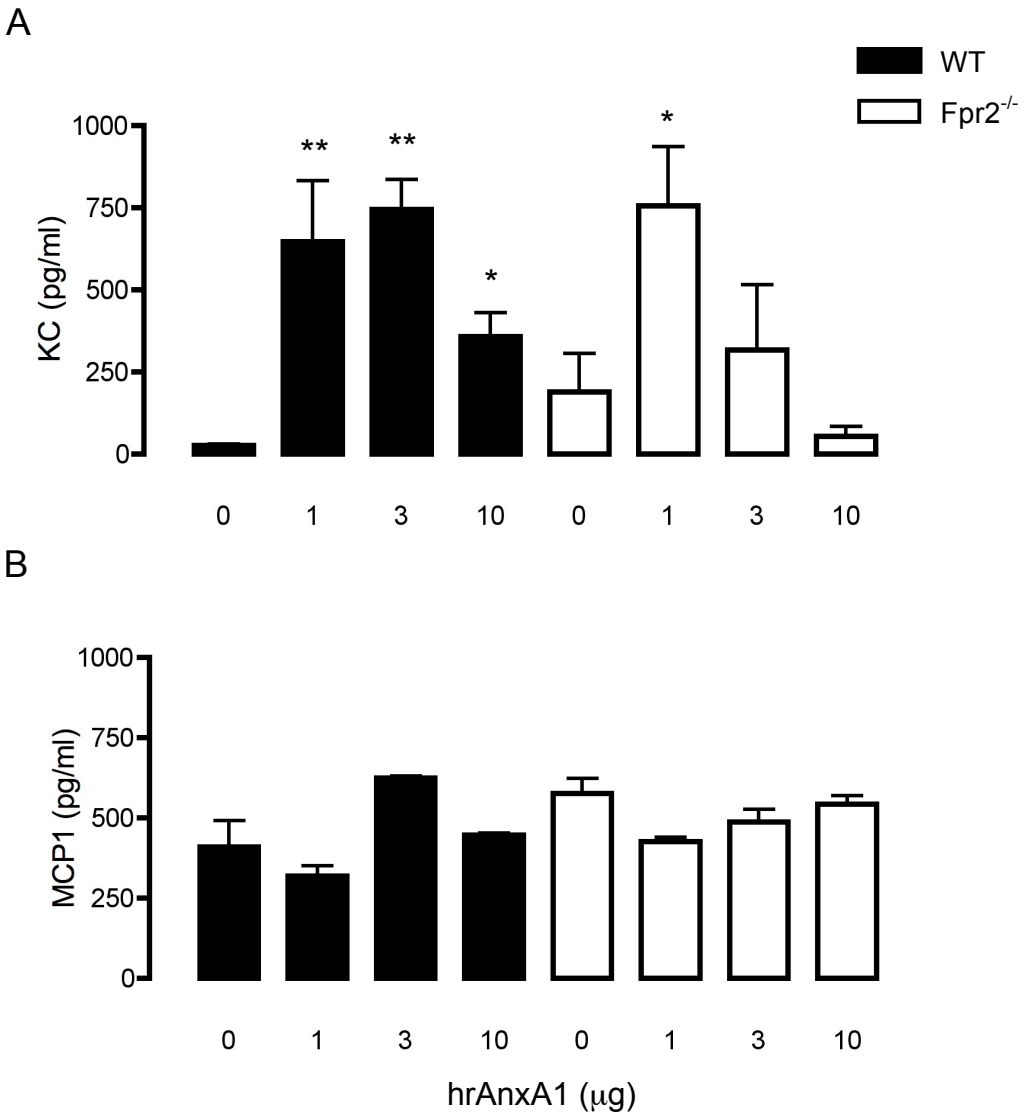


Figure 3.16. Chemokine profile following AnxA1 treatment in IL-1β-induced air-pouch. (A) KC and (B) MCP-1 levels were measured by ELISA in inflammatory exudates from air-pouches. All data is mean ± SEM n=3-4. ***P*,0.01, **P*<0.05, compared to respective vehicle treated controls by one-way ANOVA and Dunnett's post-hoc test.

3.12 The role of Fpr2 in acute leukocyte migration

To investigate Fpr2 pharmacology in acute inflammation and particularly in the context of the leukocyte transmigration a variety of pro- and anti-inflammatory ligands were assessed in both WT and $Fpr2^{-/-}$ mice.

3.12.1 Comparison of hrAnxA1 and dexamethasone in IL-1β-induced air pouch granulocyte cell infiltrate

The non-genomic anti-inflammatory effects of dexamethasone have been associated, at least partly, with rapid translocation of endogenous AnxA1 to the cell surface of neutrophil (Perretti *et al.*, 1996). Therefore it was used to compare the endogenous AnxA1 production with the direct pharmacological effects of exogenous hrAnxA1 administration.

This experiment was conducted in isolation and therefore groups are directly comparable by $GR\text{-}1^+$ cell infiltration into the air pouch. As previously established hrAnxA1 (0.5-1 μg) exhibited potent anti-migratory effects (P<0.01) in WT animals in a dose dependent manner. Dexamethasone (0.5mg/kg, s.c), administered 1 h prior to IL-1β-induced air-pouch, significantly (P<0.05) reduced PMN migration in WT mice (Figure 3.17).

$GR\text{-}1^+$ cells were not significantly affected by either hrAnxA1 or dexamethasone treatment in $Fpr2^{-/-}$ mice. This would suggest that the acute anti-inflammatory response of dexamethasone is reliant, at least in part, on

Fpr2 signalling. Altogether, these preliminary data would be in line with previous studies conducted in AnxA1$^{-/-}$ mice (Hannon *et al.*, 2003).

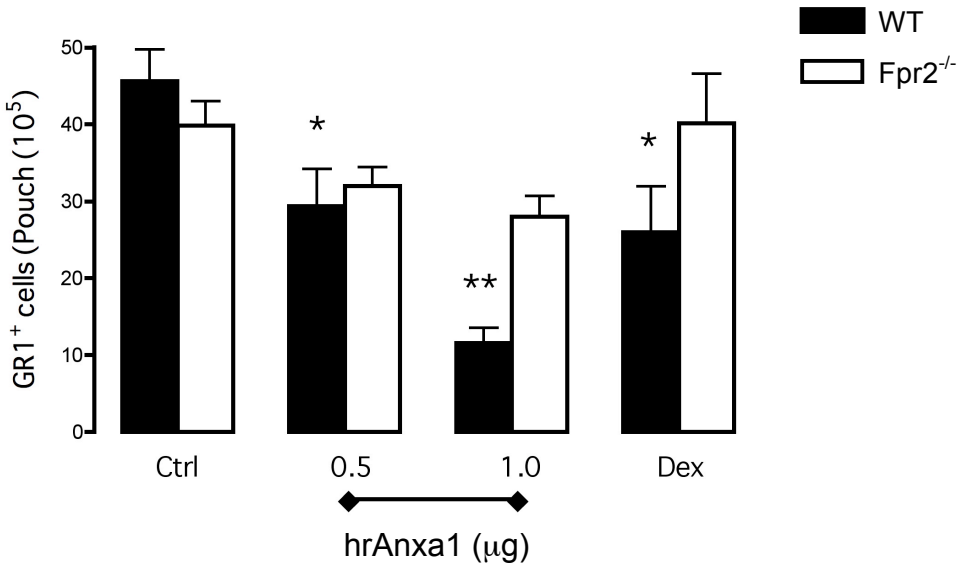


Figure 3.17. Comparative anti-migratory effects of dexamethasone and hrAnxA1 in IL-1β-induce air pouch. AnxA1 (given -10 min i.v) or dexamethasone (0.5 mg/kg, given -1h s.c) were administered prior to IL-1β (20ng) injection into the air pouch in WT (closed bars) and Fpr2$^{-/-}$ mice (open bars). All data is mean ± SEM n=5, ***P*,0.01, **P*<0.05, compared to WT untreated control by Student's t-test.

3.12.2 Comparison of Fpr2 ligands as modulator of IL-1β-induced air pouch cell infiltrate.

Similarly to my handling of the AnxA1 dose-response curve, multiple experiments are depicted here as percentage inhibition of GR-1$^+$ cell infiltrate. No direct comparison is drawn between experiments, with each data set analysed against individual experimental controls.

As observed with hrAnxA1 (1μg/mouse; *P*<0.01, 70.3%), the N-terminal peptide derivative Ac2-26 was efficacious in a dose-dependent manner when WT animals were treated with 16nmol (3μg/mouse; *P*<0.01, 52.6%) or 8nmol (1.5μg/mouse; *P*<0.05, 27.4%) when given prophylactically (Figure 3.18). Peptide Ac2-26 anti-migratory effects were not observed in Fpr2$^{-/-}$ mice at either dose tested.

LXA_4, previously found to be active in this air-pouch model (Pouliot *et al.*, 2000), was confirmed as a potent anti-inflammatory lipid, conveying significant (1μg/mouse; *P*<0.01, 52.4%) effects in WT. Again, this effect was lost in Fpr2$^{-/-}$ mice (Figure 3.18)..

C43 was originally characterised for its effects on reducing a prostaglandin E_2 and leukotriene B_4 induced-ear swelling model both via topical and systemic dosing regimes (Burli *et al.*, 2006). In the current study C43 was able to significantly inhibit leukocyte migration when a high dose was administered

p.o. (500μg/mouse; *P*<0.05, 42.6%) and, with increased efficacy, when delivered i.v. (250μg/mouse; *P*<0.001, 75.0%; Figure 3.18). This set of experiment also demonstrated that C43 did not affect leukocyte response in $Fpr2^{-/-}$ mice.

W-peptide, dosed at 12 nmols (10μg/mouse; NS, 15.0%), previously shown to be cardio-protective (Gavins *et al.*, 2005), was unable to induce a significant anti-migratory profile when in WT mice or $Fpr2^{-/-}$. W-peptide is a potent stimulator of human (Christophe *et al.*, 2001) and murine (Itou *et al.*, 2006) neutrophils *in vitro*; these actions are brought about by interaction with all members of the Fpr family including Fpr2 as reinforced by binding studies (Section 3.3). The interplay between the distinct receptors of the Fpr family *in vivo* is currently unclear.

The overall anti-migratory pharmacology of Fpr2 was contrary to the actions of SAA. The ability of SAA to instigate human and mouse neutrophil chemotaxis *in vitro* is well documented (Badolato *et al.*, 1994; Liang *et al.*, 2000). Administration of SAA (i.v). increased PMN recruitment in WT mice, complementing the pro-migratory profile of this ligand observed *in vitro* (Figure 3.6 B).

At the higher dose of 1nmol (15μg/mouse), SAA erratically increased PMN recruitment in WT mice. A more robust, and significant (*P*<0.01, -52.0%), response was observed in PMN recruitment was observed at the lower dose of 0.2nmol (3μg/mouse) in WT mice.

In $Fpr2^{-/-}$ mice, SAA administration at the top dose of 1nmol was able to significantly (P<0.01, 62.5%) inhibit leukocyte migration. This trend (NS) of an anti-migratory effect in $Fpr2^{-/-}$ mice was also apparent at the lower dose tested. This result may betray the highly promiscuous pharmacology of SAA, known to interact with a variety of receptors, however I should note there is limited literature describing anti-inflammatory properties for SAA (Renckens *et al.*, 2006; Lee *et al.*, 2006). Therefore SAA migratory properties are subverted in the absence of Fpr2, suggesting counter-regulatory mechanisms might be involved.

The cumulative experiments presented in Figure 3.18 and described above, represent a major finding of my work.

