The role of Annexin 1 in glomerular inflammation

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by

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I .... 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 in the thesis.

Acknowledgement

For everyone who believed in me.

Presentations

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Abstract

Annexin 1 is an important mediator in the active process of inflammation resolution. The protein controls leukocyte trafficking to inflammatory sites, promotes their nonphlogistic removal from the tissue and polarises infiltrating macrophages towards a pro-resolving phenotype. ANCA-associated vasculitis (AAV) is characterised by a necrotising glomerulonephritis that develops following neutrophil infiltration, accumulation of macrophages and T lymphocytes, and formation of cellular glomerular crescents. The aim of this thesis is to address whether Annexin 1 might play a beneficial role in the resolution of glomerular inflammation in AAV.

In the following experiments I examined the effect of the genetic absence of Annexin 1 in two murine models of crescentic glomerulonephritis; nephrotoxic nephritis (NTN) and anti-myeloperoxidase associated glomerulonephritis (murine experimental vasculitis/MEV). In addition, the relationship between Annexin 1 cleavage and proteinase 3 expression was investigated in neutrophils and the ability of a PR3-cleavage resistant Annexin 1 peptide (SuperAnnexinA1) to inhibit neutrophil activation was examined to understand the potential role of Annexin 1 in AAV. Annexin 1 deficiency resulted in a significant exacerbation of disease severity in the NTN model, and administration of SuperAnnexinA1 polarised infiltrating macrophages towards a less inflammatory phenotype. The disease penetrance across experimental groups in the MEV model was not substantial enough to conclude whether the absence of Annexin 1 had an effect on disease. *In vitro*, there was a significant correlation observed between an increased proportion of PR3^{high} neutrophils and abundance of Annexin 1 cleavage products in neutrophils from healthy controls and PR3-ANCA positive AAV patients. The activation of human neutrophils was inhibited in a dose-dependent manner by increasing concentrations of SuperAnnexinA1.

These data indicate that Annexin 1 plays an important protective role in experimental glomerular injury. There is a rationale established to hypothesise an increased degree of Annexin 1 cleavage within an inflamed glomerulus where neutrophils are abundant. This supports the potential for Annexin 1 peptidomimetics to be used in the treatment of glomerular injury.

Table of Contents

List of Tables

Table of Figures

Table of Abbreviations

Acronym	Definition
aa	amino acid
AAV	ANCA-associated vasculitis
AIA	antigen-induced arthritis
ANCA	anti-neutrophil cytoplasm antibody
ANOVA	analysis of variance
Anx	annexin
APC	antigen presenting cell
BALF	bronchoalveolar lavage fluid
BM	bone marrow
BSA	bovine serum albumin
BVAS	Birmingham Vasculitis Activity Score
C5aR	human C5a receptor
CFA	Complete Freund's Adjuvant
DC	dendritic cell
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell
eGPA	eosinophilic granulomatosis with polyangiitis
EMP	endothelial-derived microparticle
EVGC	European Vasculitis Genetic Consortium
FACS	fluorescence-activated cell sorting
FCGN	focal crescentic necrotising glomerulonephritis
FCS	fetal calf serum

Acronym	Definition
fMLP	N-Formylmethionyl-leucyl-phenylalanine
FPLC	fast protein liquid chromatography
FPR	formyl peptide receptor
FSC	forward scatter
GC	glucocorticoid
GCSF	granulocyte colony stimulating factor
GN	glomerulonephritis
GPA	granulomatosis with polyangiitis
GPCR	G protein-coupled receptor
GWAS	genome-wide association study
H&E	haematoxylin and eosin
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HLA	human leukocyte antigen
HLE	human leukocyte elastase
HRP	horse radish peroxidase
ICAM-1	intracellular adhesion molecule-1
IFA	incomplete Freund's adjuvant
IFN-γ	interferon gamma
Ig	immunoglobulin
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IV	intravenous
LDG	low density granulocyte
LPS	lipopolysaccharide

Acronym	Definition
MAPK	mitogen-activated protein kinase
MEV	murine experimental vasculitis
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
mMPO	murine myeloperoxidase
MP	microparticle
MPA	Microscopic Polyangiitis
MPO	myeloperoxidase
mPR3	membrane proteinase 3
MPRO	mouse promyelocytic cell line
MR	mannose receptor
mRNA	messenger RNA
NCGN	necrotising crescentic glomerulonephritis
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NK cells	natural killer cells
NMP	neutrophil-derived microparticles
NSAID	non-steroidal anti-inflammatory
NTN	nephrotoxic nephritis
NTS	nephrotoxic serum
OVA	ovalbumin
PAS	periodic acid–Schiff
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

Acronym	Definition
PFA	paraformaldehyde
PMN	polymorphonuclear leukocyte
PMP	platelet-derived microparticle
PR3	proteinase 3
PS	phosphotidylserine
RA	rheumatoid arthritis
RBC	red blood cell
ROS	reactive oxygen species
SAA	serum amyloid A
s/c	subcutaneous
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLE	Systemic Lupus Erythematosus
SNP	single nucleotide polymorphism
SSC	side scatter
TBS	Tris-buffered saline
TCR	T-Cell Receptor
TEM	transendothelial migration
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
UV	ultraviolet

Chapter 1 Introduction

This thesis investigates the role of Annexin 1 in glomerular inflammation. Chapter 1 investigates the genetic absence of Annexin 1 in a murine model of nephrotic nephritis and examines the effect of a PR3 proteolysis-resistant Annexin 1 peptide on disease. Chapter 2 describes the establishment of the murine model of anti-myeloperoxidase associated glomerulonephritis and explores the effect of the genetic absence of Annexin 1 on disease. Chapter 3 investigates human neutrophil surface expression of PR3 and abundance of Annexin 1 cleavage products *in vitro* and considers whether the activation of primed neutrophils by ANCA might be modulated by Annexin 1 peptidomimetics.

1.1 Annexin 1

1.1.1 Introduction

Annexin 1, also known as lipocortin 1, is one member of a family of calcium- and phospholipid-binding proteins termed the Annexins. Each of the Annexin proteins encompasses a core domain, comprised of a varying number of conserved 70 amino acid repeats, and an N-terminal region, which is unique to each Annexin and understood to account for the varying functions of the protein family members. It is only through binding of calcium that the functional N-terminal becomes exposed (Rosengarth and Luecke, 2003).

The initial discovery that Annexin 1 blocks phospholipase A_2 enzyme activity in the presence of calcium, highlighted the proteins' potential role in regulating inflammation. Phospholispase A_2 hydrolyses phospholipids; leading to the release of arachidonic acid; which is a vital component of prostaglandins and leukotrienes, both inflammatory mediators (Gryglewski et al., 1975). Indeed, the generation of an Annexin 1 deficient mouse strain revealed that these mice have increased levels of cytosolic phospholipase A_2 and a propensity for greater inflammatory responses (Hannon et al., 2003). Wallner *et al.* (1986) were the first to identify Annexin 1 as an anti-inflammatory substance mediating the actions of glucocorticoids (GCs) (Wallner et al., 1986) and these anti-inflammatory actions were reproduced in *in vivo* models of acute inflammation soon after (Errasfa and Russo-Marie, 1989, Cirino et al., 1989). The Annexin 1 knockout mouse generated by Hannon has partial resistance to GC treatment in a number of models of acute and chronic inflammation (Hannon et al., 2003, Yang et al., 2004).

1.1.1.1 *Expression of Annexin 1*

Annexin 1 is predominantly expressed in the cells of the innate immune system; the highest levels of Annexin 1 expression are found within monocytes/macrophages, neutrophils and natural killer (NK) cells, compared to B and T lymphocytes which express much lower levels of Annexin 1 (Morand et al., 1995, Spurr et al., 2011). Annexin 1 is expressed in a range of human tissues, the highest levels being found in tissues involved in the immune response or tolerance functions e.g. spleen and placenta. The protein is also expressed within the kidney by podocytes, collecting ducts and within epithelial cells of the Bowman's capsule (Dreier et al., 1998) (see Table 1). The expression of the Annexin 1 receptor; formyl peptide receptor type 2 or lipoxin A4 receptor, (hereafter termed FPR2/ALXR) in human cells and tissues has not been explored to the same extent as its ligand, but anecdotal reports suggest that the receptor exhibits a similar expression pattern to that of Annexin 1. FPR2/ALXR is highly expressed in monocytes, but on differentiation to macrophages the protein expression is nullified (Waechter et al., 2012).

Table 1 Expression of Annexin 1 in human cells and tissues

Cell type:	Expression of Annexin 1
	extracellular
Monocytes/macrophages	+++
Neutrophils	++
NK cells	++
B lymphocytes	+
T lymphocytes	+
	total
Tissue type:	
Respiratory tract	+++
Lymphatic tissue	++
Digestive tract	+
Hepato-pancreatic system	-

Female reproductive tract	++
Male reproductive tract	++
Urinary system	+++
Endocrine organs	+
Skin	+
Nerve tissue	+++

Tissue expression of Annexin 1 is adapted from (Dreier et al., 1998).

Annexin 1 comprises 2-4% of total neutrophil cytosolic protein (Francis et al., 1992, Rosales and Ernst, 1997) and is found predominantly within the gelatinase granules of resting neutrophils (Perretti et al., 2000). As Annexin 1 has no known signal peptide, it is believed that neutrophil-derived Annexin 1 is exported to the cell surface through the exocytosis of these granules on adhesion to the endothelium (Oliani et al., 2001) and indeed Annexin 1 itself may play a direct role in promoting exocytotic membrane fusion (Francis et al., 1992). Annexin 1 expression is partially controlled by endogenous and exogenous glucocorticoid hormones and addition of GC can result in the increased total expression of Annexin 1 in neutrophils and monocytes *in vitro* and *in vivo* (Goulding et al., 1990, Perretti and Flower, 1996).

Annexin 1 has a number of residues within the N-terminus and C-terminal core which can undergo phosphorylation to influence the protein's binding capacity and function; for example, phosphorylation of tyrosine decreases the calcium requirement for binding to phosphatidylserine (PS) (Schlaepfer and Haigler, 1987). Phosphorylation of Annexin 1 can also determine its subcellular localisation (Kim et al., 2003) and GCs have been shown to induce phosphorylation of Annexin 1 at particular sites; in some cases causing the protein's subsequent translocation to the cytoplasmic membrane (John et al., 2003, Solito et al., 2003b).

1.1.2 Anti-inflammatory and pro-resolving actions of Annexin 1

I. ↓ neutrophil migration

II. ↑ neutrophil apoptosis

III. ↑ phagocytosis

IV. ↑ efferocytosis

V. macrophage skewing
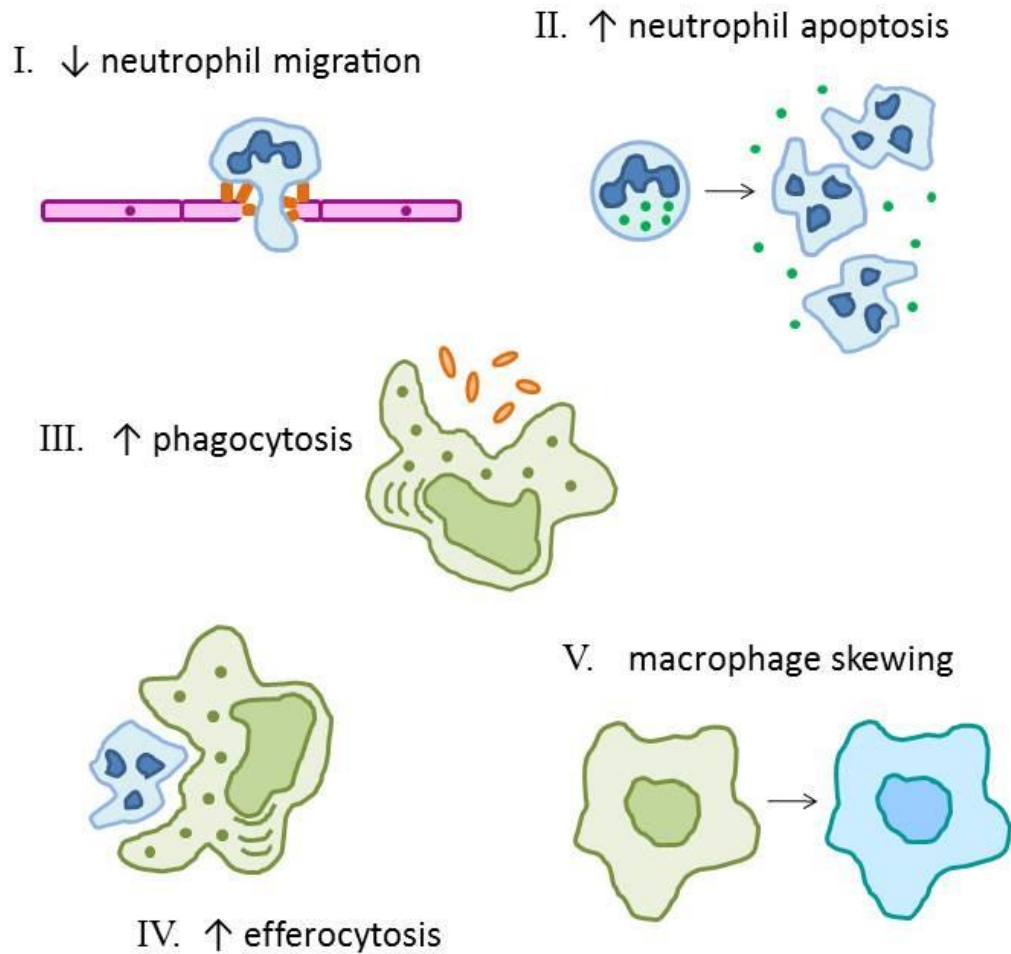
Figure 1-1 Key pro-resolving actions of Annexin 1

Annexin 1 has been implicated in a number of processes within the innate immune system that contribute to the resolution of an inflammatory reaction. **I**. Annexin 1 exposed on the neutrophil surface plays an inhibitory role in their extravasation into the tissue. **II**. Upregulation of Annexin 1 expression in neutrophils leads to an increase in pro-apoptotic pathways. **III**. Annexin 1 deficient macrophages are impaired in their phagocytic capacity. **IV**. Annexin 1 expression by BM macrophages is required for efficient phagocytosis of apoptotic neutrophils. **V**. Engulfment of secondary necrotic cells expressing Annexin 1 reduces macrophage pro-inflammatory cytokine production and exogenous Annexin 1 administration polarises M1 macrophages towards a more resolution-type phenotype.

1.1.2.1 *Leukocyte trafficking*

Annexin 1 was first shown to have an inhibitory effect on neutrophil trafficking to inflammatory sites in 1989, as exogenous administration of the protein reduced neutrophil accumulation in an *in vivo* model of inflammation (Errasfa and Russo-Marie, 1989). Following work demonstrated that recombinant human Annexin 1 could reduce the IL-1 and IL-8 dependent accumulation of neutrophils in an inflammatory air-pouch murine model (Perretti and Flower, 1993, Perretti et al., 1993) and revealed that endogenous Annexin 1 modulates monocyte and neutrophil accumulation during acute inflammation *in vivo* (Getting et al., 1997, Lim et al., 1998). Experiments in the Annexin 1 deficient mouse have also shown a markedly enhanced infiltration of neutrophils to inflammatory sites in models of acute inflammation stimulated by zymosan (Chatterjee et al., 2005) and carrageenan (Gastardelo et al., 2009).

The means by which Annexin 1 regulates neutrophil accumulation has been attributed to its inhibition on leukocyte extravasation, rather than any effect on rolling or adhesion (Mancuso et al., 1995, Chatterjee et al., 2005, Damazo et al., 2006). Under normal conditions, circulating neutrophils are thought to externalise Annexin 1 on adhesion with the endothelium, allowing Annexin 1 to act as an autocrine inhibitor, preventing neutrophil emigration through the vessel wall (Perretti et al., 1996, Perretti, 1997). However, under inflammatory conditions Annexin 1 is cleaved at the cell surface, allowing neutrophil extravasation into the tissue (Perretti and Flower, 1996, Goulding et al., 1998).

L-selectin plays an important role in mediating neutrophil-endothelial interactions and the adhesion of neutrophils to the inflamed microvasculature (Hafezi-Moghadam and Ley, 1999, Hickey et al., 2000). It has been reported that exogenous Annexin 1 promotes L-selectin shedding from neutrophils and monocytes and Annexin 1 antibodies can inhibit L-selectin shedding induced by dexamethasone treatment, providing some insight into how Annexin 1 may mediate its inhibitory effect on neutrophil extravasation into the tissue (Strausbaugh and Rosen, 2001, de Coupade et al., 2003).

Experiments investigating detachment of polymorphonuclear leukocytes (PMN) from the vascular bed have demonstrated the efficacy of administering a truncated Annexin 1 peptide; Ac2-12 in preventing diapedesis (Lim et al., 1998). Interestingly,

it is this fraction of Annexin 1 that mediates interactions with the S100 proteins; both Annexin 1 and S100A8/A9 are expressed at the neutrophil surface on activation and have been demonstrated to bind carboxylated glycans found on endothelial cells (Srikrishna et al., 2001a). These glycan interactions have been shown to be involved in the extravasation of neutrophils and monocytes into inflamed tissue (Srikrishna et al., 2001b), providing an additional putative link behind how Annexin 1 inhibits diapedesis.

Monocytes externalise Annexin 1 on adhesion to the endothelium, but not to the same extent as neutrophils (Perretti et al., 1999). Annexin 1 expressed on the surface of the monocyte is believed to interact with the α4β1 integrin on the endothelial cell surface to exert inhibitory effects on monocyte adhesion (Solito et al., 2000). However, the expression of Annexin 1 may also play an important role in the accumulation of macrophages required to resolve an inflammatory reaction as in several experimental models of peritonitis Annexin 1 deficient mice have a reduction in the number of infiltrating macrophages (Damazo et al., 2006, Chatterjee et al., 2005). Further work needs to be done to uncover the role that Annexin 1 performs in the accumulation of monocytes and macrophages within inflamed tissue.