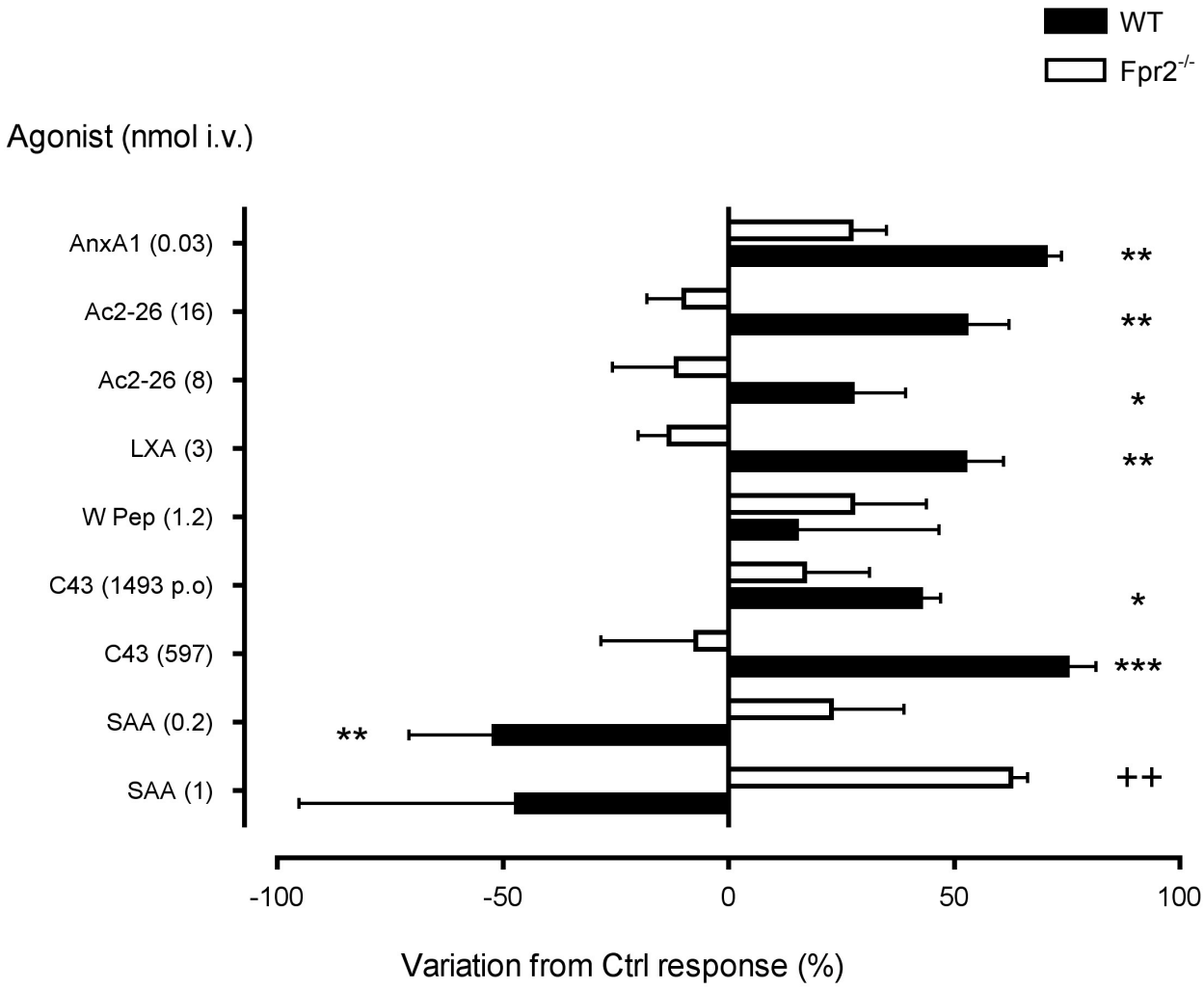


Figure 3.18. AnxA1 and other Fpr2 ligands in the air pouch model. Fpr2 ligands were given i.v. at the reported doses (nmol) with data being reported as a percentage inhibition from vehicle treated mice. The IL-1β was similar in WT and Fpr2$^{-/-}$ mice (~3 x10^{6} cells per pouch). In all cases, data are mean ± SEM of 6-12 mice per group. *P<0.05, **P<0.01, ***P<0.001 compared to respective WT control values (original numbers), ++P<0.01 compared to respective KO control values (original numbers) by Student's t-test.

3.13 Acute zymosan-induced peritonitis

Zymosan-induced peritonitis is a well-characterised model resembling the inflammatory mechanisms involved in opportunistic infection of the peritoneal cavity produced by micro-organisms in immuno-compromised patients. Acute zymosan-induced peritonitis instigates a 'classical' inflammatory cascade leading to a large increase in granulocyte cell infiltration at a 4 h time point.

3.13.1 Phenotypic response in Fpr2$^{-/-}$ mice

The role of Fpr2 pharmacology in orchestrating a complex inflammatory response was initially investigated by comparing differences between genotype following an inflammatory stimulus (1mg zymosan, i.p.) The inflammatory response observed across a number of experiments revealed a subtle but significant (32%; $P<0.05$) reduction in the ability of Fpr2$^{-/-}$ granulocyte to migrate towards zymosan at the early, 4 h, time point (Figure 3.19).

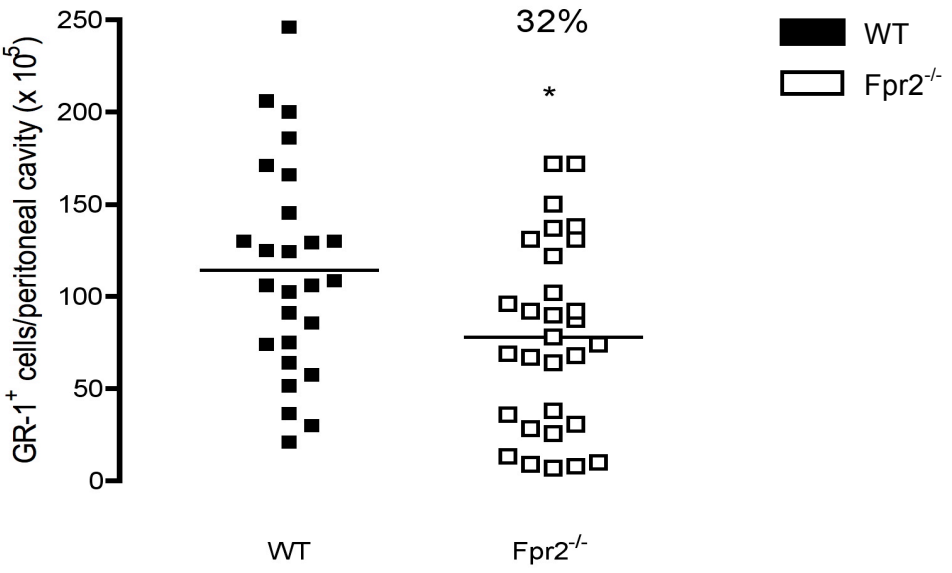


Figure 3.19. GR-1$^+$ infiltrate following 4 h zymosan-induced peritonitis. GR-1$^+$ cells counts were collated from 1mg zymosan (i.p) groups across 5 experiments (n≥26 animals). *P*<0.05 mean distrbution assessed by Student's t-test.

3.13.2 Anti-inflammatory action of AnxA1/Fpr2 interaction

To determine whether AnxA1 could modulate cellular influx to the peritoneal cavity animals were given a prophylactic dose of hrAnxA1 (1μg, i.v.)

Exogenous hrAnxA1 reduced total cells infiltrate to the peritoneum (Figure 3.20 A) with a significant (46%, *P*<0.05) reduction in GR-1$^+$ (granulocyte) cell infiltrate compared to saline-treated control animals (Figure 3.20 B). However, neither total cell infiltrate or GR-1$^+$ cell infiltrate was significantly inhibited in Fpr2$^{-/-}$ mice following hrAnxA1 treatment in respect to corresponding untreated controls (Figure 3.20 A and B).

As assessed in section 3.13 the ability of both total and GR-1^{+} leukocytes to transmigrate in vehicle treated Fpr2$^{-/-}$ animals was diminished compared to vehicle treated WT counterparts (Figure 3.19).

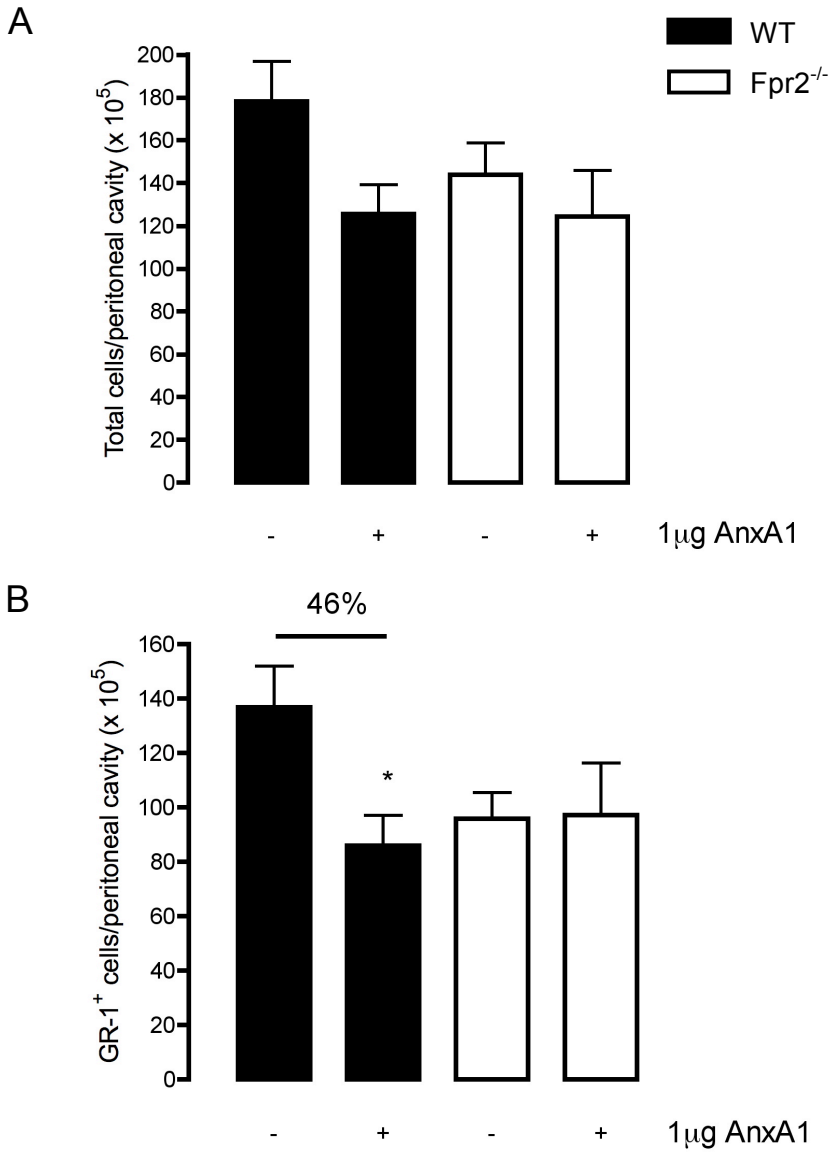


Figure 3.20. PMN (GR-1$^+$) cell infiltrate following 4 h zymosan-induced peritonitis. WT (black) and Fpr2$^{-/-}$ (white) mice were prophylactically dosed 10 min prior to zymosan with saline (-) or 1μg hrAnxA1 (+) i.v. All data is mean ± SEM n=7, **P*<0.05, compared to WT untreated control by Student's t-test.

3.14 Profile of Fpr2$^{-/-}$ in spontaneously resolving zymosan-induced peritonitis

Zymosan-induced peritonitis is equally well characterised when permitted to progress to later time points (broadly described in Section 1.2). In my thesis I have followed the progression and resolution of this model through to 120 h allowing investigation of multiple leukocyte populations in a sequential fashion to give a broader appreciation of Fpr2 pharmacology.