Recent research has suggested an alternate pro-inflammatory role for a ~33kDa C-terminal Annexin 1 fragment within neutrophil transendothelial migration. This fragment generated by calpain 1 cleavage can activate the endothelial ERK1/2 signalling pathway and is believed to promote the transmigration of neutrophils through the clustering of ICAM-1, a process which is essential for the formation of transmigratory cups required for neutrophil transendothelial migration (TEM) (Williams et al., 2010, Shaw et al., 2004).

1.1.2.2 *Promotion of apoptosis*

Programmed cell death is a vital step in the resolution of an inflammatory reaction. Apoptosis is preferable to necrosis as it avoids the release of proinflammatory mediators, contributing to any already established inflammatory reaction and this process can also avert autoimmune reactions to self-antigens.

Interaction of PMN with extracellular matrix during extravasation leads to Annexin 1 gene activation, which appears to be counter-intuitive if the only role for surface expression of Annexin 1 would be to limit diapedesis (Oliani et al., 2001). This

increased expression of Annexin 1 by extravasated neutrophils is GC-independent (Vergnolle et al., 1997).

Sakamoto *et al.* first linked Annexin 1 to influencing apoptosis in 1996, when they demonstrated that exogenous administration of Annexin 1 promotes the apoptosis of rat thymocytes and prevents cell necrosis under H_2O_2 treatment (Sakamoto et al., 1996). Subsequent work by Solito *et al.* has revealed that apoptosis can be augmented in either monocytic cells transfected with Annexin 1 or neutrophils when treated with human recombinant Annexin 1 and is associated with changes in calcium and increased caspase 3 activity (Solito et al., 2001, Solito et al., 2003a).

Recent work by Vago *et al.* has supported a pro-apoptotic role for endogenous Annexin 1 *in vivo* using LPS-induced pleurisy. The pro-resolution effect of Annexin 1 in their study was associated with an increase in pro-apoptotic pathways (Bax levels and caspase-3 cleavage) and an inhibition of pro-survival pathways (Mcl-1, ERK1/2 and NF-κB). The pro-apoptotic effect could be abolished by a pan-caspase inhibitor (Vago et al., 2012).

1.1.2.3 *Efferocytosis by macrophages*

There is a growing body of understanding that endogenous signalling molecules act to regulate the phagocytosis of apoptotic cells (Fadok et al., 1992, Moffatt et al., 1999, Hanayama et al., 2002, Anderson et al., 2003). The efferocytosis of apoptotic neutrophils can in fact lead to proresolution effects through the suppression of pro-inflammatory mediators and release of anti-inflammatory cytokines from the phagocytic macrophage (Voll et al., 1997, Fadok et al., 1998).

Experiments using macrophages from Annexin 1 null mice have revealed that Annexin 1 deficient macrophages are impaired in their ability to phagocytose non-opsonised zymosan particles *in vitro* and *in vivo* and produce more pro-inflammatory cytokines (TNF-α and IL-6). Additionally, studies using monoclonal antibodies to neutralise Annexin 1 show a requirement for Annexin 1 in the phagocytosis of apoptotic Jurkat cells, but not primary thymocytes, by macrophages *in vitro* (Yona et al., 2006, Yona et al., 2004). Interestingly Maderna *et al.* have demonstrated that Annexin 1 released by dexamethasone-treated macrophages or Annexin 1 N-terminal peptide Ac2-26 can stimulate the phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages *in vitro* (Maderna et al., 2005). In addition,

Maderna *et al.* found a reduced phagocytic capacity of bone marrow-derived macrophages from Annexin 1 deficient mice. Subsequent experiments have revealed that *in vitro* full length Annexin 1 and Annexin 1 cleaved peptides are released from apoptotic PMN, Jurkat T lymphocytes and human mesangial cells that promote their nonphlogistic phagocytosis by human monocyte-derived macrophages via actin filament reorganization. This mechanism of engulfment is totally dependent on caspase activity (Scannell et al., 2007).

Although exogenously administered Annexin 1 peptide mimetics have the ability to enhance macrophage efferocytosis *in vitro* (Dalli et al., 2013), there is no *in vivo* evidence to suggest that endogenous Annexin 1 promotes the phagocytosis of apoptotic neutrophils within the inflammatory milieu and Annexin 1 deficient mice do not exhibit a deficiency in the clearance of apoptotic neutrophils by inflammatory macrophages (Chatterjee et al., 2005). However, there is an increased accumulation of senescent neutrophils in the bone marrow of Annexin 1 deficient mice, when compared to wild-type animals which results from the fact that Annexin 1 deficient mice have a reduced capacity for the phagocytosis of apoptotic neutrophils by resident bone marrow macrophages *in vivo*. This deficiency is conferred by the absent macrophage expression of Annexin 1 (Dalli et al., 2012).

The ability to bind phospholipids externalised to the cell surface on apoptosis is common to all Annexins and research suggests that the core region of Annexin 1 can act as a bridging molecule between phosphatidylserine (PS) on the apoptotic cell and macrophage surface, permitting tethering and engulfment (Fan et al., 2004, Morand et al., 1995, Dreier et al., 1998) (Blume et al., 2009). In addition, the non-PS-binding N terminal region (aa 1-46) can promote phagocytosis through FPR2/ALXR receptor binding (Maderna et al., 2005).

In vitro evidence suggests that Annexin 1 is released from apoptotic cells as a mechanism to stimulate phagocytosis by macrophages (Arur et al., 2003, Debret et al., 2003, Scannell et al., 2007). Secondary necrotic cells also externalise Annexin 1, although the protein acts to reduce the production of proinflammatory cytokines (most notably IL-6) by macrophages on engulfment, rather than promote their efferocytosis (Blume et al., 2009). In addition, cleavage of Annexin 1 externalised by secondary necrotic cells by membrane-bound (metallo-)proteinase ADAM10 releases a 7aa N-terminal peptide which acts to promote monocyte chemotaxis in

vitro, contributing to the nonphlogistic phagocytosis of apoptotic cells (Blume et al., 2012).

1.1.2.4 Macrophage polarization

Re-defining the rigid distinctions of macrophages from M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes, pro-resolution macrophages have been identified as expressing a mixture of M1 and M2 cytokines (COX2, iNOS, IL-10, and arginase 1) and may play a role in antigen presentation, phagocytosis, lymphocyte repopulation and inhibition of leukocyte trafficking (Bystrom et al., 2008, Rajakariar et al., 2008, Stables et al., 2011). Annexin 1 peptides have been shown to augment IL-10 production *in vitro* and *in vivo* and IL-10 responses are diminished in Annexin 1 deficient mice (Damazo et al., 2005, Guido et al., 2013, Cooray et al., 2013). Recombinant Annexin 1 can also induce phosphorylated AMP-activated protein kinase (pAMPkα1) in M1 macrophages and stimulate IL-10 gene expression (Perretti *et al.* unpublished data). Current data suggest an ability of Annexin 1 to act as a phenotypic switch for the polarisation of macrophages to a resolution phenotype, but further work needs to be done to establish whether Annexin 1 can lead to the accumulation of pro-resolution macrophages in an *in vivo* setting.

1.1.2.5 Influence on the adaptive immune response

Although Annexin 1 expression is primarily found in cells of the innate immune system, all extravasated lymphocytes are positive for Annexin 1 (Perretti et al., 1999). Whereas dexamethasone upregulates Annexin 1 expression in innate immune cells, the glucocorticoid downregulates Annexin 1 expression in T cells *in vitro* and *in vivo* (Damazo et al., 2005, Damazo et al., 2006, D'Acquisto et al., 2008). Early research attributing a role for Annexin 1 in T cell responses suggested a mirroring of its anti-inflammatory actions within the innate arm of the immune response as *in vitro* studies utilising recombinant Annexin 1 and Annexin 1 peptide-derivatives attributed a role for the protein in inhibiting antigen-driven T cell proliferation and reducing pro-inflammatory cytokine production (Gold et al., 1996, Kamal et al., 2001). This hypothesis was supported by *in vivo* studies in a Th1-driven mouse model of antigen-induced arthritis (AIA); demonstrating a role for endogenous Annexin 1 in limiting chronic inflammation (Yang et al., 2004), accompanied by the inhibition of pro-inflammatory cytokine expression (IL-1β, TNFα, IL-6, and MIF). In addition, a recent paper from Weyd *et al.* describes how

externalized Annexin 1 on early apoptotic cells can suppress DC-mediated immune responses through Toll-like receptor (TLR) signalling (Weyd et al., 2013).

Conflicting evidence for the role of Annexin 1 in the adaptive immune response has pointed towards a function in positively modulating T-cell proliferation and activation and skewing the immune response to a Th1 phenotype, through TCR signalling (D'Acquisto et al., 2007a, D'Acquisto et al., 2007b). D'Acquisto's group have described how Annexin 1 affects the strength of TCR signalling and could be responsible for manipulating T cell development through the regulation of positive and negative selection (Paschalidis et al., 2010). *In vivo* evidence to support this hypothesis for Annexin 1 playing a pro-inflammatory role in the adaptive immune response comes from a murine model of multiple sclerosis. In experimental autoimmune encephalomyelitis (EAE) Annexin 1 deficient mice exhibit impaired ability to develop the disease, accompanied by a markedly reduced infiltration of macrophages and T lymphocytes and decreased T cell proliferation responses *in vitro*. Compared to the wild-type animals, the ability of Annexin 1 null cells from the draining lymph nodes to produce Th1 and Th17 cytokines was significantly reduced (Paschalidis et al., 2009).

Th2 responses play an important role in allergic reactions and autoimmune diseases such as asthma and Annexin 1 deficient mice are more susceptible to spontaneous airway hyperresponsiveness and exhibit higher antibody responses in a murine model of asthma (Ng et al., 2011). Recent research supports the idea that Annexin 1 can play a protective role in Th2-driven inflammation and inhibits the proliferation of both CD4+ and CD8+ T cell subsets (Yang et al., 2013). However, Yang *et al.*s work also confounds the evidence for Annexin 1 exacerbating Th1-driven inflammation; describing that in a Th1/Th17-driven model of collagen-induced arthritis, Annexin 1 is protective and inhibits T cell proliferation. The researchers attribute the effects of Annexin 1 to intrinsically expressed protein derived from CD4+ cells. Yang *et al.* also describe Annexin 1 as preventing Th17 activation, contrary to what was observed in the murine model of multiple sclerosis. This is supported by the recent observation that Annexin 1 deficient mice are more susceptible to an autoimmune Th17-mediated retinal inflammatory disease (Yazid et al., 2015). Interestingly, Yang *et al.* describe how Annexin 1 can prevent neutrophil, CD4+ and CD8+ cell adhesion in a T cell-dependent model of inflammation. Annexin 1 has previously been demonstrated to influence PMN extravasation, rather than rolling or adhesion and

this observation may reflect how the effects of Annexin 1 can vary as a result of the arm of the immune response involved in each disease model.

To account for the alternate roles of Annexin 1 mentioned in the above studies, it is important to recognise that the *in vitro* experiments by Gold *et al.* and Kamal *et al.*, perpetrating an anti-inflammatory role, were performed with mixed populations of PBMCs. In these studies, Annexin 1 may be having an effect on other cells, which in turn influence the behaviour of the T cells in culture. The conflicting evidence for the *in vivo* role of Annexin 1 in the adaptive immune response is perplexing but is most likely explained by the differing etiology of the experimental models used; the inflammatory stimuli employed, subtle differences in T cell subset involvement and signalling pathways involved.

1.1.2.6 *Mechanism of action: signalling through FPR2/ALXR*

Annexin 1 binds the G-protein-coupled receptor (GPCR) formyl peptide receptor (FPR) type 2 also known as the lipoxin A4 receptor, FPR2/ALXR (Perretti et al., 2002). There are 3 members of the FPR family in humans and 8 members identified in the mouse, termed FPR 1-8. Receptors FPR1 and FPR2/ALXR are orthologous in mouse and human. The murine equivalent of FPR3 is highly homologous to FPR2 and their differential expression, roles and ligands are yet to be dissected. Mouse and human neutrophils and monocytes/macrophages express FPR1 and FPR2/ALXR, whereas FPR3 is found predominantly in human monocytes/macrophages and mature DCs (Yang et al., 2002, Migeotte et al., 2005). The fomyl-peptide receptors are also found in various tissues and non-hematopoietic cell types including fibroblasts and endothelial cells (summarised in (Migeotte et al., 2006)).

The FPRs are involved in recognising peptides and lipids generated from exogenous pathogens and endogenous sources. Activation of FPRs can modulate intracellular kinase activities e.g. protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) and NADPH oxidase activation, leading to processes including the mobilization of calcium, chemotaxis and cell activation (Le et al., 1999) (reviewed in (Ye et al., 2009)).

As well as binding Annexin 1, FPR2/ALXR is also the receptor for lipoxin A_4 (LXA_4) and serum amyloid protein A (SAA) (see Figure 1-2). Unusually, FPR2/ALXR can

transduce pro- or anti-inflammatory responses, depending on the agonist engaged. For instance, like most other FPR2/ALXR ligands, LXA$_4$ mediates anti-inflammatory effects, specifically through the inhibition of NF-κB, a transcription factor responsible for the production of many pro-inflammatory cytokines and chemokines. LXA$_4$ can prevent the adhesion and transmigration of neutrophils as well as promoting the apoptosis of neutrophils and their phagocytosis by macrophages, and the eicosanoid is understood to be a key player in the resolution of inflammation (Serhan, 2005). Conversely, SAA acts as a pro-inflammatory agonist for FPR2/ALXR; it has been shown to be responsible for the secretion of IL-8 by neutrophils through NF-κB activation and the secretion of TNF-α by monocytes, as well as promoting the chemotaxis of both neutrophils and monocytes. Different ligands for FPR2/ALXR may also exhibit different potencies in terms of activating and sustaining signalling pathways (Pederzoli-Ribeil et al., 2010) and FPRs can exhibit alternate affinities for ligands, allowing the activation of distinct pathways at different agonist concentrations (Hartt et al., 1999) reviewed in (Ye et al., 2009).
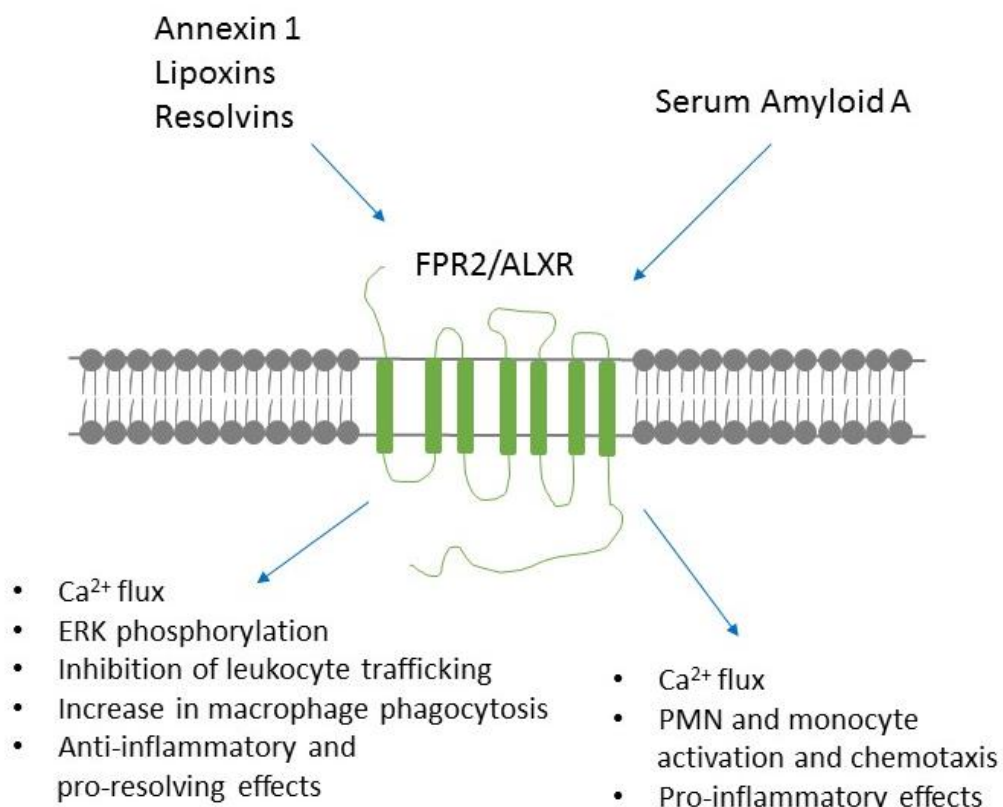
Figure 1-2 Dual nature of FPR2/ALXR

The FPR2/ALX receptor can mediate distinctive anti- or pro-inflammatory effects depending on the agonist that binds. Binding of Annexin 1 and lipoxin A_4 to FPR2/ALXR can reduce the production of pro-inflammatory cytokines through the inhibition of NF-κB, prevent neutrophil adhesion and transmigration and promote neutrophil apoptosis and their efferocytosis by macrophages. Conversely, binding of serum amyloid A to FPR2/ALXR acts as a pro-inflammatory agonist, through the activation of NF-κB, promotion of neutrophil and monocyte chemotaxis and by enhancing pro-inflammatory cytokine production from these cells.