3.14.1 Zymosan-induced peritonitis time course

Minor alterations in cell recruitment were revealed, with a significant (P<0.05) increase in total cell and monocyte (F4/80^{low}) numbers at the 72 h time-point in absence of Fpr2 (Figure 3.21 A and C). Interestingly the increase in Fpr2$^{-/-}$ monocyte numbers was counterbalanced by the reciprocal reduction (NS) in the macrophage (F4/80^{high}) population at 72 h (Figure 3.21 D). There was no significant difference in the GR-1^{+} population between the genotypes (Figure 3.21 B), however the trend of a reduced acute (4 h) response in the Fpr2$^{-/-}$ animals was again apparent (section 3.13.1).

Histogram Insets (Figure 3.21 B and D) depict the specific regions assessed when calculating specific cell populations by FACS.

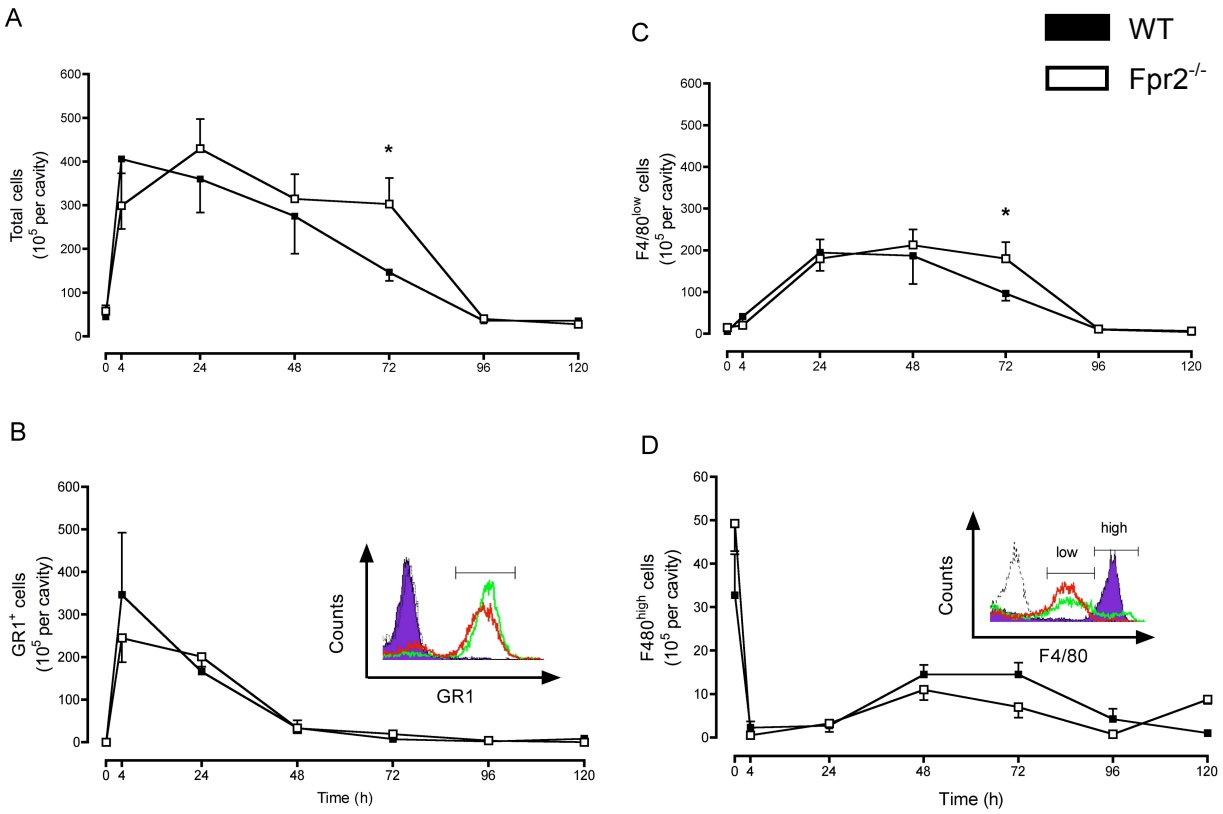


Figure 3.21. Kinetics of inflammation in the zymosan peritonitis model. WT (closed squares) and Fpr2$^{-/-}$ (open square) mice were treated with 1mg zymosan A at time 0 h. At different time-points, peritoneal lavages were analysed for cell content. Profiles of total leukocytes, Gr-1^{+} (neutrophils), F4/80^{low} (monocytes) and F4/80^{high} (macrophages) (panels A to D, respectively) are shown. Inset: representative histograms of WT (red) and Fpr2$^{-/-}$ (green) showing cell type identification of (B) GR-1^{+} staining at 4 h, (D) F4/80 staining at 72 h compared to an isotype control (open) and 0 h stained cell population (purple). Data are mean ± SEM of 4-8 mice per group. *P<0.05 compared to respective WT values.

3.14.2 Profile of inflammatory markers in spontaneously resolving zymosan-induced peritonitis

To further investigate subtle genotypic differences between WT and Fpr2$^{-/-}$ animals, the release of inflammatory markers into the inflammatory exudates were measured.

Myeloperoxidase (MPO) enzyme activity in exudates was assessed as a measure of neutrophil activity and accumulation. MPO activity peaked at 4 h in WT animals, but was delayed until 24 h in Fpr2$^{-/-}$ and was maintained at a higher level across the time course compared to the WT profile (NS; Figure 3.22 A). This profile reflects the reduced cellular infiltrate observed in Fpr2$^{-/-}$ mice at 4 h (Figure 3.19) suggesting a delay in the onset of inflammation. Furthermore the extended activity of neutrophils in the peritoneum of Fpr2$^{-/-}$ mice may contribute the persistence of both total infiltrating leukocytes and an increase magnitude in recruitment of monocytes by zymosan (Figure 3.21).

To assess whether absence of Fpr2 could modulate the local micro-environment to mediate increased cellular infiltrate, the levels of two inflammatory cytokines, KC and IL-6, were measured by ELISA.

KC, murine orthologue of Gro-α, is a potent neutrophil chemoattractant (Bozic *et al.*, 1995) that is produced acutely with levels peaking at the 4 h time point. Similar secretion profiles were established for both WT and Fpr2$^{-/-}$ mice at 4 h, with no secretion observed at any other time point during the inflammatory

response. This would suggest that there is no direct effect of Fpr2 in the production or regulation of KC following zymosan-induced peritonitis by resident cells or activated PMN.

IL-6 is traditionally considered an activator of acute phase response (Jones, 2005) and therefore a interesting candidate cytokine for conveying some of the Fpr2 mediated cell signalling. As noted with KC, IL-6 followed a very acute profile with levels rapidly increasing to peak at 4 h. Both genotypes adhered to similar profiles with marked reduction in IL-6 levels at 24 h until the experiment was terminated at 72 h (Figure 3.22).

The rapid induction and metabolism of both KC and IL-6 strongly correlate with acute neutrophilia to the peritoneal cavity and therefore suggest a distinct role for activated neutrophils in regulating cytokine production in this model of inflammatory peritonitis.

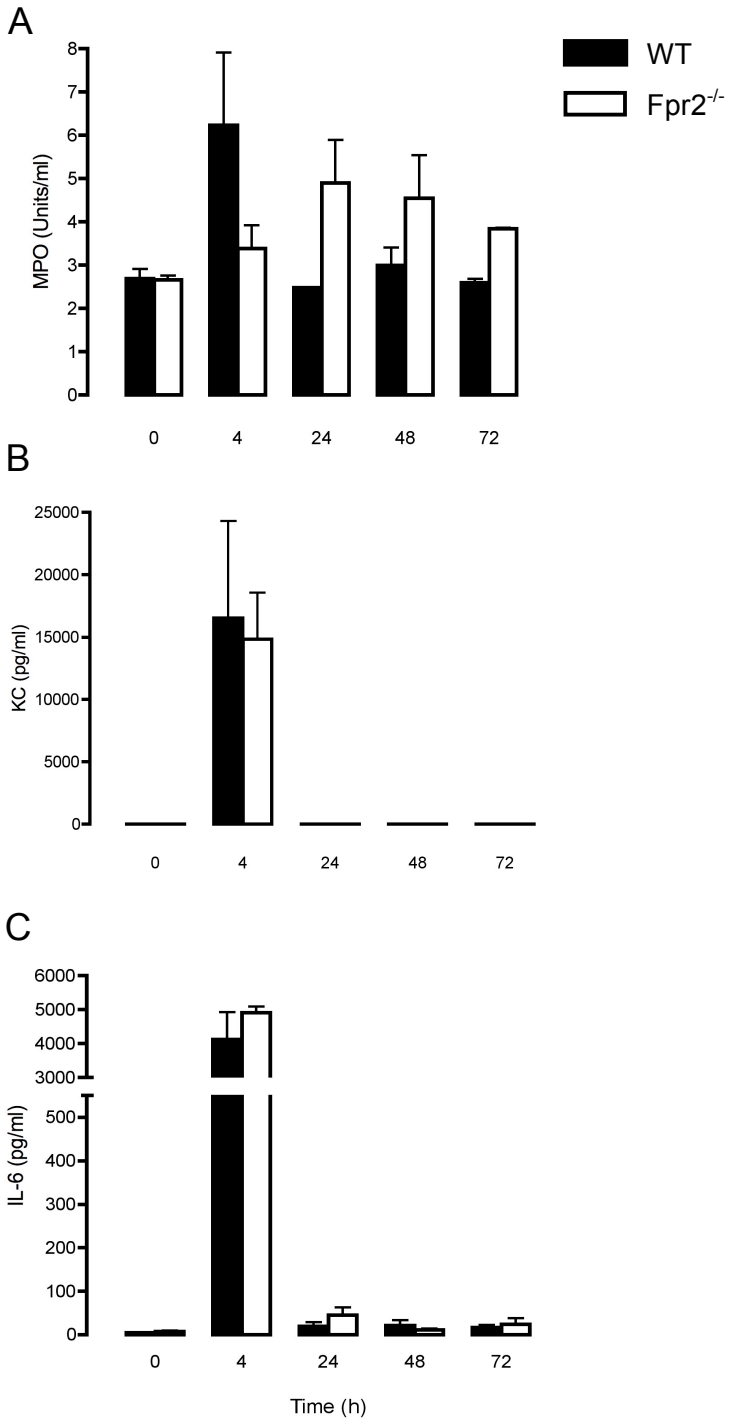


Figure 3.22. Inflammatory markers zymosan time course. Inflammatory exudates obtained from WT (black) and Fpr2$^{-/-}$ (white) animals were assessed for (A) MPO by enzyme activity assay (B) KC and (C) IL-6 by ELISA. Data are mean ± SEM of 4-8 mice per group.

3.14.3 Profile of endogenous Fpr2 ligands in spontaneously resolving zymosan peritonitis

Expression of endogenous Fpr2 ligands, SAA, LXA_4 and AnxA1, within peritoneal exudates was assessed throughout zymosan-induced peritonitis time course.

Exudate LXA_4 levels changed mildly peaking at 4 h before gradually subsiding to basal levels at 72 h. Although the level of LXA_4 are very low compared to the presences of other Fpr2 ligands measured, namely AnxA1 and SAA, they are in accordance with previously described levels in a peritonitis model (Serhan et al 2000). There were no significant differences between the genotypes during peritonitis, however naïve levels of LXA_4 in $Fpr2^{-/-}$ mice were significantly lower (-45%; $P<0.05$) than those measured in WT mice (Figure 3.23 A).

SAA was present in considerable quantities at 4 h (~7μg/cavity), and peaked at 24 h post-zymosan (Figure 3.23 B). The absence of Fpr2 led to a significant ($P<0.01$) ≥3-fold increase in SAA levels in the 24 h exudates (Figure 3.23 B). Whereas changes in plasma SAA levels have been shown to increase >1000 fold reaching as high as 1mg/ml (He *et al.*, 2008), making it an ideal diagnostic tool in the clinic, local modulation of this acute phase protein is poorly characterised.