Recent research has shed light on the mechanism by which FPR2/ALXR may exert opposing effects on inflammation depending on the agonist involved. Experiments have suggested that binding of alternate ligands to the receptor initiates conformational changes that determine downstream signalling pathways. Binding of Annexin 1, its N-terminal peptide Ac2-26 and LXA_4 leads to homodimerization of FPR2/ALXR, whereas SAA binding decreases this phenomenon. Binding of Annexin 1, Ac2-26 and LXA_4 also promotes heterodimerization of FPR1 and FPR2/ALXR. Functional studies have revealed that Ac2-26 and LXA_4 can inhibit neutrophil survival promoted by SAA through the heterodimerization of FPR1 and FPR2/ALXR, activating the JNK pathway, leading to caspase-3 activation (El Kebir et al., 2007, Cooray et al., 2013).

It is thought that, under non-inflammatory conditions, the Annexin 1 receptors are expressed on the neutrophil surface, acting in a paracrine manner to endogenously limit neutrophil infiltration into the tissues. Following initial cell activation stimulated by an inflammatory insult, there is a loss of Annexin 1 binding capacity, accompanied by the proteolytic cleavage of Annexin 1 (which has been translocated to the cell surface) and it's receptor; allowing neutrophil transmigration and full activation of the cells (Goulding et al., 1998). However, on adhesion to the endothelium, it is understood that FPR2/ALXR is translocated to the neutrophil surface as Annexin 1 binding capacity is augmented (Euzger et al., 1999). This allows renewed signalling through the receptor, with Annexin 1 binding and modulating further PMN accumulation. Patients with rheumatoid arthritis (RA), untreated or treated with non-steroidal anti-inflammatories (NSAIDs) only have significantly less binding sites for Annexin 1 on monocytes and neutrophils than healthy controls and patients with other chronic inflammatory diseases and this could explain the chronic inflammatory processes apparent in the disease as

Annexin 1 is unable to perform its endogenous anti-inflammatory role (Goulding et al., 1992).

Experiments utilizing mice deficient in FPR1 or both FPR2/ALXR and FPR3 have highlighted the receptors' roles in mediating the inhibitory effects of Annexin 1 and its N-terminal peptide Ac2-26 on PMN accumulation and limiting PMN infiltration after an inflammatory insult, in the context of acute and chronic pathologies (Perretti et al., 2001, Dufton et al., 2010, Gavins et al., 2005, Patel et al., 2012, Kao et al., 2014).

1.1.3 Cleavage of Annexin 1 by proteinase 3

Both proteinase 3 (PR3) and Annexin 1 are externalised to the plasma membrane on neutrophil activation where they co-localize allowing the cleavage of Annexin 1 by PR3 (Muller Kobold et al., 1998, Vong et al., 2007). PR3 cleaves Annexin 1 at three sites: Ala11, Val22, and Val36 (Vong et al., 2007). The shorter two peptides generated by this cleavage; 12 and 23 amino acids long, respectively, are thought to be unstable and unlikely to play a physiological role. The 33kDa peptide generated corresponds to a fragment found in bronchoalveolar lavage fluid (BALF) of patients with cystic fibrosis and can also be generated through proteolytic cleavage by human neutrophil elastase (HLE) (Smith et al., 1990, Tsao et al., 1998, Rescher et al., 2006). It is unclear whether generation of the 33kDa peptide is merely a result of proteolysis limiting the biological actions of Annexin 1 or whether the peptide has a physiological role itself. This shortened peptide has been reported to bind to monocytes with similar efficacy as the full length protein and one study suggested that it retains its ability to inhibit phospholipase A_2 activity *in vitro* (Huang et al., 1987). However, it is largely believed that the 33kDa peptide is an inactive form of Annexin 1 as the cleaved N-terminus holds much of the biological activity of Annexin 1 and the capacity for binding to FPR2/ALXR. Unlike the full length protein, *in vitro* the 33kDa peptide fails to exert anti-inflammatory effects on peritoneal macrophages and does not inhibit superoxide production in monocytes (Euzger et al., 1999, Smith et al., 1990).

Excessive Annexin 1 cleavage has been shown to be associated with PMN infiltration in an experimental model of colitis and also when comparing BALF between normal subjects and those with various lung diseases. Neutrophils are an important reservoir of PR3 and these observations suggest that neutrophil accumulation could contribute to Annexin 1 cleavage, limiting the protein's anti-

inflammatory/pro-resolution actions in these disease settings (Vergnolle et al., 1995, Smith et al., 1990).

1.1.3.1 *Serine protease-cleavage-resistant forms of Annexin 1*

A serine protease-cleavage-resistant form of Annexin 1 (SuperAnxA1 or SAnxA1) has been generated by Pederzoli-Ribeil *et al.* which is resistant to PR3 proteolysis across the three cleavage sites (Pederzoli-Ribeil et al., 2010). Their experiments using SAnxA1 demonstrate that the molecule can significantly inhibit PMN adhesion to endothelial monolayers *in vitro*. *In vivo*, SAnxA1 promotes increased and continued inhibition of cell adhesion to the postcapillary venule when compared to exogenous administration of the recombinant native protein in a model of IL-1β induced inflammation of the microcirculation. SAnxA1 also inhibits PMN accumulation when fMLP is used as an inflammatory stimulus (Pederzoli-Ribeil et al., 2010). This observation has been supported in a more prolonged model of inflammation induced by λ-carrageenan injection into mouse paw, which highlights the ability of SAnxA1 to sustain its anti-inflammatory actions, including through reduced infiltration of PMN, over longer periods than the native protein. Patel *et al.* reasoned to test the effects of SAnxA1 in a neutrophil- and macrophage-dependent mouse model of serum-induced inflammatory arthritis, due to the discovery of increased PR3 mRNA levels in the arthritic joint. Excitingly, when dosed at 1μg daily, SAnxA1 can accelerate the resolution phase of disease (Patel et al., 2012).

Anti-inflammatory actions of cleavage-resistant Annexin 1 have been reproduced more recently with a recombinant N-terminal Annexin 1 in which Val25 is replaced with a leucine residue, preventing proteolytic cleavage, termed CR-AnxA1$_{2-50}$ (Dalli et al., 2013). Experiments using this novel peptide have shown the potential for anti-inflammatory effects in murine IL-1β induced inflammation and the acceleration of the inflammation resolution phase in a model of zymosan peritonitis, mediated by the reduction of PMN migration into inflammatory sites and an increase in clearance of apoptotic cells by macrophages, respectively. Effects in a translational setting also suggest that CR-AnxA1$_{2-50}$ can enhance the inhibition of human leukocyte recruitment to endothelial monolayers at low concentrations, compared to exogenous administration of the native protein and can prevent serum amyloid A (SAA) induced-neutrophil survival. CR-AnxA1$_{2-50}$ also increases the phagocytosis of apoptotic neutrophils by human primary macrophages *in vitro*. Additionally, *in* vivo

experiments have demonstrated tissue-protective actions of CR-AnxA1$_{2-50}$ in a murine model of cardiac ischemia/reperfusion injury (Dalli et al., 2013).

These cleavage-resistant forms of the full-length Annexin 1 protein have higher affinity for FPR2/ALXR than shorter N-terminal based peptides, hence their more potent and sustained anti-inflammatory effects across *in vitro* and *in vivo* studies. Taken together, these experiments using SAnxA1 and CR-AnxA1$_{2-50}$ suggest a potential function as pro-resolution therapeutics in acute and chronic inflammatory disease settings.

1.2　Anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis

ANCA-associated vasculitis (AAV) is a chronic necrotising inflammation, predominantly affecting small blood vessel walls. Disease commonly manifests as glomerulonephritis and is typically associated with the presence of auto antibodies to proteins found in the granules of neutrophils and lysosomal compartments of monocytes; most notably myeloperoxidase (MPO) and proteinase 3 (PR3), although disease can also occur in the absence of an auto-antibody. According to the Chapel Hill Consensus (2012), the ANCA-associated vasculitides comprise three disease entities; granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (eGPA, formerly known as Churg-Strauss syndrome). These three conditions are defined by their clinical features; GPA is distinguished by the presence of extravascular granulomatous inflammation in the respiratory tract, MPA usually manifests as glomerulonephritis in the absence of upper airway involvement and eGPA is associated with eosinophilic inflammation, asthma and nasal polyps. Unlike other vasculitides, AAV is characterised by the presence of few or no immune deposits. GPA is predominantly associated with the presence of a PR3-ANCA and MPA with an MPO-ANCA. Less than half of eGPA patients have a detectable ANCA and ANCA-negative eGPA patients are least likely to have renal involvement (Jennette et al., 2013).

The annual incidence of AAV is in the range of ~13 to 20 per million (Watts et al., 2015) and the disease is predominantly found in Caucasian populations. In terms of the disease subtypes, GPA is more commonly found in northern Europe and MPA in southern Europe (Mahr et al., 2004). Global variation in disease incidence is likely partly explained by genetic factors (Lyons et al., 2012) although similar penetrance across the sexes and an increase in disease occurrence in older age suggest strong

environmental factors also contributing to disease. Environmental risk factors that have been attributed to the incidence of AAV have included UV radiation exposure (Gatenby et al., 2009), silica inhalation (Yashiro et al., 2000) and levamisole-contaminated cocaine use (Pendergraft et al., 2014). A seasonal occurrence of GPA points towards a link with infection and it has been suggested that there is molecular mimicry between human LAMP-2, a recently identified ANCA, and the bacterial protein FimH (Kain et al., 2008).

AAV is associated with considerable morbidity and mortality, with end stage renal failure developing in more than 30% of patients at 5 years (Little et al., 2004). Current treatment encompasses a general immune-suppressive approach, which is often associated with other adverse events; treatment-induced complications including severe infection now outweigh disease complication in terms of cause of death (Little et al., 2010, Calich et al., 2014). Of those patients who go into remission, there is a relapse rate of 50% over 5 years (Booth et al., 2003). This is of particular concern in GPA patients with a PR3-ANCA who are prone to relapse. There is a need to define the pathogenic mechanisms of disease more clearly and find alternative therapies not associated with current toxicities.

1.2.1 Pathogenesis of AAV

Glomerular injury in AAV is characterised by an initial influx of neutrophils followed by the crescentic accumulation of macrophages and T cells, leading to fibrosis and subsequent deterioration in renal function. Research over the last 25 years has led to the general acceptance of the following mechanism leading to disease:

When neutrophils are primed by proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) or lipopolysaccharide (LPS) there is a rapid translocation of MPO and PR3 to the cell surface where the antigens become exposed for ANCA binding (Schreiber et al., 2004, Falk et al., 1990, Charles et al., 1991, Hoshino et al., 2007). Activation of primed neutrophils by ANCA occurs through the crosslinking of ANCA antigens on the cell surface through F(ab)$_2$ engagement and is modulated through Fcγ receptor binding (Porges et al., 1994, Mulder et al., 1994, Kettritz et al., 1997). Binding of ANCA to primed neutrophils causes their activation, adherence to endothelial cells and increased transmigration into the tissue (Falk et al., 1990, Nolan et al., 2008). On activation neutrophils also degranulate, releasing lytic enzymes and reactive oxygen species (ROS) as well as proinflammatory cytokines e.g. IL-8, causing damage to the surrounding tissue, promoting further inflammation

and injury (Falk et al., 1990, Cockwell et al., 1999) (see Figure 1-3). ANCA inhibit the normal efferocytosis of apoptotic macrophages and this in turn leads to a build-up of necrotic neutrophils (Harper et al., 2000). As ANCA-binding increases the surface expression of MPO and PR3, the response is perpetuated, leading to a continual antigen-driven response.
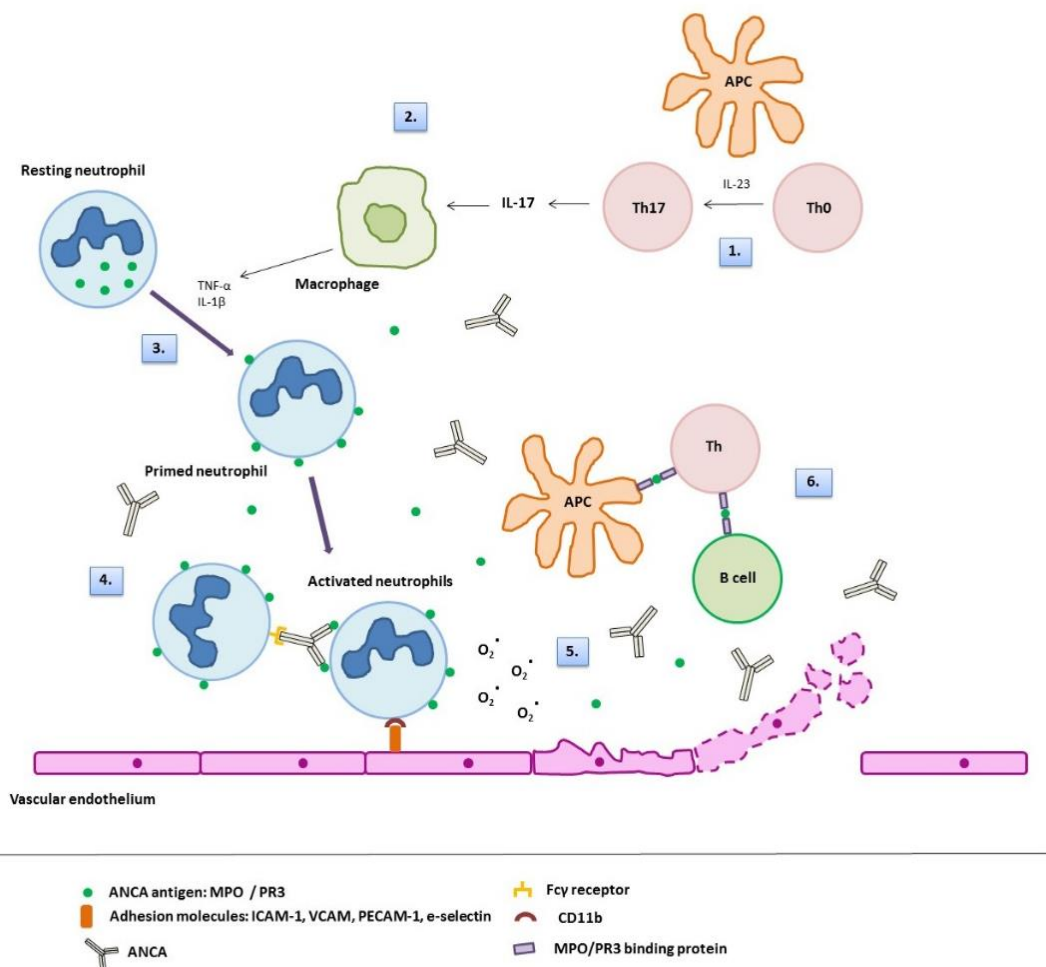
Figure 1-3 Involvement of the innate and adaptive arms of the immune response in the pathology of AAV

1. Exposure to infectious stimuli skews the adaptive immune response to a Th17 cell phenotype. 2. IL-17 produced from Th17 cells stimulates the release of TNF-α and IL-1β from macrophages. 3. Neutrophils are primed by these cytokines, leading to the exposure of ANCA antigens on the neutrophil surface. 4. ANCA binding to primed neutrophils causes full activation, degranulation and release of free radicals. 5. Adherent neutrophils activated by ANCA release proteolytic enzymes and reactive oxygen species leading to endothelial

detachment, lysis and apoptosis. 6. MPO and PR3 released by activated neutrophils is presented to Th cells by APCs, resulting in the production of ANCA from B cells.

1.2.1.1 Pathogenicity of ANCA

Circulating ANCA titres have been shown to correlate to a degree with disease activity (van der Woude et al., 1985) and the persistence of ANCA in GPA patients in remission is a strong predictor of relapse (Stegeman et al., 1994, Sanders et al., 2006). Recent observations of patients treated with rituximab, a B-cell depletion agent, have supported the pathogenic role of ANCA; Cartin-Ceba *et al.* (Cartin-Ceba et al., 2012) observed that all relapses following treatment with rituximab and corticosteroids were preceded by the reconstitution of the B-cell population and a rise in ANCA-titre. Although a rising ANCA titre has been used successfully as a predictor of relapse in AAV, the relationship is not robust and a relapse can occur more than 6 months subsequent to a rising ANCA titre (Boomsma et al., 2000, Finkielman et al., 2007). Recently Roth and colleagues (Roth et al., 2013) have described how only epitope-specific MPO-ANCA are pathogenic and these epitopes only exist in active disease. Their studies explain why MPO-ANCA can be found in healthy individuals as they lack the pathogenic epitopes. More importantly, this explains the phenomenon of MPO-ANCA negative AAV; in which detection of the circulating pathogenic auto-antibody is masked by a protein fragment. This addresses the quandary of describing ANCA as pathogenic when AAV can be diagnosed in the absence of an ANCA. A single case report of a neonate born from a mother with active MPO-associated AAV developing glomerulonephritis and pulmonary haemorrhage is direct evidence for MPO-ANCA pathogenicity, thought to have occurred through placental transfer of the antibody (Bansal and Tobin, 2004).

1.2.1.2 Genetics

Familial clustering of AAV suggests genetic risk factors for disease and Knight *et al.* have determined a 1.56 relative risk factor for first degree relatives of patients with GPA (Knight et al., 2008, Tanna et al., 2012). However, multiple cases of AAV in a family are rare due to disease incidence, so there are limited genetic associations that can be drawn from familial studies. A number of autoimmune diseases such as RA, systemic lupus erythematosus (SLE) and type 1 diabetes are strongly associated with particular human major histocompatibility complex variants. Two recent genome-wide association studies (GWAS), from the European Vasculitis Genetic Consortium (EVGC) and the US Vasculitis Clinical Research Consortium

(VCRC), as well as a number of smaller studies have revealed significant associations between several HLA SNPs and AAV, supporting a strong role of autoimmunity in disease. Interestingly, these HLA associations appear to correlate strongly with ANCA specificity, rather than clinical classifications, suggesting that the diseases have a distinct genetic background. Notable associations have been observed between PR3-ANCA and variations in HLA-DP and MPO-ANCA and particular HLA-DQ alleles (Lyons et al., 2012, Xie et al., 2013). The EVCG GWAS also revealed significant associations between PR3-positive AAV and an SNP of the promoter region of the gene encoding PR3 in addition to an SNP in a gene encoding a low-activity allele for alpha-1 antitrypsin, a major inhibitor of PR3 activity. Both of these associations support a pathogenic role for PR3 in PR3-associated AAV. Other notable genetic associations that have been identified as risk factors for AAV include a PTPN22 variant encoding lymphoid tyrosine phosphatase, involved in abnormal regulatory T cell and enhanced neutrophil function (Jagiello et al., 2005, Maine et al., 2012, Bayley et al., 2015) and an SNP in CTLA4, a glycoprotein involved in T-cell stimulation, a target for a recently trialled monoclonal antibody abatacept, which showed some efficacy in controlling disease in GPA patients (Kamesh et al., 2009, Langford et al., 2014).

1.2.1.3 Complement

Although immune deposits are not a defining clinical observation in AAV, components of the alternative complement pathway (factor B, C5a and properdin) have been detected at elevated levels in AAV patients with acute disease (Xing et al., 2010, Gou et al., 2013). *In vivo* studies investigating mice deficient in alternative complement pathway factors have revealed that factor B and C5a are essential for the development of MPO-associated glomerulonephritis in the murine model (Xiao et al., 2007, Schreiber et al., 2009). Work by Schreiber *et al.* (2009) has been crucial in hypothesising the potential pathogenic role for the alternative complement pathway in terms of AAV. Their *in vitro* experiments demonstrated that neutrophils activated by ANCA release C5a, which itself becomes a self-perpetuating inflammatory stimulus, through the further priming of neutrophils for ANCA-induced activation via the C5a receptor (C5aR). C5a has also been described as a strong neutrophil chemoattractant and C5aR stimulation upregulates PR3 on the neutrophil surface (Schreiber et al., 2009, Chen et al., 2010). There is currently a phase 2 trial recruiting to examine the efficacy of a C5aR inhibitor (CCX168) in AAV (http://www.clinicaltrials.gov, NCT01363388).

1.2.1.4 Neutrophils

Neutrophils and monocytes are traditionally seen as the main effector cells in AAV. Seminal work by Falk *et al.* (1990) suggested the mechanism by which neutrophils are not only activated by ANCA, but subsequently degranulate and release free radicals, damaging the endothelium and leading to necrotic tissue injury (Falk et al., 1990). This view is still held as one of the most central processes contributing to disease in AAV. Xiao *et al.* were the first to demonstrate the essential role for neutrophils in pauci-immune necrotising and crescentic glomerulonephritis (NCGN); as in their mouse model of anti-MPO associated glomerulonephritis, animals depleted of neutrophils are completely protected from disease (Xiao et al., 2005).

The activation of primed neutrophils and monocytes by ANCA leads to the release of cytoplasmic granule and lysosome components, encompassing proteolytic proteins; neutrophil serine proteases (NPSs) including PR3 and neutrophil elastase (NE), as well as MPO and matrix metallopeptidase 9 (MMP9). NSPs are released in inactive forms which are processed into active forms by the lysosomal cysteine protease dipeptidyl peptidase I (DPPI). DPPI-deficient and PR3/NE-double-deficient mice are protected from the development of anti-MPO induced NCGN, through the reduced production of IL-1β; highlighting the crucial role of these proteases released by neutrophils in disease (Schreiber et al., 2012). Extracellular MPO, primarily released by infiltrating neutrophils is found deposited in the glomeruli of AAV patients (O'Sullivan et al., 2015). In addition to acting as a planted auto-antigen, MPO released from neutrophils and monocytes can bind to endothelium causing oxidative damage and MPO deficient mice are partially protected from glomerular injury in a model of MPO-associated glomerulonephritis (Vargunam et al., 1992, Grattendick et al., 2002, Ruth et al., 2006).

In addition to degranulation, primed neutrophils respond to ANCA through a process termed NETosis. Neutrophils were first identified as producing extracellular traps (NETs) in a response to bacterial assault (Brinkmann et al., 2004). Lately these networks of chromatin fibres, histones and granule proteins have been identified in a number of chronic inflammatory diseases including RA, SLE and atherosclerosis (Lande et al., 2011, Khandpur et al., 2013, Knight et al., 2014). Kessenbrock *et al.* were the first to report detection of NETs in patients with AAV. They demonstrated that isolated IgG from AAV patients induced the formation of NETs from primed neutrophils *in vitro* and these NETs express MPO and PR3 that are accessible to

ANCA (Kessenbrock et al., 2009). Increased detection of NETs in AAV may result from their reduced clearance due to low levels of DNase I activity, as seen in SLE (Sallai et al., 2005, Hakkim et al., 2010, Nakazawa et al., 2014). This accumulation of NETs may cause direct damage to the endothelium, due to the excess of proteases, and could also act as a platform for ANCA antigen exposure, leading to breaking of tolerance; it has been demonstrated that NETs themselves and NET-treated DCs can induce the formation of ANCA and vasculitis in rodents (Nakazawa et al., 2012, Sangaletti et al., 2012). Recently it has been demonstrated that neutrophils from AAV patients produce more intracellular ROS and NETs when stimulated by ANCA, compared to healthy controls (Ohlsson et al., 2014).

1.2.1.4.1 Neutrophil surface PR3 expression

PR3 is an ANCA antigen closely associated with a diagnosis of GPA, which exhibits a granular cytoplasmic staining in fixed neutrophils. PR3 is released by primed neutrophils on activation by ANCA through degranulation and formation of NETs. In addition acting as an antigen for PR3-ANCA, the protease itself can cause endothelial cell detachment, lysis and apoptosis and has been demonstrated to impair the efferocytosis of apoptotic PMN and prevent phagocytic macrophages from polarizing towards a resolutive phenotype (Ballieux et al., 1994, Yang et al., 1996, Kantari et al., 2007, Gabillet et al., 2012, Millet et al., 2015) (see Figure 1-4).
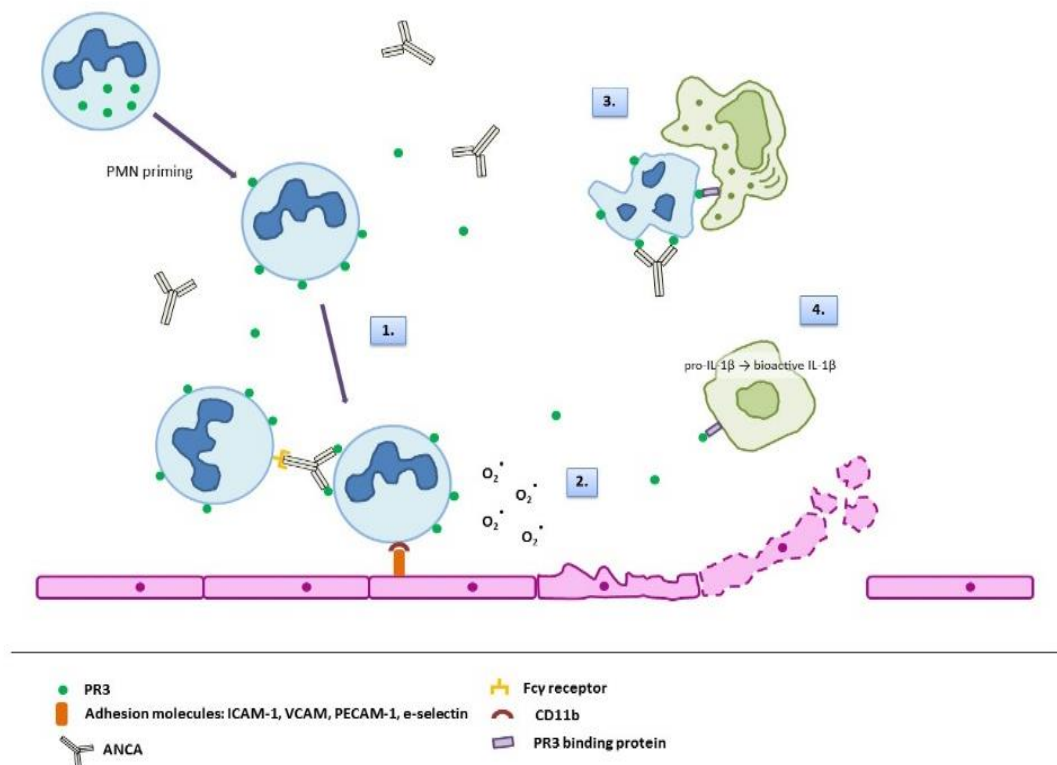
Figure 1-4 Pathogenic role of PR3 in AAV

1. PR3 acts as an ANCA autoantigen, leading to the activation of primed neutrophils and monocytes. 2. Adherent neutrophils expressing PR3 are activated by ANCA, releasing proteolytic enzymes and ROS leading to endothelial detachment, lysis and apoptosis. 3. PR3 expression on the surface of apoptotic PMN impairs macrophage phagocytosis and stimulates pro-inflammatory cytokine release, leading to further PMN and monocyte recruitment and the production of Th17 cells. 4. PR3 can process pro-IL-1β into bioactive IL-1β in monocytes/macrophages.

Csernok *et al.* (Csernok et al., 1994) originally reported PR3 expression on the neutrophil surface and it has since been established that expression is bimodal; consisting of a negative or weakly positive population of cells and a PR3 positive population. It is generally understood that PR3 expression on the neutrophil surface is genetically determined and the percentages of PR3^{low} and PR3^{high} cells are stable under resting and inflammatory conditions, although the expression of PR3 in both populations increases on activation (Schreiber et al., 2003, Halbwachs-Mecarelli et al., 1995). It has been suggested that a high percentage of membrane-expressing

PR3-positive neutrophils is a risk factor for AAV and RA (Witko-Sarsat et al., 1999) and a higher surface expression of PR3 on resting neutrophils in GPA patients correlates with risk of relapse (Rarok et al., 2002), indicating a pathogenic role for PR3. Gene expression of AAV patients has also revealed that PR3 expression correlates with disease activity and patients with a high PR3 expression are less likely to achieve remission within 6 months (Cheadle et al., 2010, Grayson et al., 2015).

1.2.1.5 *Microparticles*

Microparticles (MPs) are produced by activated and apoptotic cells as a result of disruption to the asymmetrical phospholipid cell membrane; as PS accumulates on the outer leaflet of the lipid bi-layer, there is blebbing and shedding of PS microvesicles (hereafter termed microparticles) (Zwaal and Schroit, 1997, Comfurius et al., 1990). MPs from different cellular sources can range from 0.1-1 micrometer in size and vary in their physiological function; they have been suggested to play a role in coagulation, homeostasis, cellular signalling, apoptosis, inflammation and immunity (Chou et al., 2004, Morel et al., 2006, Abid Hussein et al., 2007, Freyssinett, 2005). The production of MPs has to date been documented in platelets, erythrocytes, monocytes, granulocytes, epithelial and endothelial cells and a number of cancer cells and their cellular origin is reflected in their antigenic properties.

The first indication that leukocyte MPs could be involved in vascular injury came from *in vitro* experiments by Mesri and Altieri in 1999 in which they described activation of endothelial cells by leukocyte MPs (Mesri and Altieri, 1999). Subsequently, several studies of vasculitis patients have demonstrated significantly elevated numbers of circulating neutrophil MPs (NMPs) and platelet MPs (PMPs) in active disease (Daniel et al., 2006). Brogan *et al.* (2004) reported that in a cohort of children with systemic vasculitis, the proportion of endothelial MPs (EMPs) and PMPs were elevated in active disease; EMP numbers correlated with the Birmingham Vasculitis Activity Score (BVAS) and acute-phase reactant levels, supporting a role for EMP counts in disease monitoring and their possible involvement in perpetuating vascular injury (Brogan et al., 2004a). Interestingly, EMPs can be produced by endothelial cells acting to present superantigen to T cells, providing a potential link to vascular injury in antibody-mediated vascular disease (Brogan et al., 2004b), and PMPs can bind to and activate neutrophils *in*

vitro; relevant to neutrophil activation in AAV pathogenesis (Jy et al., 1995). Most recently, it has been demonstrated that ANCA can stimulate the release of MPs from primed neutrophils (Hong et al., 2012). These NMPs, which bind to endothelial cells via CD18 and intercellular adhesion molecule-1 (ICAM-1) interactions, have been shown to activate endothelial cells, assessed by their increase in IL-6, IL-8 and ROS production (Stassen et al., 2008). In addition, NMPs express the proteolytic enzymes MPO and PR3, allowing the capacity for direct endothelial injury (Taekema-Roelvink et al., 2000). NMPs can also exert thrombotic effects through CD11b/CD18 interactions with platelets; thromboses has been associated with disease activity in AAV, supporting a role for NMPs in mediating vascular injury in AAV (Pluskota et al., 2008).

There is a general consensus that the circulating pool of MPs consists of pro- and anti-inflammatory elements. As well as pro-inflammatory properties, NMPs have been shown to exert anti-inflammatory effects on monocyte-derived macrophages and endothelial cells *in vitro* and *in vivo* inhibit neutrophil recruitment to an inflammatory site (Gasser and Schifferli, 2004, Dalli et al., 2008, Jansen et al., 2012). The role that these anti-inflammatory microparticles might play in AAV is yet to be established.

1.2.1.6 *Monocytes and macrophages*

Monocytes contain reservoirs of MPO and PR3 in their lysosomal compartments and produce IL-8 and free radicals in response to ANCA (Charles et al., 1992, Ralston et al., 1997, Weidner et al., 2001). Human monocytes are conventionally divided into three subsets determined by their CD16 and CD14 expression; classical (CD14^{high}CD16$^{neg/low}$), intermediate (CD14^{high}CD16^{high}) and non-classical (CD14^{low}CD16^{high}). Recently a pathogenic role for intermediate monocytes has been proposed in AAV as this population is expanded in AAV patients compared to healthy controls. These intermediate monocytes have been shown to express the highest levels of MPO and PR3, compared to their classical and non-classical counterparts, and also produce the greatest amounts of IL-1β in response to monoclonal anti-MPO and MPO-ANCA purified from patients. This cytokine response is not seen with monoclonal anti-PR3 or PR3-ANCA purified from patients and is independent of Fcγ receptor binding (O'Brien et al., 2015).

Unlike neutrophils which are transient and are typically removed from an inflammatory site, infiltrating monocytes can differentiate into macrophages and persist in a chronic inflammatory setting. It has been reported that macrophages can produce NET-like structures containing proteases and MPO, so may act directly in tissue damage and auto-antigen presentation in AAV (O'Sullivan et al., 2015). Acute vascular lesions in AAV can progress from areas of necrotising neutrophils to an aggregation of monocytes, macrophages and T lymphocytes, leading to fibrosis and sclerosis, as well as leading to granulomatous inflammation, found in GPA and eGPA, which is characterised by an extravascular accumulation of monocytes, macrophages, multinucleated giant cells and T lymphocytes (Weidner et al., 2004).

Monocytes differentiate into broadly defined M1 (classical) or M2 (alternative) macrophages, depending on the surrounding stimuli. M1 macrophages are associated with the adaptive immune response, responding to cytokine stimuli such as IFN-γ, TNF-α and LPS and driving a Th1 response; activating more M1 macrophages and producing Th1 cytokines. M2 macrophages play a role in the innate immune response through IL-4, IL-10, IL-13 and TGF-β signalling and induce a Th2 response, including matrix deposition and fibrosis and the production of Th2 cytokines (IL-4, TGF-β). Serum from AAV patients has been described as polarising macrophages *in vitro* towards an M2 phenotype and M2 macrophages may predominant in focal pauci-immune necrotising GN as well as within areas of tubular injury (Ohlsson et al., 2014, Palmer et al., 2014, Zhao et al., 2015). There has been no intrinsic phagocytosis defect defined in macrophages from AAV patients although it has been suggested that ANCA may decrease the phagocytic ability of macrophages (Ohlsson et al., 2012).

1.2.1.7   T cells

T lymphocytes have been implicated in the pathogenesis of AAV as CD4+ T cells are a main component of renal and granulomatous lesions and CD4+ T cells can be detected in the urine of AAV patients, where their levels correlate with disease activity (Ronco et al., 1983, Abdulahad et al., 2009). Persistent CD4+ T cell activation is characteristic in patients, even in disease remission (Marinaki et al., 2006). *In vitro* studies have revealed that T lymphocytes from MPO- and PR3-AAV patients respond to their respective antigens *in vitro* and produce MPO-ANCA when stimulated (Griffith et al., 1996, Brouwer et al., 1994, Yoshida et al., 2005). *In vivo* models of GN demonstrate that autoreactive MPO-specific T cells are required for

the development of glomerular injury (Xiao et al., 2002, Ruth et al., 2006). The current hypothesis is that MPO-specific autoreactive effector T cells recognise deposited antigen in the glomeruli and produce a delayed type hypersensitivity response. This theory is supported by the recent discovery of an immunodominant MPO T-cell epitope that when planted in the glomeruli, induces FCGN (Ooi et al., 2012).

There is evidence for both Th1 and Th17 involvement in the pathogenesis of AAV. T cells isolated from patients with GPA produce increased levels of IFN-γ and TNF-α compared to healthy controls *in vitro* (Ludviksson et al., 1998). These Th1 cytokines have an important role in cell recruitment and activation and can upregulate neutrophil and endothelial cell surface expression of PR3 (Baudeau et al., 1994, Csernok et al., 1994). Neutrophils and monocytes are a potential source of IL-12, with increased cytokine levels identified in monocytes from GPA patients with active disease as well as in remission, which could potentiate a Th1 immune response (Romani et al., 1997, Ludviksson et al., 1998). Serum levels of IL-17A and IL-23 are elevated in AAV patients with acute disease, compared to healthy controls, and IL-23 levels correlate with disease activity. Elevated numbers of circulating PR3-specific IL-17 producing T cells are found in PR3-positive GPA patients supporting a role for the Th17 axis in disease (Abdulahad et al., 2008, Nogueira et al., 2010). IL-17A is a strong neutrophil and monocyte/macrophage chemoattractant and activator, connecting both the innate and adaptive arms of the immune response (Jovanovic et al., 1998, Laan et al., 1999, Shahrara et al., 2009). Notably, IL-17A deficient mice are protected from developing a delayed-type hypersensitivity response to deposited MPO in a mouse model of anti-MPO focal necrotising glomerulonephritis, accompanied by the absence of neutrophil and macrophage infiltration and a reduction in CCL5 levels (Gan et al., 2010). When mouse neutrophils are stimulated with MPO-ANCA *in vitro* they produce IL-23, favouring a Th17 response (Hoshino et al., 2008).