Unfortunately there is no ELISA available for quantitative measurement of murine AnxA1 so levels of production by each genotype were monitored comparatively using western blotting. Secretion of AnxA1 into inflammatory exudate was present in large amounts at both 4 and 24 h post-zymosan in both WT and $Fpr2^{-/-}$ mice. Interestingly AnxA1 secretion appeared slightly elevated (NS) in $Fpr2^{-/-}$ exudate, which might suggest a subtle modulation in AnxA1 release through Fpr2. As with inflammatory cytokine production (Figure 3.22), AnxA1 secretion closely mapped the infiltration and clearance of leukocytes to the peritoneum (Figure 3.23C).

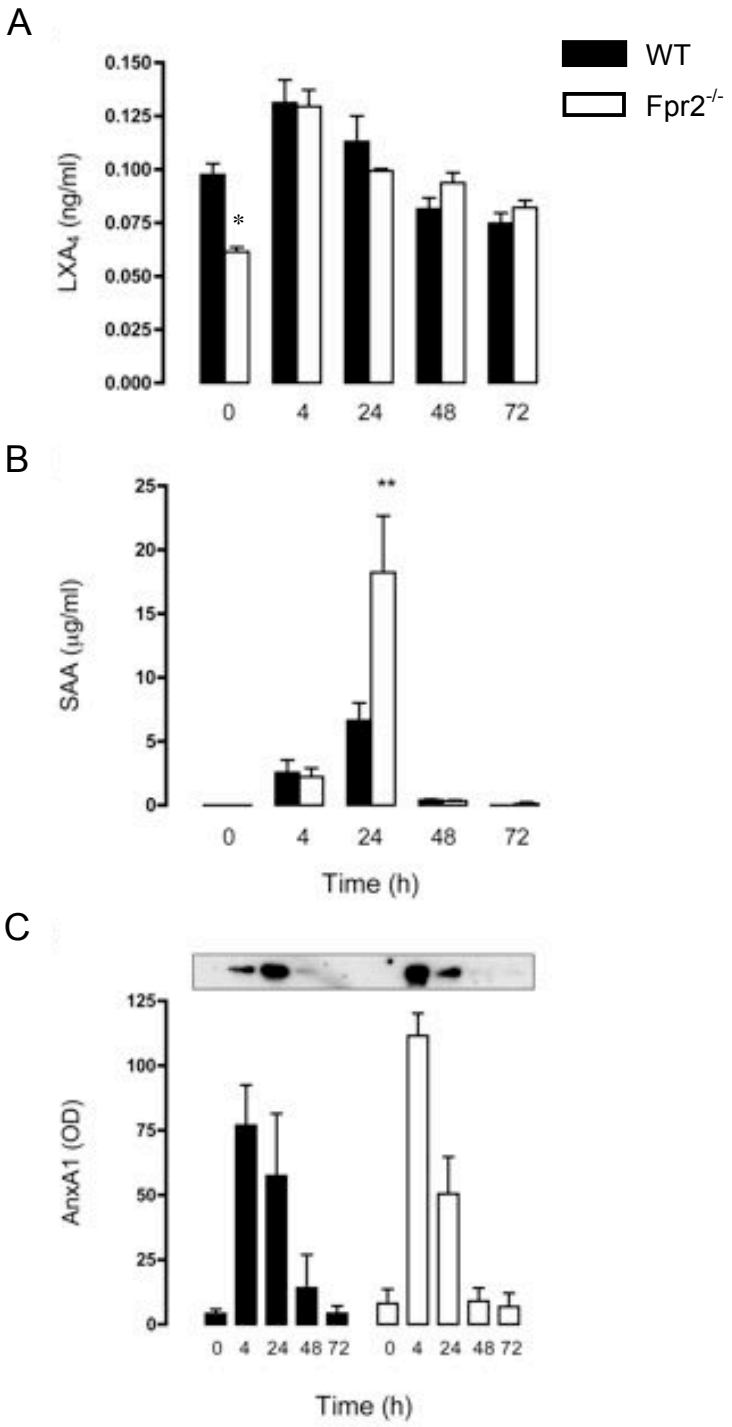


Figure 3.23. Evidence for subtle Fpr2-related circuits in peritoneal inflammation. Exudate LXA4, SAA, and AnxA1 were detected by ELISA, EIA and western blotting with densitometric analysis, respectively (panels A to C). Data are mean ± SEM of 4-8 mice per group. ***P*<0.01, **P*<0.05 by student's t-test compared to respective WT group.

3.14.4 Modulation of Fpr2 circuitry in inflammation

The presence of SAA within the peritoneal exudate, augmented by Fpr2 deficiency, led us to hypothesise that local SAA expression could be modulated via Fpr2 regulated feedback.

SAA, administered to WT animals (1μg; i.p) 10 h post zymosan, resulted in a significant (*P*<0.01) increase in endogenous SAA measured in the peritoneal lavage at 24 h (Table. 3.5), attaining similar levels to those observed in $Fpr2^{-/-}$ mice (Figure 3.23 B). Following the increase in endogenous SAA, there was a significant increase in both total cell (*P*<0.01) and macrophage ($F4/80^{high}$; *P*<0.05) numbers at 72 h post zymosan (Table 3.5).

To assess whether this could be attributed to a direct or indirect effect of SAA I monitored IL-6 levels, since this cytokine has been proposed to afford the switch between PMN and Monocyte/Macrophages in acute inflammation (Romano *et al.*, 1997). Exudate IL-6 levels were measured by ELISA following pharmacological intervention with Fpr2 ligands, as a potential way to impact on the resolution phase of zymosan-induced peritonitis.

At the time of local SAA or C43 treatment (10 h; 1μg i.p.) IL-6 levels had diminished from its peak at 4 h (Figure 3.22) and further declined in both WT and $Fpr2^{-/-}$ animals in a time-dependent fashion. WT IL-6 showed a trend (NS) to be reduced following C43 treatment, with SAA mediating a mild increase

(NS) in IL-6 levels compared to control (Figure 3.24). $Fpr2^{-/-}$ exhibited no notable fluctuation in IL-6 production following either treatment.

Treatment	Exudate SAA	Leukocyte Number ($x10^5$; 72 h)			
	(24 h; µg/ml)	Total	GR-1$^+$	F4/80^{low}	F4/80^{high}
Vehicle	8.5 ± 1.7	39.0±1.7	11.3±0.9	17.3±1.7	2.3±0.5
SAA	20.2 ± 2.70**	74±13**	7.3±0.7	29.5 ± 5.6	7.3±1.9*

Table 3.5. Effects of exogenous SAA on delayed cell recruitment in the zymosan peritonitis model. Animals were injected with either vehicle (100 µl) or SAA (1µg i.p) 10 h post-zymosan (1 mg at time 0). Inflammatory exudates were harvested from vehicle- and SAA-treated groups at 24 h (to assess local SAA levels by EIA) and at 72 h (to monitor recruitment of leukocyte populations). Data are mean ± SEM of 4 mice per group. *P<0.05, **P<0.01 compared to respective vehicle values.

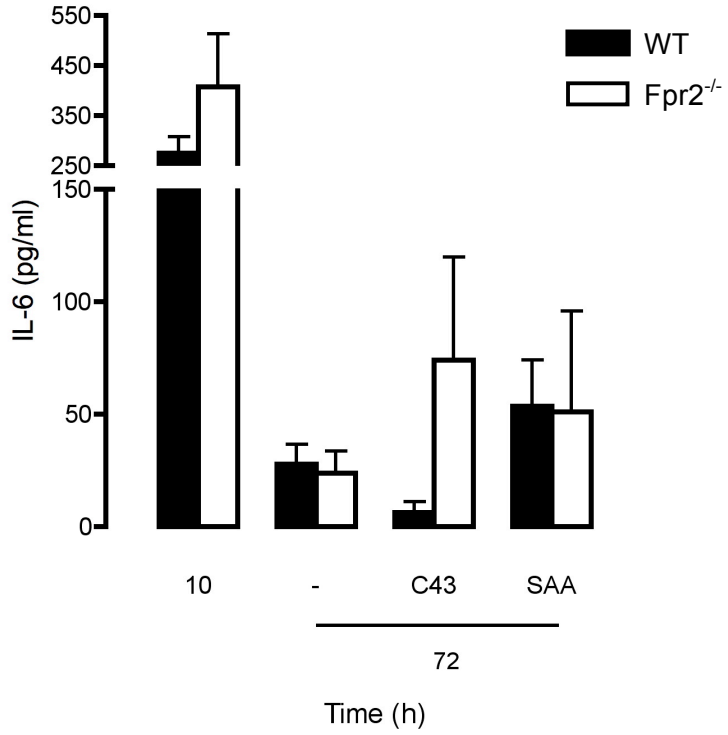


Figure 3.24. Effect of Fpr2 ligands on IL-6 production within zymosan-induced inflammatory exudates. IL-6 levels were assessed by ELISA prior (10 h) or subsequent to local (i.p) administration of vehicle, 1μg C43 or 1μg SAA (72 h). Data are mean ± SEM of 4 mice per group.

3.15 K/BxN-induced arthritis in Fpr2$^{-/-}$ mice

To Investigate the role of Fpr2 pharmacology in a more chronic model of inflammation, arthritis was induced by administration of K/BxN serum, which provokes a rapid arthrogenic response highly reliant on innate immunity cells (Ji *et al.*, 2002). This model induced disease within the first five days of treatment, with a peak of disease occurring at day 7 (Figure 3.25A). The pathology steadily begins to resolve over the subsequent ~15 d and experimental measurements were ended after 30 days.

Neutrophils and myeloid cells have been shown to be integral to the disease pathogenesis, with selective neutrophil (Wipke *et al.*, 2001) or Mϕ depletion (Solomon *et al.*, 2005) making mice resistant to arthritis. This model was therefore a logical progression from having noted a disregulation in both acute neutrophil infiltration and monocyte/Mϕ activation during resolving peritonitis.

Fpr2 animals were significantly (P<0.05) more susceptible to arthritic disease following administration of K/BxN serum (Figure 3.25B). Although the disease profiles of WT and Fpr2$^{-/-}$ genotypes showed similarities, Fpr2$^{-/-}$ mice developed significantly (P<0.05) exacerbated symptoms and prolonged disease (Figure 3.25 A). The inflammatory response in WT animals subsided by 18 d, but remained high in Fpr2$^{-/-}$ mice until termination of the experiment at 30 d (Figure 3.25B).