T regulatory cells (Tregs) are responsible for maintaining immune tolerance to self-antigens (Sakaguchi, 2004). A number of studies have shown that there is a functional defect in Tregs in AAV patients and patients with higher proportions of Tregs are most likely to respond to treatment (Abdulahad et al., 2007, Morgan et al., 2010, Free et al., 2013). The depletion of Tregs in a mouse model of MPO-associated glomerulonephritis exacerbates the severity of disease and shows enhanced autoantibody responses to MPO (Tan et al., 2013). Tregs can inhibit T

cell expansion and prevent their migration into inflamed tissue and have also been reported to suppress Th17 cells (Enarsson et al., 2007, Fletcher et al., 2009, Tanriver et al., 2009). Current consensus is that a defect in Tregs in AAV may predispose towards a Th17 response, leading to the recruitment of macrophages to the inflammatory site, perpetuating a delayed-type hypersensitivity reaction.

1.2.1.8 B cells

Most of the research on the pathogenic mechanisms in AAV has focused on neutrophils, T cells and ANCA. Since the identification of the anti-CD20 monoclonal antibody rituximab as an effective agent in the induction of remission in AAV patients, studies have begun to address the contribution of B cells to disease (Jones et al., 2010, Specks et al., 2013, Stone et al., 2010). There is B cell infiltration apparent in granulomatous lesions in GPA, of which the persistence may be associated with relapse (Ferraro et al., 2008, Voswinkel et al., 2008).

Regulatory B cells (Bregs) are able to suppress CD4+ T cell functions through IL-10 signalling and may inhibit Th1/Th17 responses (Bouaziz et al., 2010, Carter et al., 2011). Bregs can also inhibit TNF-α production by activated monocytes *in vitro* (Iwata et al., 2011). Decreased serum levels of IL-10 have been found in AAV patients, correlating with an increased risk of relapse (Hruskova et al., 2009, Sanders et al., 2006) and a numerical deficiency in Bregs has been reported in active and quiescent AAV which may be associated with increased Th1 activation through reduced IL-10 production (Todd et al., 2014, Wilde et al., 2013).

1.3 Animal models of AAV

Key in the elucidation of pathological mechanisms involved in AAV has been the development of rodent models of disease. Two murine models have been used in the following experimental work; a mouse model of nephrotoxic nephritis (NTN) and a model of murine experimental vasculitis (MEV). NTN is a model of immune-complex mediated crescentic glomerulonephritis that shares many features with the crescentic GN often seen in antibody-mediated vasculitis. The disease model is characterised by glomerular thrombosis and crescent formation, associated with macrophage and T cell infiltration. Two alternative models of MEV are used in the following work; both are mouse models of myeloperoxidase anti-neutrophil cytoplasm antibody disease. The first MEV model is induced by the passive transfer of anti-MPO antibodies into recipient mice and results in a pauci-immune vasculitis,

mimicking human MPA. The second is generated through the immunisation of human MPO into recipient mice, followed by administration of sub-nephritogenic anti-glomerular basement membrane antibody, directing the immune response to the kidney. These two alternative models of MEV provide the opportunity to investigate both the cellular and the humoral arms of the immune response in AAV. All models were set up de novo in our laboratory.

1.3.1 Nephrotoxic Nephritis

NTN is a model of immune-complex mediated glomerulonephritis. The disease induced shares a common pathology with human glomerulonephritis and has been widely used to model glomerular thrombosis and crescent formation. Antibodies to mouse glomerular basement membrane are typically raised in sheep or rabbit by the injection of isolated glomeruli in adjuvant. Injection of this resulting nephrotoxic serum (NTS) into the mouse causes acute heterologous injury followed by an adaptive immune response to the deposited antigen, termed the autologous phase. The adaptive immune response to the deposited antigen varies between mouse strains; C57BL/6 mice exhibit delayed-type hypersensitivity to the NTS, with crescentic accumulation of macrophages and T cells and deposition of fibrin, characteristic of the Th1 type response seen in AAV, whereas BALB/c mice develop a Th2 polarised response, with a glomerular accumulation of complement, with less T cell and macrophage infiltration (Huang et al., 1997). Pre-immunisation of animals with serum from the host species in which the nephrotoxic serum is generated elicits a more rapid response to the NTS. This model is termed accelerated NTN (see Figure 1-5).
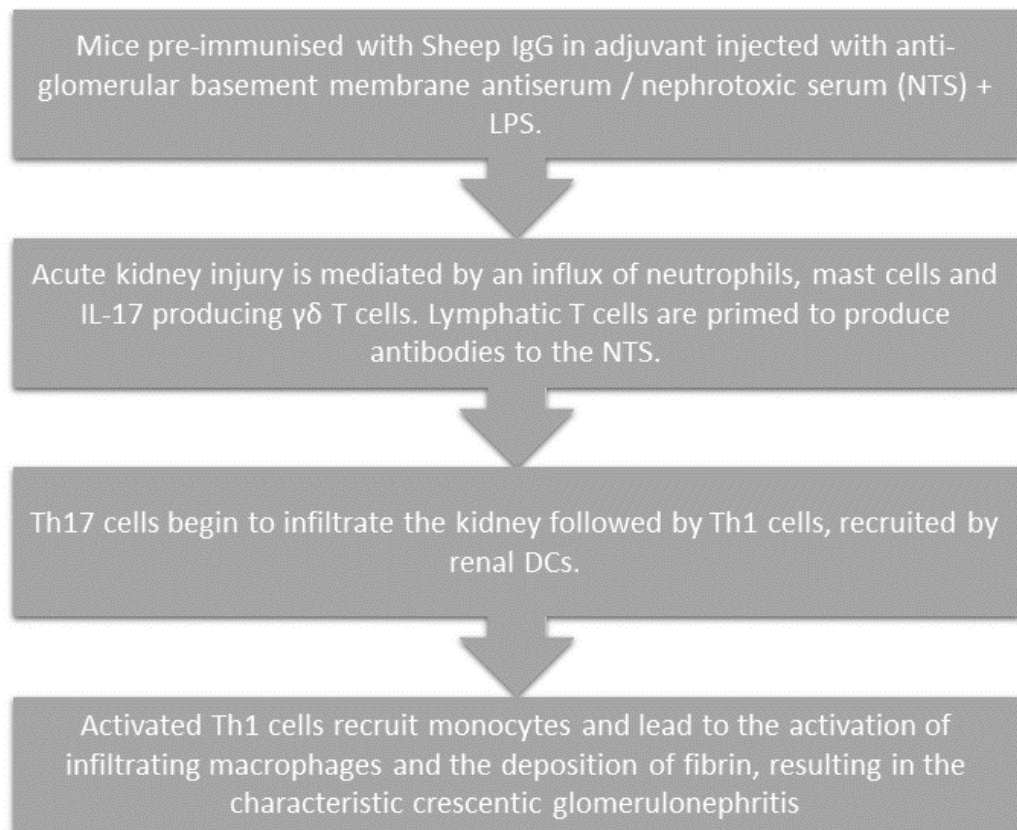
Figure 1-5 Cellular immune response in accelerated nephrotoxic nephritis in C57BL/6 mice

Accelerated NTN is a murine model of immune-complex mediated glomerulonephritis. Pre-sensitised mice elicit a rapid cellular response to injected nephrotoxic serum, followed by an adaptive immune response to the deposited antigen, leading to a crescentic accumulation of macrophages and T lymphocytes and deposition of fibrin. DCs, dendritic cells.

Studies using the murine model of NTN have allowed the dissection of key processes contributing to the formation of glomerular thrombosis and crescent formation. Crescentic GN can develop in the absence of B cells and immunoglobulin (Li et al., 1997) and it is the balance of T helper cell subset activation that determines the severity of the resultant glomerular injury (Huang et al., 1997). Th1 and Th17 subsets both contribute to renal injury, but their respective cytokine responses can inhibit the other subset; IL-17 can exacerbate acute kidney injury but plays a protective role in established disease through the inhibition of the Th1 response (Paust et al., 2009, Odobasic et al., 2011, Turner et al., 2012).

Experiments using the murine model of NTN have shown the capacity of particular cytokines to attenuate (IL-4, IL-10) or exacerbate (IL-12) the development of crescentic glomerulonephritis by modulating the T cell response to the deposited antigen (Kitching et al., 1999b, Kitching et al., 2000, Tipping et al., 1998, Tipping et al., 1997). Interferon-gamma (IFN-γ), produced by Th1 cells, directs cell-mediated renal injury (Kitching et al., 1999a) and tumour necrosis factor (TNF), also a key Th1 cytokine, is essential in the upregulation of adhesion molecule expression (ICAM-1 and VCAM-1) and the recruitment of inflammatory cells after the injection of NTS, leading to the formation of glomerular crescents (Le Hir et al., 1998).

In addition to infiltrating leukocytes, intrinsic renal cells play an important role in the development of renal injury in crescentic glomerulonephritis; IL-12-producing non-bone marrow derived cells significantly contribute to the glomerular accumulation of leukocytes (Timoshanko et al., 2001) and CD40-expressing intrinsic renal cells are involved in the development of disease through the induction of Th1 cytokines (Ruth et al., 2003, Timoshanko et al., 2002). Major histocompatibility complex class II (MHC II)-expressing intrinsic renal cells are also essential for the development of glomerular injury via their interaction with CD4+ T cells (Li et al., 1998).

Experiments using the NTN model first described the vital role of Fc receptors in the acute phase of crescentic GN (Suzuki et al., 1998). The function of circulating leukocytes in the disease manifested in NTN has shown to be mediated through Fcgamma receptor I and III interactions (Tarzi et al., 2002, Tarzi et al., 2003) and a role has been revealed for the mannose receptor (MR; a pattern recognition receptor) in the Fc-mediated functions of macrophages and their survival, promoting glomerular injury, independent of the adaptive immune response (Chavele et al., 2010).

1.3.2 Murine experimental vasculitis

Several murine models have been developed in an attempt to replicate MPO-associated AAV. The models used in the following experiments successfully reproduce a pauci-immune crescentic glomerulonephritis as seen in the human disease (see Figure 1-6).
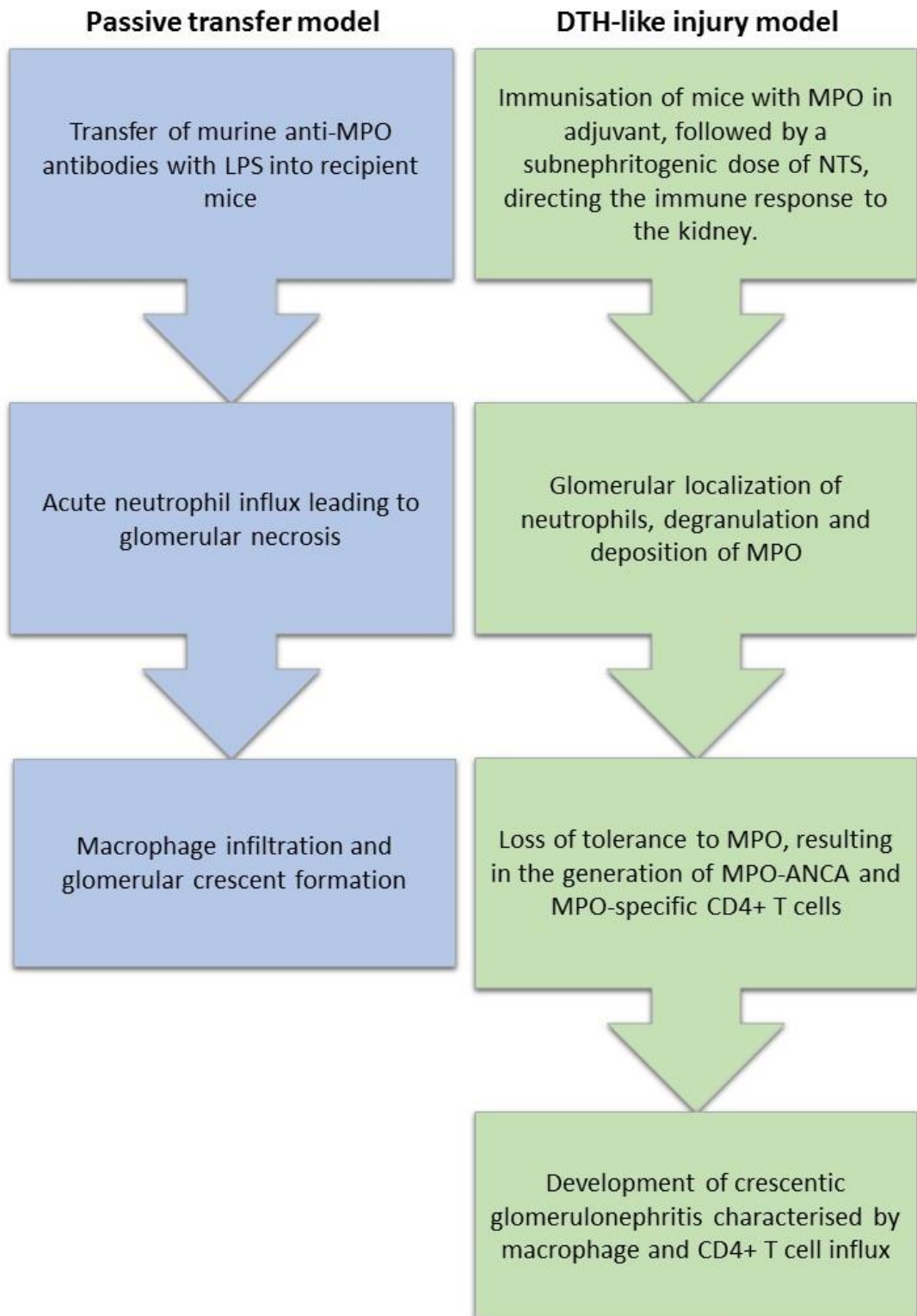
Figure 1-6 Cellular effectors in two models of anti-MPO associated glomerulonephritis

Two murine models of anti-MPO associated glomerulonephritis. The passive transfer model is generated by an acute cellular response to injected anti-MPO IgG combined with adjuvant. This leads to glomerular injury and the crescentic accumulation of macrophages and T lymphocytes. The DTH-like injury model is initiated by the injection of MPO and low dose NTS, causing glomerular accumulation of neutrophils, leading to a breaking of immune tolerance to MPO and subsequent adaptive immune response.

1.3.2.1 Rodent models of myeloperoxidase anti-neutrophil cytoplasm antibody disease

Xiao *et al.* published the first example of a murine model of severe focal necrotising and crescentic glomerulonephritis caused by the transfer of anti-MPO antibodies into wild-type mice (Xiao et al., 2002). The disease induced by purified IgG transfer is pauci-immune in nature, lacking glomerular immune deposits, and is histologically identical to the human disease, making it an ideal experimental model. The disease severity in this model of anti-myeloperoxidase antibody-induced crescentic glomerulonephritis can be enhanced by the administration of granulocyte colony stimulating factor (GCSF) and bacterial LPS, the latter mimicking the effect of an infection (Huugen et al., 2005, Freeley et al., 2013). Neutrophils are essential effector cells in this murine model of glomerulonephritis and mice depleted of neutrophils are completely protected from disease (Xiao et al., 2005). The model has been used to demonstrate the critical role of the alternative complement pathway in disease and has led to the development of a clinical trial testing the efficacy of a C5aR inhibitor in AAV (Xiao et al., 2007, Xiao et al., 2014). A modification of this model, where MPO knockout (Mpo−/−) mice that are immunized with mouse MPO are irradiated and bone marrow (BM) is replaced with Mpo+/+ or Mpo-/- BM has revealed the essential expression of MPO on haematopoietic cells for disease development as mice engrafted with Mpo-/- bone marrow do not develop disease (Schreiber et al., 2006).

An alternative model of myeloperoxidase anti-neutrophil cytoplasm antibody disease has been developed by the group of Holdsworth and Kitching (Gan et al., 2010, Ruth et al., 2006). In this model, mice are injected with human or mouse MPO in adjuvant and subsequently administered a subnephritogenic dose of NTS, directing the immune response to the kidney. This model is unique in replicating the DTH-like response to planted MPO, seen in AAV. CD4+ T cells are a significant contributor to crescentic renal injury in this model, a mechanism which is independent of ANCA.

Experiments using this model have revealed the essential role of IL-17 in disease induction; IL-17 deficient mice are significantly protected from disease and show a marked reduction in neutrophil and macrophage accumulation independent of antibody titres (Gan et al., 2010). The importance of TLR stimulation as a co-stimulus for disease induction has been demonstrated in this model, supporting a link between infection and disease (Summers et al., 2011).

The homology between human and rat MPO has allowed the development of a rat model of crescentic pauci-immune glomerulonephritis through the immunization of Wistar-Kyoto (WKY) rats with human MPO in adjuvant. Cross-reactivity between the anti-human MPO antibodies and rat MPO induces crescentic nephritis and lung haemorrhage. This model of disease is more reproducible than murine models of anti-neutrophil cytoplasm antibody disease and has highlighted the contribution of innate immune stimuli (within the adjuvant) and genetic background to disease (Little et al., 2005, Little et al., 2009). However, rat models do not have the advantage of being transferrable to such a wide range of genetic modifications that are available in mice.

1.3.2.2 Murine models of anti-proteinase 3 anti-neutrophil cytoplasm antibody disease

Although there is a substantial body of evidence to indicate the direct pathogenicity of MPO-ANCA in animal models, a lack of homology between human and murine PR3 and an absence of surface expression of PR3 on mouse neutrophils has hindered the development of a murine model of anti-PR3 anti-neutrophil cytoplasm antibody disease. There is one mention of a direct pathogenic role of PR3-ANCA recently described by Little *et al.* in 2012 (Little et al., 2012). In Little *et al.*'s model, irradiated NOD-*scid-IL2Rγ−/−* mice are rescued with the infusion of human haematopoietic stem cells. These chimeric mice develop a mild to severe glomerulonephritis with lung haemmorhage when injected with IgG from PR3-ANCA vasculitis patients. Although this model is crucial in demonstrating a direct pathogenic role for human PR3-ANCA, there is no current reproducible model of the granulomatous inflammation found PR3-associated vasculitis and this model has not been replicated since.

1.4   Project hypothesis

Annexin 1 has emerged as a key mediator in the resolution of inflammation (see Figure 1-7). The protein plays a regulatory role in neutrophil trafficking to inflammatory sites and promotes PMN clearance in a nonphlogistic manner, promoting the return of the tissue to homeostasis. Annexin 1 deficient mice are more susceptible to a number of acute and chronic models of inflammation and Annexin 1 derived peptides can resolve an established inflammatory reaction.
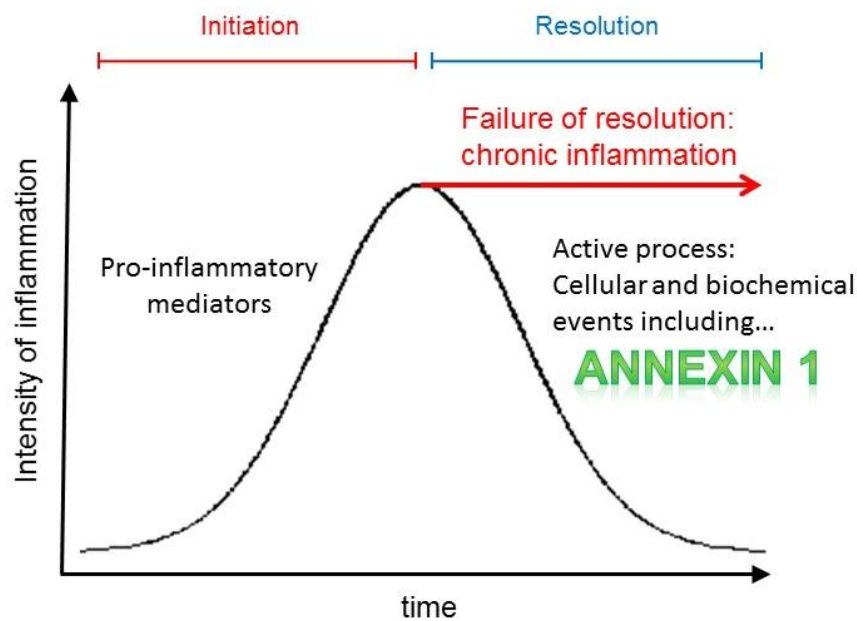
Figure 1-7 Annexin 1 is a key player in the active process of inflammation resolution

Resolution of inflammation is understood to be an active process involving several endogenous protein and lipid mediators including lipoxins, resolvins and Annexin 1. Dysregulation of these resolution mediators may contribute to chronic inflammatory states.

AAV is characterised by a necrotising glomerulonephritis, driven by the accumulation of neutrophils and macrophages leading to chronic, non-resolving inflammation. Current treatment strategies in AAV encompass a general immune-suppressive approach with treatment-associated complications and relapse incidence of significant concern. 'Resolution'-based therapy may offer a new avenue of treatment options.

Recent understanding of the pathogenesis of AAV has demonstrated an important role of PR3 in disease. A higher neutrophil expression of PR3 correlates with a risk of relapse and is an indicator for poor treatment response. PR3 cleaves Annexin 1 and my hypothesis is that an abundance of PR3 expressing neutrophils at the inflammatory site in AAV leads to an increase in Annexin 1 cleavage, preventing the proteins' pro-resolution actions, contributing to the chronic inflammatory state.

1.4.1 Thesis aims

The primary aims of this thesis are:

- To examine the effect of the genetic absence of Annexin 1 on the disease manifested in a murine model of immune-complex mediated crescentic glomerulonephritis. **Chapter 3.**

- To investigate whether cleavage–resistant forms of Annexin 1 might modulate disease in a murine model of immune-complex mediated crescentic glomerulonephritis. **Chapter 3.**

- To determine whether any effects of glucocorticoid treatment in a murine model of immune-complex mediated crescentic glomerulonephritis are mediated by Annexin 1. **Chapter 3.**

- To examine the effect of the genetic absence of Annexin 1 on the disease manifested in murine models of anti-MPO associated glomerulonephritis. **Chapter 4.**

- To compare
    i.   the neutrophil surface expression of PR3
       and
    ii. proportion of intact Annexin 1 compared to its cleavage products in neutrophils

  between healthy controls and PR3-positive GPA patients and observe any correlations between the two. **Chapter 5.**

- To investigate whether cleavage–resistant forms of Annexin 1 might modulate the ANCA-induced activation of human neutrophils. **Chapter 5.**

Chapter 2 Materials and Methods

2.1　Reagents, Antibodies and Animals

2.1.1　Reagents

All oligonucleotides were from Sigma.

Immunodominant MPO peptide was synthesised by Activotec, Cambridge, UK with acetyl/amide termini.

Table 2 Reagents

Company	Reagent	Catalogue number
Andwin Scientific, Illinois, U.S.A.	Diff-Quick stain kit	NC9943455
AppliChem GmbH, Connecticut, U.S.A.	Cetyltrimethyl ammonium bromide	A0805
BD Biosciences, California, U.S.A.	BD Horizon Fixable Viability Stain 450	562247
	BD CompBeads anti-Rat IgG	552844
	Annexin V FITC	556419
Biolegend, California, U.S.A.	1X RBC Lysis Buffer	420301
Bio-Rad Laboratories, California, U.S.A.	Recombinant human TNF-α	PHP051
Citifluor, London, UK	Antifadant mount solution	AF1
GE Healthcare, Buckinghamshire, UK	HiTrap Protein G Sepharose columns	GZ17040403
	HiTrap ConA 4B sepharose columns	28-9549-01 AA
	PD-10 columns	17-0851-01
KPL, Maryland, U.S.A.	LumiGlo chemiluminescent substrate system	54-61-00

Company	Reagent	Catalogue number
Life Technologies, California, U.S.A.	Microplate Immuno MaxiSorp 96 well flat bottom polystyrene plate	DIS-971-010P
	Trypan Blue Solution, 0.4%	15250-061
	DMEM with GlutaMAX™	31966047
Merck Millipore, Darmstadt, Germany	Human myeloperoxidase	475911
Newmarket Scientific, Newmarket, UK	Polink-2 HRP Plus kit	D46
PAA Laboratories, Somerset, UK	Iscove's Modified Dulbecco's Media (IMDM)	E15-819
PeproTech EC, London, UK	GM-CSF	315-03-1000
Sartorius Stedim Biotechnology, Surrey, UK	Vivaspin centrifugal concentrator	85030-511-57
Siemens AG, D-91052 Erlangen, Germany	Multistix 8 SG urine urinary dipsticks	2146
Sigma-Aldrich, Poole, Dorset, UK	Lipopolysaccharide from Ecoli serotype 026:B6	L8274
	Complete Freund's Adjuvant	F5881
	Incomplete Freund's Adjuvant	F5506
	Sheep IgG	I5131
	Fetal Calf Serum	F9665
	10% neutral buffered formalin	HT501128
	REDTaq® ReadyMix™ PCR Reaction Mix with $MgCl_2$	R2523
	SIGMA*FAST*™ OPD (*o*-Phenylenediamine dihydrochloride) tablets	P9187
	p-nitrophenyl phosphate tablets	N2770-50SET

Company	Reagent	Catalogue number
	Dexamethasone 21-phosphate disodium	D1159
	Percoll	P1644
	Histopaque 1119	11191
	5-sulphosalycyclic acid dihydrate	247006
	Cytochalasin B from Drechslera dematioidea	C2743
	N-Formyl-Met-Leu-Phe (fMLP)	47729
Thermo Fisher Scientific, Illinois, U.S.A.	Detoxi-Gel Endotoxin Removing Gel	PN20344
	BCA Protein Assay Kit	23227
	OCT	SDLAMB/OCT
	Thermo Scientific Spectra Multicolor Broad Range Protein Ladder	26634
	Alpha-methyl-d-mannopyranoside	10023170

Table 3 Antibodies

Company	Reagent	Catalogue number
Kind gift from Professor M. Perretti, Queen Mary University of London, London, UK	Monoclonal mouse anti-human Annexin 1 antibody	
Kind gift from Professor P. Heeringa, University of Groningen, Groningen, The Netherlands	Murine MPO and anti-MPO antibody	
ABCAM, Cambridge, UK	Rat anti-mouse Ly6G (for immunostaining)	AB25273
Bio-Rad Laboratories, California, U.S.A.	Rat anti-mouse CD68	MCA1957GA
BD Biosciences, California, U.S.A.	Mouse anti-human CD54 (ICAM-1) – PE	560971
	Mouse IgG1k PE isotype	556650
	Rat anti-mouse Ly6G – PE	551461
	Rat IgG2a k PE isotype	553930
	Rat anti-mouse CD11b – PerCP/Cy5.5	550993
	Rat IgG2b k PerCP/Cy5.5 isotype	550764
	Rat anti-mouse Ly6C – APC	560595
	Rat IgM k APC isotype	400810
Biolegend, California, U.S.A.	Mouse anti-human CD15 - APC	323007

	Mouse IgG1 k APC isotype	400122
	Rat anti-mouse PE/Cy7 - F4/80	123114
	Rat IgG2a k F4/80 isotype	400521
	Rat anti-mouse CD41 PE	133905
Bio-Rad Laboratories, California, U.S.A.	Rat anti-mouse CD45 - FITC	MCA1031FA
	Rat IgG2b FITC isotype	MCA1125FT
Santa Cruz Biotechnology, Texas, U.S.A.	Mouse anti-human PR3 FITC	sc-52716
	Normal mouse IgG1 FITC	sc-2855
Sigma-Aldrich, Poole, Dorset, UK	Monoclonal mouse anti-goat/sheep IgG FITC	F4891
	Goat anti-mouse IgG – alkaline phosphatase antibody	A9316

2.1.2 Animals

C57BL/6-Annexin-/- were obtained from Charles River Laboratories (Margate, UK). B6.129X1-Mpo^{tm1Lus}/J mice were obtained from The Jackson Laboratory (Maine, USA). Mice used in the experiments were between 8-15 weeks of age and gender matched. Controls used were either littermate wild-type mice or C57BL/6 mice bred in-house (strain originally from Charles River Laboratories). All mice were housed under pathogen-free conditions and kept according to the animal facility guidelines. Experiments were carried out under project licence number 40/3228 and followed the regulations set out by the UK Home Office Animals (Scientific Procedures) Act, UK (1986). Animal group sizes for experiments were minimised by the use of a sample size calculation based on preliminary results.

2.2 *In vivo* techniques

2.2.1 Induction of accelerated nephrotoxic nephritis

NTS was generated from the injection of sheep with isolated mouse glomeruli emulsified in Complete Freund's Adjuvant (CFA), followed by two boosters of

glomeruli in Incomplete Freund's Adjuvant (IFA). Then a terminal bleed was performed and serum isolated. NTS was precipitated by Professor Alan Salama, UCL Centre for Nephrology, UCL, London, using ammonium sulphate. To induce NTN, mice were pre-immunised with a subcutaneous injection of 0.25mg sheep IgG (Sigma) on day -5. 1 part sheep IgG was mixed with 4 parts CFA and 3 parts sterile 0.9% saline. On day 0 mice were given an IV tail injection of 200µl NTS, diluted 1:1 in sterile 0.9% saline, mixed with or without varying doses of LPS. Mice were sacrificed on Day 7 or 8.

2.2.2 Generation of anti-MPO antibodies in MPO-/- mice

MPO-/- mice over 6 weeks of age were given a total dose of 40µg mMPO over 36 days (injections on day 0, 21 and 36). The first injection of 20µg was given IP in CFA, the second and third injections of 10ug were given IP in Incomplete Freund's Adjuvant (IFA). Animals were sacrificed on day 42 and blood was obtained by cardiac puncture. Blood was collected into untreated eppendorfs and kept on ice before obtaining serum by centrifuging at 5000rpm for 10 mins at 4°C. Serum was stored at -20°C prior to antibody isolation.

Additional dosing of MPO immunodominant peptide

A subset of MPO-/- mice were injected with mouse immunodominant peptide $MPO_{409-428}$ (PRWNGEKLYQEARKIVGAMV) (Ooi et al., 2012). This peptide was administered at a dose of 50µg with the first and second injections of mMPO, within the emulsion.

2.2.3 Induction of anti-myeloperoxidase associated glomerulonephritis

Either C57BL/6 wild-type mice or Annexin-/- mice were injected with 100µg/g anti-MPO IgG by IV tail injection day 0, t=0hr, followed by an IP injection of 0.1µg LPS at t=1hr. Additional IP injections of 26µg GCSF were administered at day -4, 0 and +4.

2.2.4 Assessment of disease

2.2.4.1 *Measurement of haematuria and proteinuria*

Urine was collected over 18 hours using murine metabolic cages. Haematuria was estimated using urine dipstick analysis (Siemens) and urine was collected into eppendorfs for subsequent measurement of proteinuria, serum urea and creatinine. Proteinuria was measured using a sulphosalicyclic acid (Sigma) assay against a

standard curve of BSA and read at 450nm. Urine samples were measured in triplicate and diluted at 1:10 in water or 1:100 if nephrotic (classified as +++ on a urine dipstick scale of proteinuria). Serum urea and creatinine were measured by Clinical Chemistry, MRC, Harwell.

2.2.4.2 Preparation of histology

After sacrifice, the murine circulation was flushed with ice cold PBS using a peristaltic pump by injection into left ventricle, after cutting the inferior vena cava, to remove any circulating leukocytes. Hemi-kidney, liver and spleen were fixed in 10% neutral buffered formalin (Sigma) and hemi-kidney and spleen were snap-frozen in liquid nitrogen with OCT on a cork disc for subsequent cryo-sectioning.

Fixed sections were processed and stained with periodic acid Schiff (PAS) and haematoxylin-eosin (H&E) by The Department of Cellular Pathology, The Royal Free Hampstead NHS Trust.

2.2.4.3 Histological assessment

Histological assessment was carried out blinded to the experimental conditions. PAS stained sections were scored on a scale of 0-2 (mild, moderate or severe) for the degree of glomerular proliferation and a score of 0-4 for the number of thrombotic quadrants. 25 glomeruli were scored per sample.

2.2.4.4 Cryosectioning

Frozen samples were cut into 6µm sections using a cryostat and were left to air dry, protected from light, overnight before being stored at -80°C until subsequent staining.

2.2.4.5 Immunostaining of frozen tissue sections

Direct immunofluorescence was performed on frozen kidney sections to detect sheep IgG. Samples were fixed in acetone for 10 minutes, before rehydrating in PBS and incubating with monoclonal mouse anti-goat/sheep IgG FITC (Sigma) at 1/200 dilution for 1hr at room temperature in a humidified chamber. Sections were then washed in PBS and mounted using an anti-fadant mount solution (Citifluor).

Indirect immunostaining to detect CD68 and Ly6G was performed on frozen sections using a Polink-2 HRP Plus kit (Newmarket Scientific). Tissue was fixed in pre-cooled acetone for 10 minutes before incubating in 0.3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase activity. After blocking in 10% semi-skimmed dry milk powder (Marvel) for 30 minutes, slides were incubated with rat anti-mouse CD68 (Bio-Rad Laboratories) at 1/200 or rat-anti mouse Ly6G (ABCAM) at 1/200, diluted in PBS with 10% normal goat serum for 1hr at room temperature in a humidified chamber. Control sections were incubated in PBS with 10% normal goat serum only. Spleen was included as a positive control for CD68. The subsequent steps followed the manufacturers' instructions and colour development was stopped after identifying positive staining on the spleen by microscopic visualisation. Slides were counterstained with Mayer's haematoxylin and mounted using DePeX mounting medium.

2.2.4.6 *Assessment of immune response to sheep IgG (ELISA)*

Systemic immune response to Sheep IgG was measured by ELISA using serum samples. Flat-bottomed 96-well plates (Nunc Maxisorb, Life Technologies) were coated with sheep IgG at 0.1mg/ml, diluted in borate buffered solution (BBS; 100mM boric acid, 25mM sodium tetraborate anhydrous, pH 8.3-8.5) or BBS alone, overnight at 4°C. Plates were washed three times in PBS/0.075% Tween-20 and blocked with 3% BSA in PBS/0.075% for 1 hr at 37°C. Total IgG was measured by incubating samples at a 1:1000 dilution for 1 hr at 37°C, followed by incubation with an alkaline phosphatase secondary antibody (goat anti-mouse IgG, Sigma) at a 1 in 1000 dilution for 1 hr at 37°C. Plates were developed with *p*-nitrophenyl phosphate (Sigma), 50µl per well and read at 405nm on an ELISA plate reader.

2.3 *In vitro* techniques

Informed written consent was obtained from patients and controls included in the following experiments. Clinical sample collection and use received ethical approval from the Royal Free Hospital, London.