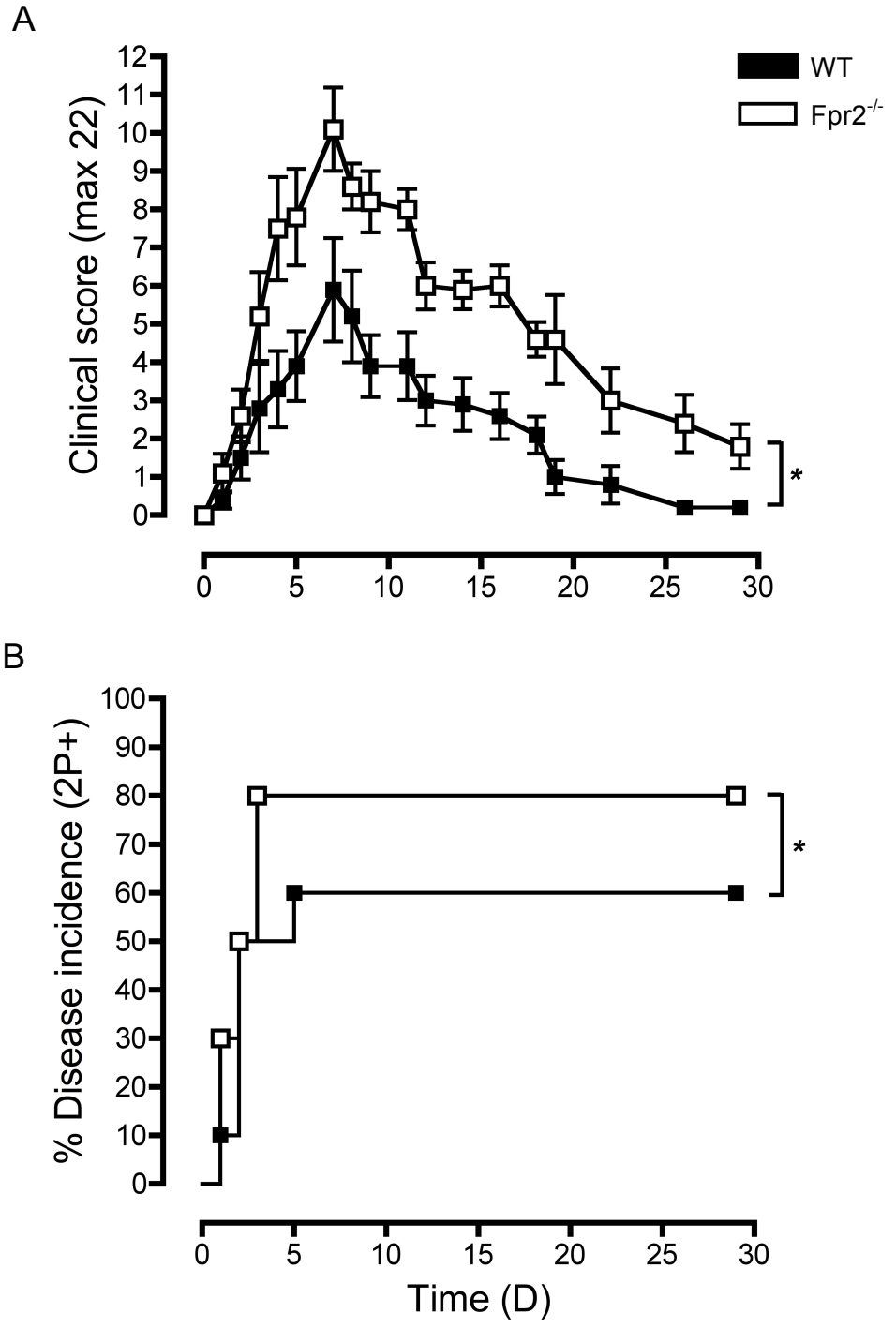


Figure 3.25. Passive serum induced arthritis: exacerbation in Fpr2$^{-/-}$ mice. Mice received 200 μl i.p. of arthrogenic K/BxN serum. (A) Time-course of the clinical arthritic score in wild type (n=6) and Fpr2$^{-/-}$ (n=6) mice; mean ± SEM; *P <0.05, 2-way ANOVA; (B) Cumulative disease incidence (cut off score ≥3); **P*<0.05, Log rank test.

Chapter 4.

Discussion

The formyl peptide receptor family is emerging as a group of key regulators in both host defence and tissue homeostasis. The novel characteristic of multiple ligand interactions has pushed the field forward, prompting the generation of pharmacological tools and transgenic strategies, both partly exploited in this thesis. Primarily I have focused on the initial characterisation of a novel colony of Fpr2 null mice, validated in both cell based assays as well as acute and chronic patho-physiology to decipher the pharmacology of this receptor.

In line with the aims of my thesis I have described modulation of *fpr2* promoter activity *in vitro* and *in vivo,* which corroborates appearance of an inflammatory phenotype in $Fpr2^{-/-}$ mice. Furthermore I have tested the multifaceted actions of both endogenous and synthetic ligands, with functional crossover between human FPR2/ALX and murine Fpr2. This is an important initial characterisation of a unique transgenic tool that could represent a significant advance for screening Fpr2-selective therapeutics.

4.1 Naïve $Fpr2^{-/-}$ phenotype

To validate our novel transgenic we confirmed incorporation of the vector and specificity for silencing *fpr2* by southern blot and PCR. To determine any compensatory mechanisms in the $Fpr2^{-/-}$, the closely homologous *fpr1* expression was compared between genotypes. Both initial characterisation and subsequent comparisons of *fpr1* and *anxa1* mRNA expression in naïve

and inflamed WT and Fpr2$^{-/-}$ mice revealed no palpable compensatory mechanisms within the AnxA1/FPR pathway in our transgenic.

The absence of Fpr2 had little observable effect on the phenotype under naïve conditions. Healthy litters were produced with expected Mendelian ratio, mice grew normally with no adverse effects on spontaneous infection, size or weight gain. The assessment of generic antigen markers and proportions of circulating immune cells were unaffected, with observations matching those in the Fpr1$^{-/-}$ colony (Gao *et al.*, 1999). Furthermore in a more mature tissue-specific cell population from the peritoneum there was no difference between genotype. Although detailed studies on gene expression have not been undertaken on either of these transgenic colonies this preliminary evidence would imply that the *fpr* gene family is not crucial to healthy development within a pathogen-free environment. A comparable assessment undertaken in the AnxA1$^{-/-}$ transgenic mouse revealed a similar conclusion (Hannon *et al.*, 2003); however a stringent examination revealed increased expression of pro-inflammatory enzymes COX-2, cPLA$_2$ and iNOS in lung and thymus (Wells *et al.*, 2004). Future studies may address the potential subtle impact of Fpr2 deletion on the naïve phenotype.

4.1.1 fpr2 *promoter activity*

A number of question marks still surround the functional similarities between the human FPR and murine Fpr family. Initial studies of FPR2/ALX noted a similar expression profile to FPR1, potentially attributable to cross reactivity of

the human antibody used (Becker *et al.*, 1998). Although there is considerable homology between human (FPR1 and FPR2/ALX) and mouse receptors (Fpr1 and Fpr2, respectively) currently there are no murine antibodies commercially available. Tissue and cellular expression is therefore restricted to the investigation of mRNA extraction by RT-PCR, cellular expression profiles are largely restricted to human.

In the mouse, the *fpr* gene cluster (on chromosome 17) has undergone differential expansion, so that currently seven genes have been identified (Le *et al.*, 2002). Many of the additional murine members remain orphan receptors with little currently know about their expression or function. To address the question of Fpr2 promoter activity in mouse the colony was developed using a target/reporter strategy following the successful development of the AnxA1$^{-/-}$ mouse containing a LacZ reporter construct (Hannon *et al.*, 2003). For the Fpr2$^{-/-}$ mouse a target vector was generated incorporating a GFP construct 'in-frame' within the promoter region. This fluorescent approach has two distinct advantages over the more common LacZ constructed applied in the AnxA1$^{-/-}$ mouse. Primarily fluorescence can be monitored within living cells using sensitive flow cytometry technique, therefore not requiring time consuming histological analysis. Furthermore it offers the potential to develop AnxA1/Fpr2 null double transgenic colony without compromising the specificity of either tool.

With our novel target/reporter transgenic I was able to further confirm the presence of Fpr2 promoter activity in both naïve, inflamed and *ex vivo*

proliferation of Mϕ. It was striking to note that within peripheral blood the predominant population of GFP^+ cells were granulocytes. An observation underlined by promoter activity increasing proportionately to granulocyte infiltration to the peritoneum following zymosan stimulation. Interestingly, despite the apparent increase in proportion of leukocytes with *fpr2* promoter activity, there was no significant change in the intensity of promoter activity during acute inflammation. There are a number of possible scenarios that could contribute to this outcome. Firstly there is constitutive *fpr2* promoter activity *in vivo* in mature granulocytes, as both naïve peripheral blood and activated infiltrating PMN had similar GFP expression. Fpr2 shares traits with classical chemotactic receptors, such as its relative Fpr1, and therefore is prone to heterologous and homologous desensitisation (Tiffany *et al.*, 2001). It may be reasonable to speculate that translocation, internalisation and recycling of Fpr2 may play a more dramatic role in functional regulation of Fpr2 by PMN than the contribution of nuclear transcription.

Secondly there is the possibility that *fpr2* regulates its own expression. Therefore I decided to investigate *fpr2* promoter activity in a naïve myeloid cell population of bone marrow to ascertain whether we could synthetically modulate promoter activity during maturation towards mature Mϕ. This population was chosen as a robust model of cell maturation as well as containing a high proportion of pre-granulocyte cells that could address pre-disposition for a given phenotype. Promoter activity was shown to gradually increase across the maturation time-course within the Mϕ population. This data revealed that promoter activity could indeed be modulated within a

discrete cell population, identifying myeloid cell differentiation as an important aspect of Fpr2.

Mϕ *fpr2* promoter activity could be induced or reduced by LPS or prednisolone respectively. Both LPS and GC have previously been shown to directly modulate TNF-α by regulating NFκB signalling, with a noted augmentation of TNF-α release from AnxA1$^{-/-}$ Mϕ, suggesting a regulatory role for AnxA1 (Yang *et al.*, 2009). Both LPS and TNF-α have been shown to up-regulate *fpr1* mRNA expression in murine peritoneal Mϕ, an action not shared with IL-1β or IL-6 (Mandal *et al.*, 2005). Furthermore LPS and TNF-α have been demonstrated to up-regulate *fpr2* mRNA in microglia (Cui *et al.*, 2002a) and fibroblast-like synoviocytes (O'Hara *et al.*, 2004). The data obtained would corroborate the underling themes that murine Mϕ are sensitive to LPS and TNF-α-induced NFκB activation to influence *fpr2* gene transcription. However we did not note a disproportionate production of TNF-α in the absence of Fpr2. Overall these observations would implicate the presence of Fpr2-independent regulation of promoter activity within this cell type.

The action of prednisolone in this assay is contrary to a previous study using the human peripheral blood mononuclear cells, which revealed a ~2 fold increase in FPR2/ALX mRNA expression 4 h post treatment (Sawmynaden *et al.*, 2006). This discrepancy may imply differential species or cell-specific regulation, which could underlie the physiological complexity of Fpr2. Despite this anomaly, collectively these data technically validated our approach *in vitro*.

Thirdly it has been noted by other groups that although GFP is a stable fluorescent protein it emits at a relatively weak intensity particularly if the promoter of interest is not ubiquitous within the cell (Ikawa *et al.*, 1998). The weak signal detected in granulocytes could therefore be masked by auto-fluorescence often attributed to intracellular vesicles, prominent in granulocyte morphology. Furthermore fluorescence intensity of GFP is strongly reliant on protein conformation (Tanudji *et al.*, 2002), which could not be guaranteed within our model. To confront the possibility that one or all of these processes may interfere with the detection of subtle changes in GFP fluorescence I used an antibody-based approach to specifically stain total cellular GFP by permeabilistion of the cell membrane. Staining using an anti-GFP antibody increased the sensitivity of our FACS data again revealing preferential *fpr2* promoter activity in granulocytes. However, even with this protocol, I could not show a significant change in induction within naïve or inflammatory leukocyte populations.

The culmination of this data would imply a key role for *fpr2* in granulocyte homeostasis as well as within the development of immature myeloid cells to a mature Mϕ phenotype.

4.1.2 Fpr2 expression and Fpr family compensation

Having assessed the expression profile and naïve phenotype associated with silencing *fpr2* gene transcription I also wanted to demonstrate the presence of

cell surface Fpr2 on Mϕ. My primary aim, in this section, was to establish the absence of a functional Fpr2 receptor in $Fpr2^{-/-}$ cells and mice; subsequently, I investigated possible compensatory mechanisms associated with disregulation of the Fpr family.

The loss of cell surface Fpr2 expression was demonstrated by radio-ligand binding assays. The ^{125}Iodine-labelled W-peptide (WKYMVm), a synthetic peptide in the dextro-conformation, has previously been shown to bind all three human receptors expressed on transfected cell lines (Christophe *et al.*, 2001). This commercially available radio-ligand not only revealed a predilection for Fpr2 binding on Mϕ, but also a secondary low affinity binding sites on WT Mϕ, conceivably Fpr1 (Strouse *et al.*, 2009). The absence of binding in $Fpr2^{-/-}$ Mϕ at high concentrations reflected the specificity of our transgenic technique and addressed a lack of functional compensation by Fpr1 binding. This observation, together with minimal modulation of Fpr1 mRNA in both naïve and inflammatory environments, suggest that the absence of Fpr2 has little effect on the expression and functionality of Fpr1.