2.3.1 Cellular techniques

2.3.1.1 *Generation of murine MPO from MPRO cell line*

Induced-differentiated mouse promyleocyte cell line (MPRO; ATCC CRL-11422) was cultured at 37°C, 5% CO_2 in Iscove's Modified Dulbecco's Media (IMDM, PAA

Laboratories) with 20% heat-inactivated fetal calf serum (FCS), 1% penicillin and streptomycin (p/s) and supplemented with either 6-10ng/ml GM-CSF (PeproTech) or 10-15% HM-5 conditioned medium (filter-sterilised). MPRO cells were grown to approximately 2×10^6 cells/ml before harvesting and freezing down in Buffer A (6.7mM sodium dihydrogen phosphate pH 6, 1mM $MgCl_2$, 3mM NaCl, protease inhibitor, $_{dd}H_2O$). Pellets were frozen and stored at -80°C prior to MPO extraction. HM-5 cells were maintained at 37°C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™ (Life Technologies), 10% heat-inactivated FCS, 1% p/s and cultured for 5-6 days past 100% confluency to produce conditioned medium containing GM-CSF. Cells were initially cultured personally and then grown by Dr Margaret Goodall, School of Immunity and Infection, University of Birmingham, UK.

2.3.1.2   Isolation of leukocytes from murine whole blood

Blood from cardiac puncture was anti-coagulated using 1:5 ratio of 3.8% tri-sodium citrate solution. Red cells were lysed for 10 minutes at room temperature using 2ml 1X RBC Lysis Buffer (Biolegend) per 100ul whole blood. Cells were washed in FACS wash buffer (0.1% sodium azide, 1% heat-inactivated fetal calf serum (FCS) in PBS) before counting and staining for FACS analysis.

2.3.1.3   Isolation of microparticles from murine whole blood

Blood from cardiac puncture was anti-coagulated using 1:5 ratio of 3.8% tri-sodium citrate solution. Platelet-poor plasma was isolated from anti-coagulated blood by centrifuging at 2400rpm for 5 minutes, followed two further centrifugations at >10,000rpm for 5 minutes to purify the supernatant containing the microparticles. Plasma was stored at -80°C prior to staining for FACS analysis.

2.3.1.4   Separation of mononuclear cells from kidney

After sacrifice, the murine circulation was flushed with ice cold PBS using a peristaltic pump by injection into left ventricle, after cutting the inferior vena cava, to remove any circulating leukocytes. Kidney was cut into small pieces and placed in cold sterile PBS before being transferred into a 0.5mg/ml collagenase solution for digestion. The tissue was then digested for 25 minutes at 37°C before further mashing and an additional digestion time of 5 minutes. Digests were then sieved through 70µm and 30µm cell strainers before washing and staining for FACS analysis.

2.3.1.5 PMN isolation from human blood

5-7mls of peripheral blood anticoagulated with EDTA was layered onto 6ml Histopaque 1119 (Sigma) and centrifuged at 800g for 20 minutes with no brake. The lower, reddish layer containing the granulocytes was washed in PBS (centrifuged at 300g for 10 min) and transferred onto a discontinuous Percoll (Sigma) gradient made up of 2mls each of the following percentages; 85%, 80%, 75%, 70%, 65%. After centrifugation at 800g for 20 minutes with no brake, the layers between 65% and 85% were collected, washed and counted for subsequent manipulation or analysis. Viability was ≥95%, assessed by trypan blue exclusion (Life Technologies).

2.3.1.6 Generation of whole cell lysates

Neutrophils were resuspended at 10×10^6 cells / ml in a non-denaturing lysis buffer (20mM Tris-HCl pH8, 137mM NaCl, 10% glycerol, 1% Triton-X 100, 2mM EDTA) on ice for 30 minutes in the presence of protease inhibitors. Cells were centrifuged at ≥10,000g for 10 minutes at 4°C and supernatants were stored at -20°C.

2.3.1.7 Purity assessment of PMN population by cytospin and subsequent staining

To assess purity of isolated PMN population, cells were resuspended at a concentration of 0.75×10^6 cells / ml in PBS and 100µl was added to the cytospin apparatus. Slide chambers were spun at 450rpm for 6 minutes before disassembling and leaving to air dry for 10 minutes. Cells were then fixed in 100% ethanol for 10 minutes before staining with Diff-Quick (Andwin Scientific); 30 seconds in solution I (red) followed by 30 seconds in solution II (blue).

2.3.1.8 FACS techniques

2.3.1.8.1 Immunofluorescent staining of isolated cells

Leukocytes from blood or kidney single cell suspensions were washed in FACS wash buffer (0.1% sodium azide, 1% fetal calf serum (FCS) in PBS) and resuspended at a concentration of 1×10^6 cells in 100µl wash buffer for staining. When monocytes or macrophages were included in the cell suspension, cells were stained in the presence of 5% FCS to reduce non-specific staining. Antibodies were used at the recommended concentration, or titrated to an optimum level of staining before use, and incubated with the cells for 30 minutes on ice before washing,

fixation with 1% paraformaldehyde and storage at 4°C prior to acquisition within 1 week.

2.3.1.8.2 Viability assessment of cells by FACS

Cell viability was assessed using a fixable viability stain (BD Biosciences) that allows the discrimination between live and dead cells based on the fluorescence intensity at 450nm. Cells were stained as directed by the manufacturer.

2.3.1.8.3 FACS acquisition

BD CompBeads (BD Biosciences) were used for compensation controls. Data was acquired using the BD LSRFortessa and between 100,000 and 1×10^6 events were collected per sample.

2.3.1.9 In vitro activation of human neutrophils

by patient ANCA

Human neutrophils were resuspended at 2.5×10^6 cells / ml and primed with cytochalasin b (Sigma) and TNF-α (Bio-Rad Laboratories) at a concentration of 5µg/ml and 2ng/ml respectively for 15 minutes at 37°C, with or without a specified concentration of SuperAnnexinA1. Subsequently, 100µl of cells were delivered to wells of a U-bottomed 96-well plate and incubated with either PBS, 1µM fMLP (Sigma), endotoxin-depleted ANCA or normal human IgG, both at 0.2mg/ml, for 15 minutes at 37°C. Then, the plate was spun at 1500rpm for 5 minutes before transferring 75µl of the supernatant to a flat-bottomed 96-well plate for measurement of MPO release.

by TNF-α

Human neutrophils were resuspended at 10×10^6 cells / ml in HBSS and plated in a 48-well plate. Cells were incubated with/without TNF-α at 10ng/ml for 30min at 37°C.

2.3.2 Molecular Biology

2.3.2.1 Genotyping of mice

Genomic DNA was obtained from the digestion of ear clips overnight in 100ul DNA lysis buffer (100mM Tris HCl pH 8.5, 5mM EDTA, 0.2% sodium dodecyl sulphate

(SDS), 200mM NaCl) at 55°C with 100µg/ml proteinase K. Samples were then diluted with 300µl of distilled water and heat inactivated for 15 minutes at 75°C.

PCR was carried out using REDTaq® ReadyMix™ PCR Reaction Mix with $MgCl_2$ (Sigma) and 2µl of the crude lysate template, using the standard protocol.

MPO-/- genotyping

See Appendix Figure 6-3 for MPO-/- genotyping example

Primers: MPO1 (5' TGA-CAC-CTG-CTC-AGC-TGA-AT 3'), MPO2 (5' TGC-AGG-CAG-CTG-GTC-TCG-CA- 3'), MPO3 (5' CTA-CCG-GTG-GAT-GTG-GAA-TGT- 3').

PCR conditions:

95°C for 5 minutes, 1 cycle

94°C for 1 minute, 35 cycles

60°C for 1 minute, 35 cycles

72°C for 1 minute, 35 cycles

72°C for 5 minutes, 1 cycle

10°C hold

2.3.2.2 Gel electrophoresis

PCR products were analysed by gel electrophoresis on a 2% agarose gel with 1µl ethidium bromide (10mg/ml) per 50ml volume. Gels were run at 100V for 30 minutes and visualised using a UV light source.

2.3.3 Protein Biochemistry

2.3.3.1 Degranulation assay / MPO release

75ul of supernatants from neutrophil activation assays were incubated 100µl MPO substrate (SIGMA*FAST*™ OPD tablets, Sigma) per well for 30 minutes in the dark at room temperature. The reaction was stopped by adding 100µl acetic acid and the OD was read at 450nm on a plate reader.

2.3.3.2 Assessment of protein concentration

Total protein concentration of cell lysates was determined by a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Bovine serum albumin (BSA) standards were made up in the lysis buffer used to create cell lysates and standards and samples were run in triplicate. Standard protocols were followed.

2.3.3.3 SDS-PAGE

10% SDS polyacrylamide gels were made and equal amounts of total protein per sample were loaded and separated by electrophoresis alongside a multicolour broad range protein ladder (Thermo Fisher Scientific). For straightforward protein visualisation, gels were stained with Coomassie blue.

2.3.3.4 Western blotting

Western blotting was used to examine Annexin 1 cleavage products in neutrophil whole cell lysates. Protein was transferred from SDS gels using a semi-dry technique to a nitrocellulose membrane and blocked overnight in blocking buffer (5% semi-skimmed dry milk powder (Marvel), 1X TBS, 0.1% Tween-20). Membranes were washed and incubated with the primary antibody at appropriate concentrations in blocking buffer for 2 hours at room temperature and then washed and incubated in the appropriate secondary antibody conjugated to horse radish peroxidase (HRP) for 1 hour at room temperature in blocking buffer. LumiGlo chemiluminescent substrate system (KPL) was used to visualize the protein.

2.3.3.5 Purification of human and mouse IgG

Human IgG containing ANCA or mouse IgG containing anti-MPO IgG was isolated from plasma exchange fluid or serum, respectively, using a Protein G Sepharose column (GE Healthcare) on a fast protein liquid chromatography FPLC machine. Columns were washed with 10 column volumes of binding buffer (20mM sodium phosphate pH 7.0) before applying the sample, adjusted with an equal volume of binding buffer. Unbound protein was washed through the column with further volumes of binding buffer until no material appeared in the effluent. IgG was eluted in 1 ml fractions with 0.1M glycine-HCl pH 2.7 into eppendorfs containing 40µl Tris-HCl pH9.0 to neutralise the solution. The IgG concentration of each fraction was determined by the absorbance at 280nm and selected fractions were pooled and

dialysed against PBS overnight. Final concentration was determined by the above mentioned method and samples were stored at -20°C for future use.

2.3.3.6 Removal of endotoxin

LPS-depletion of IgG preparations was achieved by applying the samples to a polymyxin B column (Detoxi-Gel Endotoxin Removing Gel, Thermo Scientific). Columns were firstly regenerated with 1% sodium deoxycholate, before equilibrating with pyrogen-free water and applying the sample. IgG was incubated for 1 hour on the column to maximise endotoxin depletion efficiency, eluted with pyrogen-free water and stored at -20°C.

2.3.3.7 Anti-MPO IgG ELISA

NUNC maxisorb plates (eBioScience) were coated with 0.5µg/ml mMPO in coating buffer (0.1M carbonate-bicarbonate pH9.6) overnight at 4°C. Plate washed 5 times with wash buffer (10mM Tris, 75mM NaCl, 0.05% Tween 20, pH8) before blocking in 1% BSA in PBS for 1hr at room temperature. Wash x5. Samples diluted 1:100 in incubation buffer (PBS with 0.2% BSA, 0.05% Tween-20) and added to plate. Standard and samples pipetted across the plate in a 4-fold titration curve. Incubated for 1hr at room temperature before wash x5. Incubated with alkaline phosphatase labelled goat anti-mouse IgG diluted 1/000 in incubation buffer for 1hr at room temperature before wash x5 and developed with p-nitrophenyl phosphate (Sigma). Plates scanned at 405nm and reaction stopped by adding 100µl/well NaOH 5M when a plateau for the lower dilutions is reached.

2.3.3.8 MPO extraction from MPRO cell line

All steps were carried out on ice to avoid protein degradation. MPRO cell pellets were resuspended in 10mls Buffer A (see Appendix) (0.5mM PMSF) per 1ml cell pellet and lysed using dounce homogenisation. Vesicles were then ruptured by mixing cell pellets end-over-end with 1% cetyltrimethyl ammonium bromide (CTAB) (AppliChem GmbH) for 2h. Insoluble material was removed by centrifugation at 20,000g for 20 minutes at 4°C. Soluble material was dialysed against Buffer B (see Appendix) overnight at 4°C. The following day, MPO was purified using fast protein liquid chromatography over a HiTrap ConA 4B sepharose column (GE Healthcare) and eluted using 750mM methyl-d-mannopyranoside (Thermo Fisher Scientific). Fractions were analysed using a spectrophotometer and measured at 280nm and

430nm to determine purity before being pooled. Pooled fractions were buffer exchanged into sterile PBS using PD-10 columns (GE Healthcare) and concentrated to 1mg/ml using Vivaspin centrifugal concentrator (Sartorius Stedim Biotechnology).

2.3.3.9 MPO activity assay

The MPO content of the MPRO cells was monitored over time using an MPO activity assay. A standard curve of MPO was made with human MPO (Merck Millipore), diluted in PBS. MPRO cells were lysed with a non-denaturing lysis buffer (as mentioned in section 2.3.1.6) on ice for 30 minutes. Serial dilutions of test samples were added to wells in duplicate. The samples were incubated with OPD substrate (Sigma) and H_2O_2 for 30 minutes at room temperature, protected from light. The reaction was stopped with sulphuric acid and read at 490nm on a plate reader.

2.3.4 Statistical analysis

2.3.4.1 FlowJo

FACS files were analysed using FlowJo version 7.6.5 - 10.0.7 (Tree Star Inc., Ashland, Oregon, USA).

2.3.4.2 Prism

Statistical analysis was performed using GraphPad Prism 4.0 - 6.0 (GraphPad software, San Diego, CA, USA). Data were assessed for normality using a D'Agostino & Pearson omnibus normality test to choose the appropriate statistical test. A student's t-test was used to determine any difference between two parametrically distributed sets of unpaired data. A Mann-Whitney test was used when unpaired data were non-parametrically distributed. Paired data were analysed using a paired t-test when data were parametrically distributed and a Wilcoxon matched-pairs signed rank test when data were non-parametrically distributed. A Kruskal-Wallis test was used to compare two or more groups of non-parametrically distributed data when there was a single independent variable and a two-way analysis of variance (ANOVA) was used to compare two or more groups of non-parametrically distributed data when there were two independent variables.

2.3.4.3   ImageJ

Image J was used to calculate densitometry measurements on Western blot (National Institutes of Health, USA).

Chapter 3 The effect of Annexin 1 in a murine model of accelerated nephrotoxic nephritis

3.1 Introduction

The murine model of nephrotoxic nephritis (NTN) is used successfully as a model of crescentic glomerulonephritis to determine the underlying pathology of glomerular thrombosis and crescent formation that can be found in AAV. The disease generated in C57BL/6 mice is characterised by a Th1-response; dependent on the glomerular accumulation of macrophages and T lymphocytes and deposition of fibrin leading to renal injury.

Annexin 1 has been shown to play a protective role in a number of Th1-driven models of inflammation; Annexin 1 deficient mice exhibit exacerbated disease phenotypes in mouse models of antigen- and collagen-induced arthritis, accompanied by increased cellular infiltration, T cell proliferation and expression of proinflammatory cytokines within the synovial joint (TNF-α, IL-1β, IL-6 and MIF). In T cell-mediated dermal contact hypersensitivity there is an increase in neutrophil and CD4+ T cell adhesion in the absence of Annexin 1, accompanied by increased T cell proliferation and IL-17A mRNA. In ovalbumin (OVA)-induced dermal delayed type hypersensitivity (DTH) response there is also increased T cell proliferation and IFN-γ and IL-17A levels detected in Annexin 1 deficient mice (Yang et al., 2004, Yang et al., 2013).

Steroid treatment is an important element in the management of AAV and dexamethasone has been shown to attenuate disease in NTN (Nagai et al., 1982). A number of the anti-inflammatory actions of glucocorticoids have been attributed to Annexin 1 (Hannon et al., 2003, Wallner et al., 1986) and Annexin 1 deficient mice are resistant to the pro-resolving effects of dexamethasone in two models of inflammatory arthritis (Patel et al., 2012, Yang et al., 2004). A serine protease-cleavage-resistant form of Annexin 1 (SuperAnxA1) has been shown to inhibit the adhesion and infiltration of neutrophils in an *in vivo* model of inflammation (Pederzoli-Ribeil et al., 2010) and has been used successfully to accelerate the resolution of inflammation in a model of serum-induced inflammatory arthritis (Patel et al., 2012), revealing the potential for Annexin 1 peptidomimetics in the treatment of chronic inflammation.

Microparticles (MPs) have been implicated in the pathogenesis of AAV; neutrophil-derived MPs can activate endothelial cells and platelet-derived MPs can activate neutrophils *in vitro*. In addition, levels of neutrophil-, platelet- and endothelial-derived MPs are elevated in active disease. Microparticle numbers have not been investigated in murine models of crescentic glomerulonephritis, and with a potentially emerging role in vascular injury it is important to characterise them in these models and examine any parallels between their abundance in murine and human vascular disease.

3.2   Aim

This chapter investigates the role of Annexin 1 in the murine model of accelerated NTN. Disease was compared between wild-type and Annexin 1 deficient mice, while the effect of SuperAnxA1 and dexamethasone on disease was examined. In addition to histological readouts for disease, neutrophil and macrophage infiltration into the kidney was analysed, peripheral and infiltrating leukocytes were phenotyped for inflammatory markers and microparticle numbers were determined in blood plasma.