4.2 Inflammatory Fpr2 phenotype

The notable promoter activity of *fpr2* in granulocytes led us to assess the potential role of Fpr2 in acute, neutrophil-driven pathologies. PMN recruitment is a hallmark of the inflammatory response (Nathan, 2006b; Nathan, 2002), exquisitely susceptible to inhibition by endogenous Fpr2 ligands including LXA_4 (Chiang *et al.*, 2006) and AnxA1 (Perretti *et al.*, 2009).

The air pouch model is a simple and highly effective method of inducing stimulus-specific leukocyte chemotaxis *in vivo*. IL-1β was a chosen as both a robust activator of immune cells eliciting acute PMN chemotaxis (Perretti *et al.*, 1993d). Studies have suggested the activation of endothelium, release of IL-8 (Bittleman *et al.*, 1995) and involvement of kinins, such as substance P (Perretti *et al.*, 1993a) and bradykinin (Ahluwalia *et al.*, 1996), are integral mediators of IL-1β-induced neutrophil migration *in vivo*. Apart from some variation between experiments there was no difference in neutrophil migration in the absence of Fpr2, when compared to values of WT mice. Therefore, endogenous Fpr2 does not control the leukocyte influx promoted by IL-1β.

Contrary to the similarities in neutrophil numbers in the air pouch of either genotype, there was a mild reduction in the ability of neutrophils to migrate to the peritoneum following zymosan treatment. Zymosan is a non-specific inflammogen able to instigate a classical, PRR-mediated, response. As with the air pouch, the majority of PMN recruitment occurs in the acute phase peaking around 4 h post challenge. The ability of $Fpr2^{-/-}$ PMN to infiltrate the peritoneum at this early time point was consistently impaired compared to the response in WT mice. Regulation of *fpr2* expression is known to be directly influenced by PRRs, TLR2 and NOD2 (Chen *et al.*, 2008), therefore it would be reasonable to assume that Fpr2 is involved in their counter-regulation. These interactions are particularly important in priming resident cells to shape the inflammatory environment. Notably zymosan-induced neutrophil migration is strongly modulated by TNF-α (Volman *et al.*, 2002) and LTB_4 (Petersen *et*

al., 1990). Consequently the lack of Fpr2 expression may interfere with the ability of resident cells to respond to pathogen efficiently further cementing its status as a pattern recognition receptor.

Systemic loss of Fpr2, initially identified as a chemotactic receptor by its high homology (81%) to Fpr1 (Su *et al.*, 1999), may also contribute to the inability of circulating granulocytes to rapidly migrate in the early phase of inflammation. It is interesting to note that human FPR2/ALX can form hetero-dimers with the leukotriene B_4 receptor, at least in artificial cellular system (Damian *et al.*, 2008). The proposition that Fpr2 can complex with different receptors is a fascinating possibility of GCPR pharmacology with wide-ranging cogitations for ligand recognition and intracellular signalling.

The role of receptor desensitisation is an unanswered subject, however it should be noted that unlike Fpr1, Fpr2 internalisation is dependent on β-arrestin (Gripentrog *et al.*, 2008). It has also been shown that dysregulation of β-arrestins inhibits chemotaxis in a mouse model of allergic allergy (Walker *et al.*, 2003). The functional impact of Fpr desensitisation will be revisited later in the chapter (Section 4.3).

Numerous related transgenic mice have been investigated using an acute model of zymosan-induced peritonitis making the reduction of acute granulocyte migration an intriguing consequence of Fpr2 deletion. An FPR2/ALX over-expressing myeloid transgenic, assessed under similar conditions, revealed a reduction in PMN migration profile (Devchand *et al.*,

2003). Additionally, the AnxA1$^{-/-}$ mouse had an exacerbated inflammatory phenotype with a significant increase in PMN recruitment (Chatterjee *et al.*, 2005). Perhaps most importantly no modulation of PMN recruitment was observed in the Fpr1$^{-/-}$ (Perretti *et al.*, 2001a). Together these data imply a distinct role of Fpr2 in the homeostasis of PMN activation and migration *in vivo* that is not shared within Fpr family as a whole.

In my experiments I allowed this model to progress over a 120 h time course: Fpr2$^{-/-}$ revealed an exacerbated leukocyte infiltration at a later stage of inflammation. This delayed onset was mirrored in neutrophil activation measured by MPO, but not chemokine or cytokine secretion. When assessing the potential regulatory roles of Fpr2 on endogenous ligands it was striking to note a considerable dysregulation of SAA, but only subtle changes in the more selective ligands AnxA1 and LXA_4. The roles of each of these ligands, notably the pro-resolving effects of both AnxA1 and LXA_4 in the removal of apoptotic leukocytes by Mϕ (Scannell *et al.*, 2007), and the multiple receptor interactions of SAA will be addressed later in this chapter (Section 4.3).

The prominence of monocytes in this late phase response and reduction in mature Mϕ population in the absence of Fpr2 creates an interesting paradigm. The initiation of *fpr2* promoter activity observed *in vitro* Mϕ maturation may reflect an inability for peripheral monocytes to mature to tissue-specific Mϕ. Therefore Fpr2 is not only an important mediator of acute phase host-defence but also might act as a regulatory receptor throughout the evolution of an acute/resolving pathology influencing both cell migration and differentiation.

To exemplify the role of infiltrating leukocytes in disease pathology I decided to test the effect of Fpr2 deletion in a longer-lasting resolving model of inflammation using K/BxN serum. Injection of this serum promotes a rapid arthritic response that lasts up to 28 days; the etiology of the disease is reliant on neutrophils (Kim 2006) and resident Mϕ (Solomon *et al.*, 2005). Furthermore it amalgamates common traits of both acute models employed, being IL-1-dependent as well as relying on a strong contribution by TNF-α (Ji *et al.*, 2002). Interestingly TNF-α stimulates a marked early phase (first 5 h) secretion AnxA1 from RA patient synovial fibroblasts but IL-1β peaked 24 h post stimulation (Tagoe *et al.*, 2008), this may have a significant bearing on the pathology observed in all three models.

Increased susceptibility and prolonged clinical arthritis was perceived in $Fpr2^{-/-}$ mice, corroborating data from the extended model of zymosan-induced peritonitis. It is notable that intra-articular injection of methylated BSA in $AnxA1^{-/-}$ mouse induced an increased histological score at day 28 compared to WT (Yang *et al.*, 2004). Overall the moderate difference between models and indeed slight divergence with previously characterised transgenic mice emphasises some of the inherent subtlies associated with gene manipulation.

Taken together these data would suggest that $Fpr2^{-/-}$ mice have a distinct pro-inflammatory phenotype compared to WT mice. Hypothetically I would propose the reduced ability of granulocytes to migrate to Fpr2-dependent stimuli, such as zymosan, is due to the absence of a classical chemotactic

pathway, but also impairment of resident cells to be fully primed. The subsequent delay in inflammation sparks a cascade that lacks Fpr2-mediated regulation resulting in the unchecked increase of pro-inflammatory mediators such as SAA. Therefore culminating in the inability of $Fpr2^{-/-}$ mice to completely resolve in extended models of inflammation.

4.3 Ligand biased Fpr2 pharmacology

The promiscuity of Fpr2 for structurally diverse ligands is now well established (Migeotte *et al.*, 2006), however the pharmacology responsible for mediating the myriad of contradictory pathways is far from understood. Throughout this thesis I have employed a number of synthetic and endogenous agonists to decipher common modes of action.

I employed principally peptides as pharmacological tools throughout my thesis for a variety of reasons. Firstly they represent the majority of agonists that recognise Fpr2 (Le *et al.*, 2007). Secondly they are often robust agonists, less susceptible to degradation and easier to handle than their parent compounds. Third, and perhaps most crucially, these peptides are commercially available and therefore are comparable across a range of studies and techniques. I also assessed the action of three non-peptidic ligands, the lipid, LXA_4, protein, hrAnxA1, and the pyrazolone derivative, C43.

To evaluate the role of Fpr2 in mediating a specific intracellular signalling cascade, I assessed four ligands for their capacity to elicit phosphorylation of

ERK1/2 in Mϕ. Three of the four, W-peptide, C43 and Ac2-26 all induced a concentration-dependent Fpr2-specific ERK phosphorylation response however SAA was capable of activating ERK in the both the presence and absence of Fpr2. Of the four ligands only Ac2-26 and C43 transduced anti-migratory effects in WT mice whereas W-peptide was inactive in the IL-1β-induced air pouch. Furthermore SAA induced a pro-migratory response which is in line with its strong pro-inflammatory profile (Song *et al.*, 2009).

The actions of W-peptide have almost exclusively been characterised *in vitro* studying chemotaxis, $Ca2^{+}$ flux and NADPH activation with human purified leukocytes or transfected cell lines (Karlsson *et al.*, 2006). However its physiological actions have rarely been assessed *in vivo* with one isolated study describing cardioprotection (Gavins *et al.*, 2005). The signalling cascade induced by W-peptide ligation has been investigated in the context of human monocyte survival, revealing a PKC-dependent Akt pathway, but not ERK pathway, was responsible for monocyte survival (Bae *et al.*, 2002). Further studies have noted that ERK1/2 phosphorylation is directly mediated by G-protein and is not dependent on internalisation of the receptor (Gripentrog *et al.*, 2005).

A second high-throughput agonist C43, developed by Amgen, was the only synthetic chemical compound assessed within this study. Data generated with $Fpr2^{-/-}$ mice confirmed the initial characterisation of C43 as an Fpr2 specific potent anti-migratory compound (Burli *et al.*, 2006). The differential pharmacology of these two highly selective Fpr2 ligands suggests an

underlying difference in intracellular signalling cascades. It is also possible that W peptide may have unfavourable PK in my experimental conditions. Together these data suggests that ERK phophorylation is clearly a readout for G-protein activation following Fpr2/ligand interaction, but it may be unreliable as for functional prognostic for Fpr2-mediated *in vivo* physiology.

To determine the chemotactic profile of Fpr2 signalling I utilised both Fpr1 and Fpr2 ligands. Human FPR1 is classed as a classical chemotactic receptor (Campbell *et al.*, 1996), therefore activation of this receptor *via* fMLF interaction was used as a 'gold standard' in this assay. WT Mϕ showed a distinct chemotactic response to fMLF stimulation, within a previously defined range of concentrations (Hartt *et al.*, 1999a), with approximately 100-fold lower affinity than human FPR1. The reduced affinity of fMLF in mouse may reflect the different pathogens presented to each species during evolution (Gao *et al.*, 1993). Fpr2 was shown to be responsible for imparting some of the chemotactic response at higher concentrations of fMLF confirming the notion of a hierarchical action of fMLF in the activation of Fpr receptors (Herrmann *et al.*, 2007). The desensitisation of FPR1 is largely regulated by G-protein uncoupling, a process mediated by receptor phosphorylation (Maestes *et al.*, 1999). Therefore the activation of a member of the FPR family, and possibly its murine couterpart, may be enough to evoke considerable changes in non-specific FPR ligand signalling.

The ability of SAA to rapidly stimulate the phosphorylation of ERK1/2 in both genotypes confirms contemporary studies associating SAA with a multitude of

receptors to regulation MAPK pathways (Baranova *et al.*, 2005; Cheng *et al.*, 2008; He *et al.*, 2003). This promiscuity was not reflected in the exclusive role for Fpr2 in mediating its chemotactic response in Mϕ. Furthermore I noted in the IL-1-induced air pouch model, that SAA was able to inhibit leukocyte migration in $Fpr2^{-/-}$ mice. An isolated study describes the ability of exogenous SAA to diminish the migratory response of neutrophils to gram-negative bacterial infection (Renckens *et al.*, 2006). Interestingly SAA has been shown to opsonise gram-negative bacteria resulting in increased phagocytosis and production of pro-resolving mediators such as IL-10, however this biological effects could be distal from Fpr2 (Shah *et al.*, 2006). During acute inflammation in our transgenic model, which could be extrapolated as an artificial model of Fpr2 receptor desensitisation following infection, SAA transduces anti-migratory actions *via* an as yet undefined receptor. This may represent the extent to which the Fpr family rely on activation and desensitisation in a hierarchical manner to regulate ligand functionality.

Having assessed two known pro-inflammatory FPR family ligands, I undertook comparative studies for the chemotactic profile of AnxA1 and Ac2-26, both well-established anti-migratory mediators *in vivo* (Perretti *et al.*, 1993b; Perretti *et al.*, 2001b). Full-length hrAnxA1 was shown to induce a mild locomotive response in both genotypes, whereas its peptide derivative was unable to provoke Mϕ locomotion *in vitro*. Additionally I observed some residual efficacy of hrAnxA1 in an *in vivo* chemotaxis model using the IL-1β-induced air pouch in the absence of Fpr2, which was not apparent with Ac2-26. However the action of exogenous hrAnxA1 was exclusively Fpr2-mediated

in an acute model of zymosan-induced peritonitis, which would support stimulus-specific effects noted when assessing the inflammatory phenotype of $Fpr2^{-/-}$ mice (Section 4.2).

Many studies have reviewed the action of Ac2-26 and full length AnxA1 as interchangeable, however it is important to note that Ac2-26 has two orders of magnitude lower potency than its parent protein (Perretti *et al.*, 1993d). The hrAnxA1 utilised in my thesis may have a number of caveats relating to its specificity and action. Primarily the full-length human sequence of the protein, comprising of 346 amino acids, is liable to impose spatial and conformational constraints on the protein (Hu *et al.*, 2008). Classically the convex side of the molecule binds the surface membrane prior to inducing conformational changes to enable the membrane-bound protein to interact with cellular protein or in the case of aggregation a second cell membrane (Gerke *et al.*, 2002). Furthermore the active cleavage of AnxA1 by proteases, e.g PR3 (Vong *et al.*, 2007), may give rise to numerous active N-terminal peptides with a range of specificities for the Fpr family (Walther *et al.*, 2000). Finally I cannot exclude the possibility that the C-terminal tail also has the potential to initiate multiple facets of the host defence pathway.

Despite the residual effects of hrAnxA1 to diminish leukocyte migration in $Fpr2^{-/-}$ mice following IL-1β stimulation, hrAnxA1 was not able to reduce PGE_2 production in these mice. Inhibition of PGE_2 is a common trait of AnxA1 action and may underpin the efficacy of dexamethasone in this model. The rapid translocation of endogenous AnxA1 to the cell membrane is an integral

mechanism of action of GC, particularly dexamethasone (Perretti *et al.*, 2009). Importantly, when an intermediate dose of dexamethasone was used, the steroid was ineffective in preventing leukocyte migration in $Fpr2^{-/-}$ mice. Although the anti-inflammatory properties of GC are not exclusively reliant on AnxA1 (Clark, 2007), it is compelling evidence that reduced efficacy in AnxA1 deficient mice (Damazo *et al.*, 2006; Hannon *et al.*, 2003) can be functionally linked with a specific receptor. This observation also supports the logical hypothesis that endogenous AnxA1 has a greater specificity than its human counterpart in the murine experimental setting.

In spite of the discrepancies in efficacy the anti-migratory actions of endogenous AnxA1, hrAnxA1 and its peptido-mimetics share their ability of provoking the "leukocyte detachment phenomenon". This idea was first postulated to explain the ability of exogenous AnxA1 to provoke the detachment of adherent neutrophils from inflamed post-capillary venules (Lim *et al.*, 1998). This working hypothesis is supported by increased emigration of leukocytes in cremaster model of transmigration in $AnxA1^{-/-}$ animals (Chatterjee *et al.*, 2005) as well as data obtained using ischemia reperfusion of the mesentery in $Fpr2^{-/-}$ mice (Brancaleone V, personal communication).

As an addition to this established mechanism of action, Ac2-26 directly inhibit Mϕ in a Fpr2-dependent manner: pre-treatment of these cells with Ac2-26 markedly inhibited SAA-mediated chemotaxis. Many studies have detailed the ability of different FPR ligands to compete for receptor binding (Perretti *et al.*, 2002), receptor internalisation or desensitisation of secondary ligand

interactions. In the latter case, high dose fMLF treatment of monocytes has been shown to completely abolish secondary Ca^{2+} mobilisation with subsequent SAA stimulation (Su *et al.*, 1999). A close peptide analogue, Ac1-25, has also been shown to have similar effect on fMLF and W-peptide mediated Ca^{2+} flux (Ernst *et al.*, 2004), whether Ac2-26 instigates a common desensitisation pathway as fMLF is currently uncertain.

This novel observation that two Fpr2-specific agonists can transduce counter-regulatory pathways adds weight to a new field of ligand-biased pharmacology. A recent study investigating the signalling cascade of the β1-adrenergic receptor revealed changes conformation rearrangements of the receptor/G-protein complex when receptor bound full, partial or inverse agonists (Galandrin *et al.*, 2008). The capability of Fpr2 to undergo conformation changes during ligand interaction would corroborate the identification of multiple binding domains within human FPR2/ALX (Le *et al.*, 2005).

As inflammation progresses beyond the acute phase the influence of leukocyte migration gives way to active resolution of inflammatory and cellular debris. Two endogenous Fpr2 ligands, AnxA1 and LXA_4, are strongly linked with inducing pro-resolution pathways (Scannell *et al.*, 2006). Both are associated with modulating gene transcription, for example promoting IL-10 (Souza *et al.*, 2007), and suppressing pro-inflammatory gene expression (Jozsef *et al.*, 2002).

During this phase, Mϕ engulf apoptotic PMN (Serhan *et al.*, 2005) facilitating their final egress from the site of inflammation *via* the lymphatic system (Schwab *et al.*, 2007). Phagocytosis, and therefore efficient clearance, of apoptotic neutrophils can be a directly measured as an indication of a pro-resolution phenotype (Scannell *et al.*, 2006). Pre-treatment of Mϕ with either Ac2-26 or LXA_4 was able to significantly enhance phagocytic capacity for apoptotic neutrophils in an Fpr2-dependent manner. This data confirmed a distinct role for AnxA1/Fpr2 interaction, supporting previous experiments utilising the $AnxA1^{-/-}$ transgenic (Yona *et al.*, 2006).

LXA_4 portrays similar functional effects as AnxA1, both with high affinity for Fpr2, inducing a potent anti-migratory affects in the WT mice using the air pouch model. Equally its ability to increase phagocytosis of apoptotic neutrophils would suggest correlation between ligand pharmacology; this is not the case. LXA_4 has been shown to interact with a distinct domain, distal from both peptide and protein binding sites (Le *et al.*, 2005). Its action is unique as does not induce Ca^{2+} mobilisation (Bae *et al.*, 2003) and can inhibit ERK phosphorylation mediated by secondary cell activation (El Kebir *et al.*, 2007). These characteristics imply that despite LXA_4 high affinity for Fpr2, LXA_4 lacks full agonistic activity (Ye *et al.*, 2009).

Lipoxins are also known to share affinity for a ligand-activated transcription factor nuclear receptor, AhR (Nebert *et al.*, 2008). The physiological actions of this interaction are currently uncertain; in this study we did not demonstrate

any potential LXA_4/AhR action within either phagocytosis or regulation of Fpr2 dependent leukocyte migration.

During the extended peritonitis time course AnxA1 and LXA_4 secretion followed similar profiles, with rapid induction within the first 4 h and subsequent catabolism until the experiment was terminated. This data would suggest Fpr2 is not necessary in regulating the production of either of these endogenous ligands, both strongly reliant on leukocyte/endothelium interaction (Chiang *et al.*, 2005). The exacerbation of cellular infiltrate in zymosan-induced peritonitis and K/BxN-induced arthritis would implicate Fpr2 as an important mediator of phagocytosis during the resolution of inflammation, potential via both AnxA1 and LXA_4-dependent mechanisms.

In contrast to the pro-resolving actions of AnxA1 and LXA_4, the acute phase protein SAA is a pro-inflammatory Fpr2 ligand. The mechanisms ascribed to specific biological actions of SAA are complex, and the precise receptors uncertain (Shah *et al.*, 2006), however our data indicate conclusively that *in vitro* and *in vivo* stimulation of cell locomotion by SAA occurs through Fpr2.

SAA is an atypical acute phase protein, produced both locally and by hepatocytes, with levels reaching 80mM during overt inflammation (He *et al.*, 2009). I observed a significant elevation of local SAA in WT mice, peaking at 24 h post zymosan-induced peritonitis. The induction of SAA was exaggerated (~3-fold) in the absence of Fpr2 providing evidence for a novel regulatory circuit. To further assess the modulation of SAA production during

inflammation I injected SAA 10 h post zymosan in WT animals. The influence of an systemic increase of SAA lead to a positive feedback in the mouse with SAA levels mimicking those seen in the $Fpr2^{-/-}$ mouse at 24 h. This is the first study to identify an autonomous feedback loop for SAA as well as highlighting a clear role of SAA/Fpr2 interaction in self-regulation.

The consequence of high levels of SAA in this model show correlation with increased mononuclear cellular infiltrate at this later time point. In light of the concentrations reached by SAA at 24 h it is unlike to be continuing to function as a chemotractant. Studies have demonstrated that SAA is a potent stimulator of granulocyte-colony stimulating factor (G-CSF) and peripheral blood neutrophilia via TLR2 (He *et al.*, 2008). Furthermore it is also notable that SAA can convey an apoptosis-delaying action in human neutrophils (El Kebir *et al.*, 2007), which could be extrapolated to the delayed profile of neutrophil activity, measured by MPO, in $Fpr2^{-/-}$ mice.

The hallmark of the exacerbated inflammatory $Fpr2^{-/-}$ phenotype was an increase in infiltration of $F4/80^{low}$ monocytes. This was counterbalanced by a reduction in the number of $F4/80^{high}$ Mϕ at 72 h post zymosan. Despite the subtlety of these changes, they were reproducibly obtained in separate experiments suggesting that this represents a meaningful pathway. This phenomenon was mirrored by an increase in both populations following exogenous SAA administration in WT animals. Furthermore this response was independent of IL-6, a well-documented proponent of immunological switching (Jones, 2005), previously shown to be a potential pathogenesis in

RA synoviocytes (Koga *et al.*, 2008). Studies have also noted that SAA induces differential IL-12 and IL-23 release from human monocytes (He *et al.*, 2006). The cascade of IL-23 to produce IL-17 secretion would concur with contemporary studies noting the ability of IL-17 to prolong inflammation (Maione *et al.*, 2009). Together this data suggests a subtle Fpr2-specific role in shaping the resolution phase response by controlling maturation of monocytes to Mϕ, which may underpin many of the physiological processes investigated in this thesis.

4.4 Limitations of experimental models

Animal models are essential for studying patho-physiological processes of disease particularly with respect to complex inflammatory responses. However no animal model can unequivocally reproduce human disease pathology, although all respond to clinically effective anti-inflammatory drugs. This thesis describes three models used as representatives of fundamental aspects of the inflammatory process.

The air pouch model was initially characterised as a model of the synovial cavity. This has been modified to function as a model of transmigration *in vivo*. There are a number of caveats to this model in transgenic mice particularly in the assumption that the structure of the air pouch would be identical between genotypes. I was unable to perform histology of the lining tissue within my thesis relying on similar control responses as a guide of comparative pathologies. FPR2/ALX has been identified as potential mediator

of angiogenesis *in vitro* (Lee *et al.,* 2006), which could an impact on the ability of cells to infiltrate *in vivo*.

Zymosan peritonitis was investigated to deduce the involvement of Fpr2 as a pattern recognition receptor. Zymosan has conserved motifs that have some similar characteristics to infectious pathogens, which would be more applicable to the pathology of human peritonitis.

The final model assessed in this thesis was the K/BxN serum-induced arthritis. This model is potentially the *in vivo* model most relevant to human disease, auto-antibodies are key players in the development of rheumatoid arthritis. The serum transferred to initiate the disease contains antibodies that recognise the self-antigen glucose-6-phosphate isomerase, the formation of antigen-antibody complexes trigger joint specific inflammation. This model of arthritis would represent an ideal extension of the more acute models, since highly reliant on cells of innate immunity (response driven by neutrophils, mast cells and macrophages).

Since the completion of my thesis I have continued to investigate alternative models of inflammation, which have re-inforced my overall conclusions that Fpr2 is an anti-inflammatory receptor. Two models, carrageenan-induced paw oedema and ischemia reperfusion injury have subsequently been included in a publication Dufton *et al*., (Journal of Immunology *in press*; Figure 4.1).

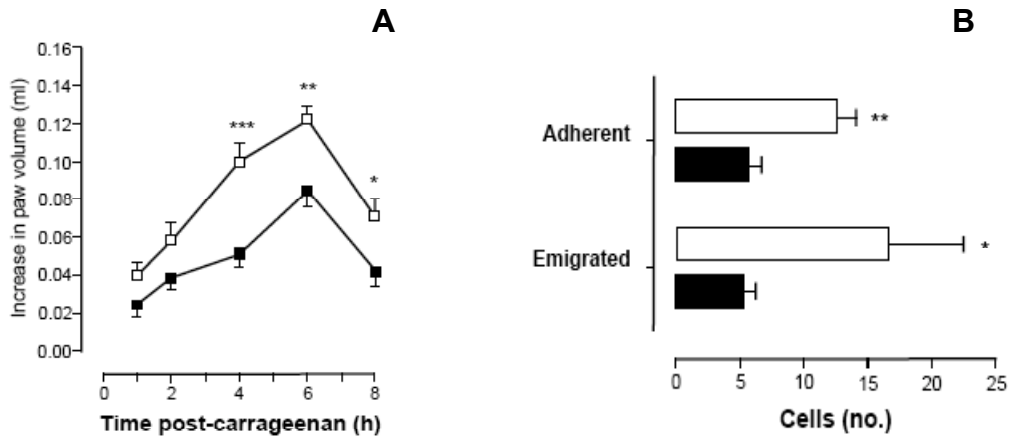


Figure 4.1. Figures from subsequent publication Dufton *et al.* (A) Carrageenan-induced paw oedema: exacerbation in Fpr2$^{-/-}$ mice. Mice paws were injected with 50µl of 1% carrageenan solution. Time course of the paw swelling in wild type and Fpr2$^{-/-}$ mice. Data are shown as mean ± SEM of n=15 animals; * P<0.05, ** P<0.01, ***P<0.001, Student T-test. (B) Mesenteric ischemia reperfusion injury of WT and Fpr2$^{-/-}$ mice. Mesenteric circulation was subjected to 30 min ischemia followed by 90 min perfusion. Cells were counted within the vessel spanning 100µm in length and surrounding 50µm of tissue either side of the vessel wall. Data are mean ± SEM of three fields per mouse n=5 mice per group; **P<0.01, *P<0.05 compared to WT by student T-test.

4.5 Conclusion

This study describes, for the first time, the functions of Fpr2 in murine development, cell maturation, mobility and finally experimental models of inflammation. Collectively these data highlight anti-inflammatory effects conveyed by Fpr2 mediated ligation, exemplified by the inability of dexamethasone to be efficacious in Fpr2$^{-/-}$ mice. The Fpr family plays a significant role in modulating both the acute and resolution phase of inflammation. In my thesis I have confirmed a specific function of Fpr2 in host-defence including a previously unreported action in monocyte/macrophage recruitment and differentiation.

In the acute phase of inflammation (≤8 h) Fpr2 regulates the activation and transmigration of PMN to home towards an inflammatory stimuli. The actions of endogenous anti-inflammatory ligands, AnxA1 and LXA_4, are probably most notable during the interaction of leukocytes with inflamed endothelium. Each ligand competes for Fpr2 to counterbalance pro-migratory actions of locally secreted SAA. Throughout *in vivo* inflammation I would propose SAA acts largely as a chemokinectic factor, although it certainly has influence as a chemotractant (Figure 4.1 A).

During the later phase of inflammation and resolution (≥48 h) the actions of Fpr2 are integrated within the site of inflammation. Again AnxA1 and LXA_4 are produced by leukocyte interactions promoting the apopto-phagocytosis response. At variance with this action, SAA prolongs survival of inflammatory infiltrate at well as promoting Mϕ maturation. This could identify a stepping-stone action of SAA retaining a required inflammatory response prior to the priming monocytes and Mϕ to influence the resolution phase (Figure 4.1 B). The deletion of a functional receptor disrupts the regulation of this integrated pharmacology leading to an exaggerated inflammatory phenotype in the $Fpr2^{-/-}$ mouse.

As yet no common structural motif of peptide, protein and lipid ligands has been identified, the multiple ligand recognition domains identified on human FPR2/ALX may be responsible for dictating the novel ‘ligand-biased’ actions of this receptor (Chiang *et al.*, 2000). The versatility GPCR functions within the body is truly astonishing, the Fpr family is no different. Unrelated to host

defence, the Fpr2 receptor has been documented in hair follicles, protecting mice from experimental alopecia (Tsuruki *et al.*, 2007), and as a candidate chemosensory receptor in the vomeronasal organ (Liberles *et al.*, 2009). The current momentum of research to explain this physiological diversity implicates shape recognition (Kortagere *et al.*, 2009) and conformational changes (Galandrin *et al.*, 2008). It would be conceivable that conformational changes in ligand/Fpr2 complexes could dictate specific intracellular signalling, e.g PKC, PI3K and Akt. This study has only begun to scratch the surface of the complex pharmacology of this unique receptor, substantiating the idea that ERK1/2 is a common feature of agonist/Fpr2 interaction but not a determinant of Fpr2-mediated cell function.

The capability of FPR2/ALX to influence other receptor signalling cascades is also an intriguing aspect of FPR-family biology. Both FPR1 and FPR2/ALX are functionally regulated by homologous and heterologous desensitisation. Here I identify dual roles of SAA in the presence and absence of Fpr2 that could have wide ranging implications on its perceived biological actions.

One plausible explanation for these opposite actions could lie with the capability of GPCRs to heterodimerise with receptors at the cell surface (Terrillon *et al.*, 2004). Indeed FPR2/ALX has been shown to form hetero-dimers with the leukotriene B_4 receptor (Damian *et al.*, 2008). Therefore our transgenic could be an ideal comparative tool to assess the possibilities of ligand-specific homo- and/or hetero-dimerization processes (Bosier *et al.*, 2007).

In conclusion this thesis has been able to validated sufficient functional crossover of ligand interactions with murine Fpr2 receptors. This represents a significant milestone in investigating the pharmacology of promiscuous receptor in physiological conditions and disease models. Our research, lead strongly via annexin A1, has identified a number of patentable peptide sequences termed Ac2-26 with ongoing collaborations with both Unigene and Compugen (Hecht *et al.*, 2008). Furthermore evidence provided in my thesis describes successfully screening of Fpr2-selective anti-inflammatory actions of C43 *in vivo*, a compound developed for its affinity to the human receptor. These data therefore provide a compelling rationale for developing novel anti-inflammatory therapeutics depicted on agonists to Fpr2 and its human counterpart FPR2/ALX (Burli *et al.*, 2006; Hecht *et al.*, 2009), offering considerable scope to assess future therapeutic compounds.

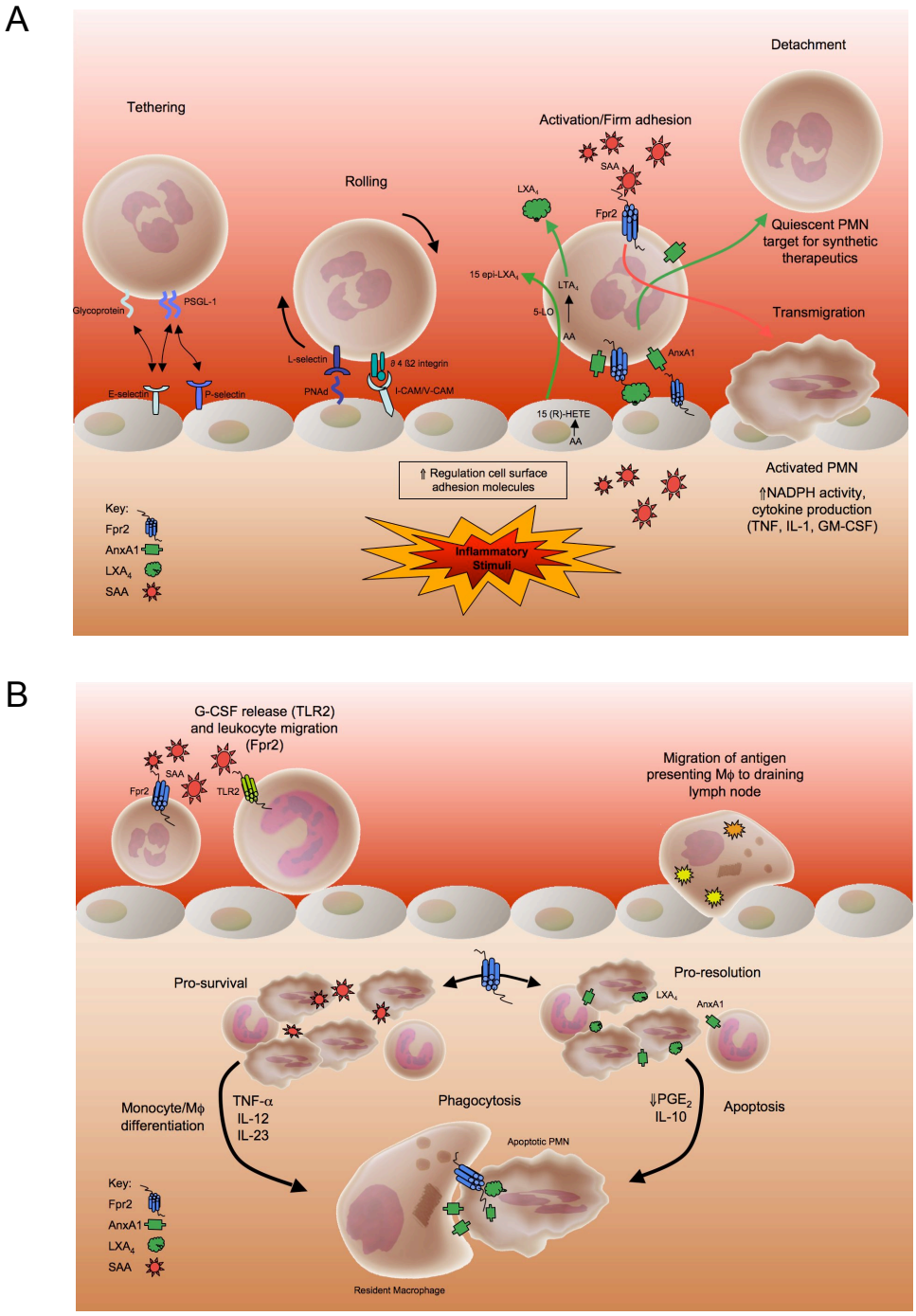


Figure 4.2. Hypothetically Fpr2-mediated mechanisms of action in inflammation. The roles of Fpr2 are both complex and varied throughout (A) acute and (B) chronic inflammation. Fpr2 was initially characterised as a chemotactic receptor, cell migration can be mediated on interaction with SAA as well as exquiste regulation of diapedisis on interaction with the endothelium. Once leukocytes have infiltrated the site of inflammation Fpr2 instigates a second 'ligand-bias' profile. Pro-resolution mediators AnxA1 and LXA_4 induce PMN apopotosis and clearance by phagocytosis. Contraty to this SAA is a pro-survival factor important in full maturation of Mϕ. Therefore SAA may also participate in priming the resolution response.

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