3.3   Experimental Design

C57BL/6 and Annexin 1 -/- mice used were sex- and age-matched and between 8-15 weeks of age. Initial experiments to generate NTN in wild-type mice were performed with varying doses of LPS. Disease severity was assessed by renal function; haematuria and proteinuria estimated by urinary dipstick, proteinuria measured by sulphosalicyclic acid method and serum urea and creatinine measured by Clinical Chemistry, MRC, Harwell. Glomerular thrombosis was scored on PAS stained kidney sections. Neutrophil and macrophage infiltration into the intra-glomerular space was quantified by Ly6G and CD68 staining of frozen kidney sections, respectively. Phenotyping of blood leukocytes and renal macrophages was achieved using FACS. Blood plasma samples were stored for microparticle analysis. The systemic immune response to pre-immunisation with Sheep IgG was assessed by ELISA.

Statistical analysis was performed using GraphPad Prism.

3.4 Results

3.4.1 Establishing the murine model of accelerated nephrotoxic nephritis

Murine models are notoriously variable in terms of the degree of disease induced when generated in different animal units with varying degrees of pathogen levels and when different sources of adjuvant or stimulus are used.

The aim was to replicate the model of accelerated NTN in a new animal house and establish a moderate degree of disease that could be influenced by treatment with particular reagents or the genetic absence of specific proteins.

Nephrotoxic serum (NTS) used in the following experiments had been used successfully to induce disease at the Hammersmith Hospital Campus in the presence and absence of additional endotoxin. NTS was diluted 1:1 in sterile saline for injection.

PAS-stained kidney was scored blinded, 25 glomeruli were counted per section.

Experiments to establish the mouse model of NTN are summarised in Table 4.

Table 4 Table describing the preliminary experiments performed to establish the nephrotoxic nephritis model

Experiment ID	Experimental groups	Number of mice in experimental group	Dose of NTS and route administered	Dose of LPS and route administered	Thrombosis score (0-4)
1	Control	2	N/A*	None	0, 0
	NTN	3	200µl IP	None	0, 0, 0
2	Control	2	N/A	None	0, 0
	NTN	3	100µl IV	None	2.7, 0.6 , 0
3	Control	6	N/A	None	0, 0, 0, 0, 0, 0
	NTN	6	100µl IV	None	0, 0, 0, 0, 0, 0
4	Control	8	N/A	None	0, 0, 0, 0, 0, 0, 0, 0
	NTN	9	100µl IV	None	0, 0, 0, 0, 0, 0, 0, 0, 0
5	NTN	8	100µl IV	None	0, 0, 0, 0, 0, 0, 0, 0
6	Control + 0.025µg LPS	3	N/A	0.025µg IP	0, 0, 0
	Control + 0.5µg LPS	6	N/A	0.5µg IP	0, 0, 0, 0, 0, 0

	NTS + 0.025μg LPS	1	100μl IV	0.025μg IP	1.56
	NTS + 0.5μg LPS	1	100μl IV	0.5μg IP	0
7	Control + 0.5μg LPS	4	N/A	0.5μg IP	0, 0, 0, 0
	Control + 1μg LPS	3	N/A	1μg IP	0, 0, 0
	NTS + 0.5μg LPS	3	100μl IV	0.5μg IP	0, 0, 0
	NTS + 1μg LPS	3	100μl IV	1μg IP	0, 0, 0
8	NTN	4	100μl IV	0.625μg IV with NTS	3.76, 3.80, 2.16, 3.60

* Control animals were injected with an equivalent volume of sterile saline

Attempts to establish NTN with NTS alone

The first experiment to establish the NTN model (experiment ID 1, see Table 4) was trialled through the intraperitoneal (IP) injection of 200µl NTS alone, into mice pre-immunised with Sheep IgG, as a straightforward route of administration. When proteinuria was estimated by urinary dipstick 7 days after NTS, there was some proteinuria evident in all three animals that were given NTS; however no glomerular disease was evident on PAS-stained kidney sections. The second experiment (experiment ID 2) replicated the protocol previously established at the Hammersmith Hospital Campus and involved the injection of 100µl NTS intravenously (IV). One of the three animals that was administered NTS displayed an elevated level of haematuria when estimated by urinary dipstick, 7 days after NTS, and a thrombosis score of 2.7 when glomerular disease was quantified (see Figure 3-1 for scoring classification); however the other two animals that received NTS displayed little or no glomerular disease. Repeats of this protocol (experiment IDs 3, 4 & 5) to account for any shortcomings in the IV injection route yielded little or no proteinuria and no haematuria when estimated by urinary dipstick, 7 days after NTS. Glomerular disease was not evident in any animals when visualised on PAS-stained kidney sections.
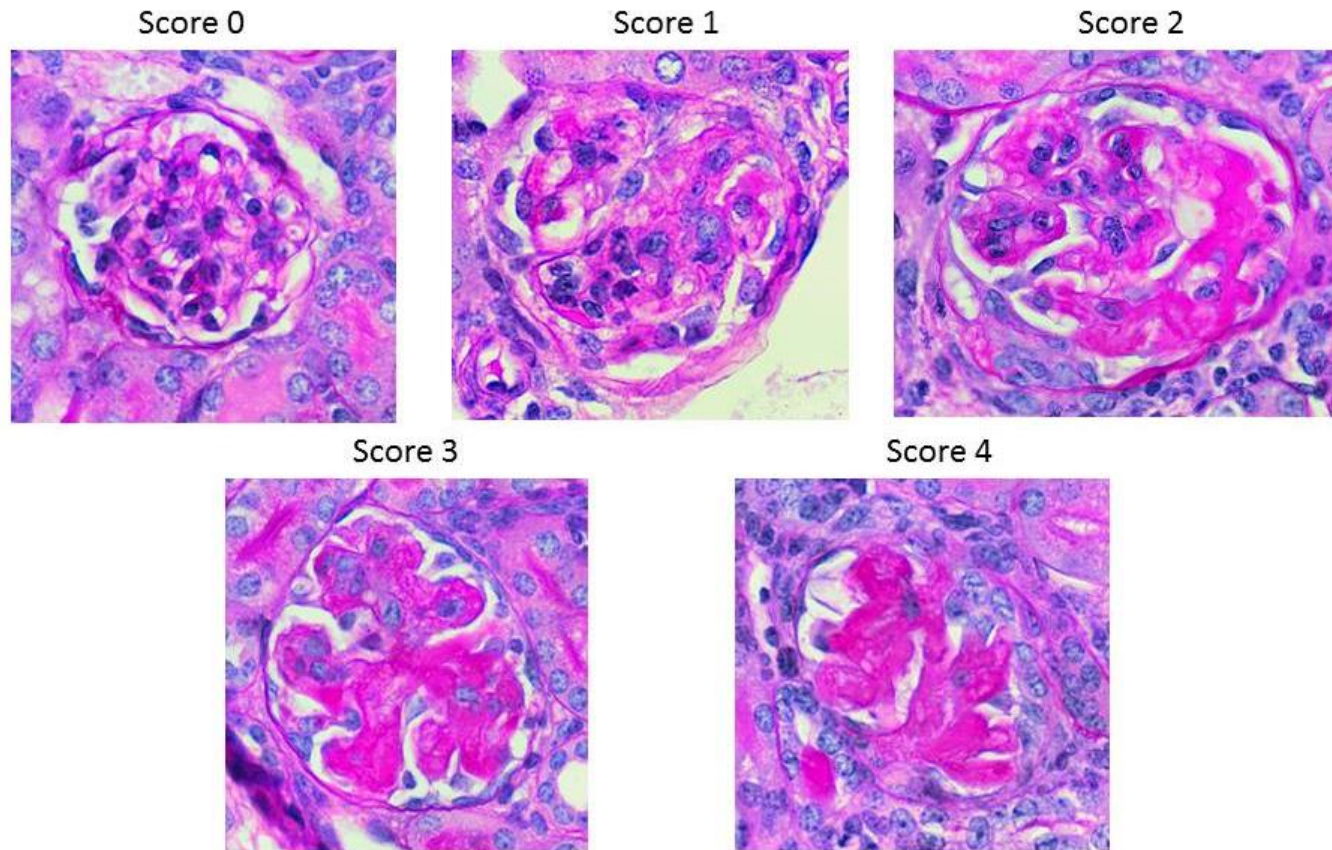
Figure 3-1 Images to demonstrate glomerular thrombosis scoring system

PAS stained kidney sections were visualised at x630 magnification and 25 individual glomeruli were scored per section. Score 0: capillary loops intact within glomerulus. Score 1: glomerular structure disrupted but no evidence of thrombosis. Score 2: glomerular thrombosis detected. Score 3: majority of glomerulus thrombosed, some structure still intact and cells still visible. Score 4: glomerulus totally thrombosed, minimal cells visible.

<u>Determination of LPS dose to exacerbate response to NTS</u>

Lipopolysaccharide (LPS) has historically been administered alongside NTS to exacerbate disease in the murine model of NTN. A pilot experiment to determine a dose of LPS that caused neutrophilia was undertaken using mice pre-immunised with CFA 5 days previously. Mice were injected IP with either 0.025μg or 0.05μg LPS alone. FACS analysis of peripheral blood samples stained with anti-Ly6G antibody 18 hours after administration of LPS revealed that a dose of 0.05μg LPS compared to 0.025μg LPS resulted in an increased median neutrophil percentage compared to total leukocyte events (median 22% versus 13%, Figure 3-2).
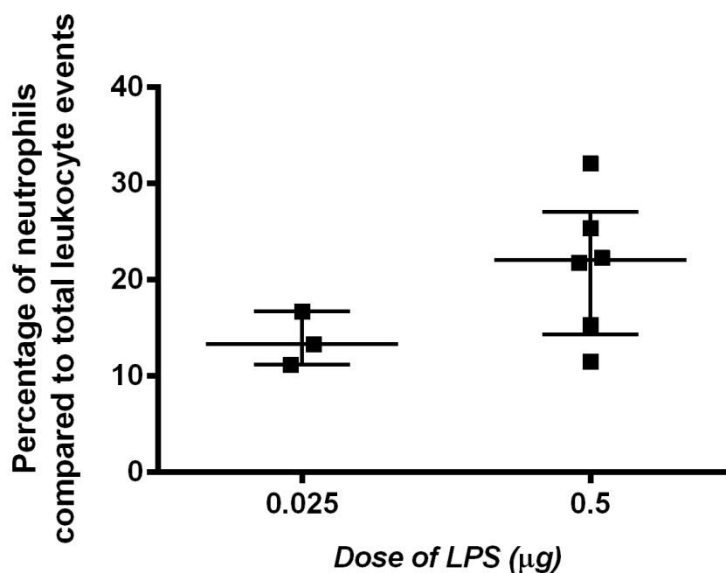
Figure 3-2 LPS administration leads to an increase in the percentage of neutrophils compared to total leukocytes in the peripheral blood

FACS analysis of tail bleeds from mice pre-immunised with CFA alone that had been administered 0.025μg and 0.5μg LPS IP revealed an increased median percentage of neutrophils, compared to total leukocyte events, 18hrs after LPS, when a higher dose of LPS was given. Total leukocytes were determined by FSC/SSC profile and neutrophils were determined by Ly6G^{+} expression. Data are expressed as median with interquartile range.

When mice pre-immunised with CFA and sheep IgG were injected IP with 0.05µg LPS, alongside NTS IV, 4 hours after administration the percentage of neutrophils in the peripheral blood was increased when compared with the injection of 0.05µg LPS in the absence of NTS (median 86% versus 79%) (Figure 3-3).
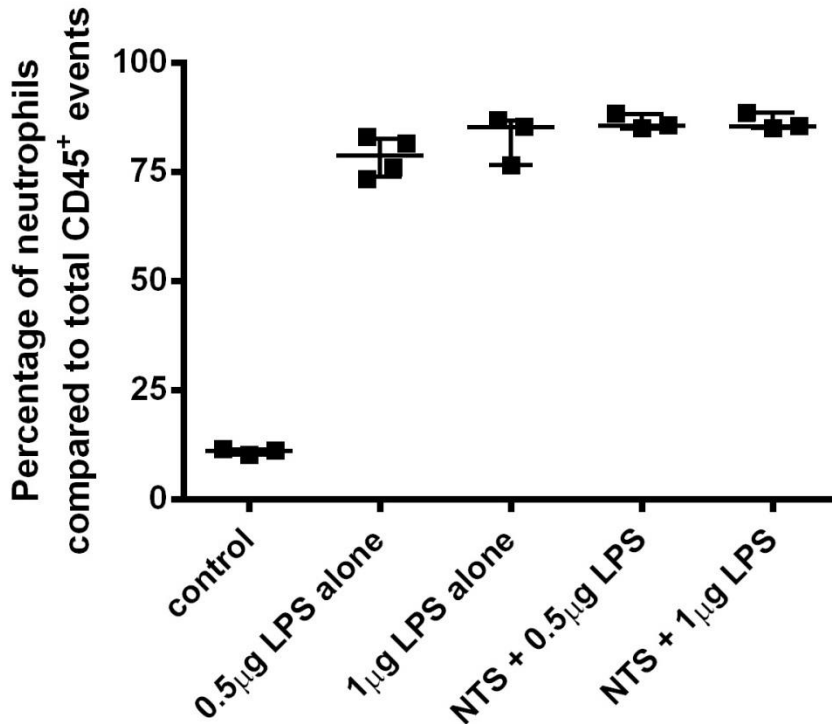
Figure 3-3 NTS administration alongside LPS leads to an increase in the percentage of neutrophils compared to total leukocytes in the peripheral blood

FACS analysis of tail bleeds from mice pre-immunised with CFA and sheep IgG, 4 hrs after IP administration of saline alone, 0.5µg or 1µg LPS alone or in combination with 100µl NTS IV. Total leukocytes were determined by FSC/SSC profile and neutrophils were determined by Ly6G^{+} expression. There was an increased percentage of neutrophils, compared to total leukocytes, when LPS was administered compared to saline alone. When NTS was administered in parallel with 0.5µg LPS, compared to 0.5µg LPS alone, there was an increase in the percentage of neutrophils, compared to total CD45^{+} events. Data are expressed as median with interquartile range.

Although the neutrophil proportion was increased within the total leukocyte population 4 hours after the administration of LPS, in mice where LPS was injected IP alongside the IV injection of NTS there was no haematuria evident when estimated by urinary dipstick and on examining PAS stained kidney sections there was no glomerular disease evident apart from a single animal with a small amount of glomerular thrombosis, 7 days after NTS injection.

A subsequent experiment replicating an LPS dose given in combination with NTS established at the Hammersmith Hospital Campus involved the addition of 0.625µg LPS to the NTS solution and the injection of this suspension IV. 7 days after NTS/LPS proteinuria and haematuria were strongly elevated when estimated by urinary dipstick in all 3 mice tested. Glomerular thrombosis score ranged from 2.2-3.8 in all 4 mice when quantified on PAS stained kidney sections.

3.4.2 The absence of Annexin 1 exacerbates disease in a mouse model of nephrotoxic nephritis

The Annexin 1 knockout mouse developed by Hannon *et al.* (Hannon et al., 2003) is more susceptible to a number of models of inflammatory disease (Yang et al., 2004, Yang et al., 2013, Liu et al., 2015). I previously described the establishment of the accelerated NTN model in wild-type mice with a dose of 0.625µg LPS given in combination with NTS IV. This dose resulted in a relatively severe disease phenotype when scored on glomerular thrombosis. My hypothesis was that Annexin 1 would attenuate disease in NTN and therefore a lower LPS dose of 0.1µg was administered in the following experiments to avoid potential lethality of disease in Annexin 1 deficient mice.

To investigate whether the absence of Annexin 1 in C57BL/6 mice confers a greater susceptibility to the development and severity of glomerular inflammation in a mouse model of immune complex glomerulonephritis, I induced NTN in wild-type (WT) and Annexin 1 deficient (Anx-/-) mice. Animals pre-immunised with Sheep IgG 5 days previously were injected with 0.1µg LPS in combination with NTS IV on day 0. A subset of WT and Anx-/- mice (n=3 per group) were culled on day 3 to allow the investigation of early effects of the absence of Annexin 1. The remaining mice were culled equally across experimental groups on day 7 or 8 and end-point data were combined from two experiments; n=8 per group and n=5 per group, respectively. Two WT animals and one Anx-/- animal died between day 1 and day 3 of unknown causes.

In this model of NTN, FACS analysis of cells isolated from cardiac puncture blood on day 3 did not reveal a difference in the absolute leukocyte counts or total number of neutrophils between WT and Anx-/- mice (Figure 3-4).

(i)

(ii)
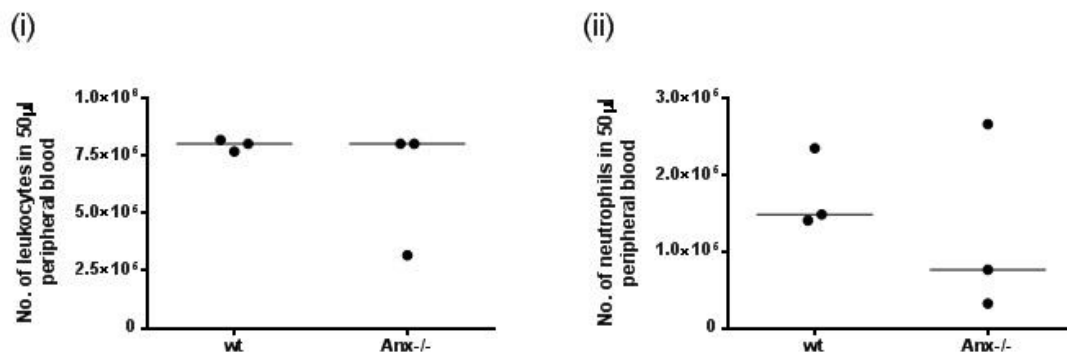
(iii)

Strain	Sample	Average cell count per 50µl blood	Percentage neutrophils / CD45$^+$ events	Number of neutrophils per 50µl blood
wt	A	816667	28.7	234383
	B	800000	18.5	148000
	C	766667	18.3	140300
Anx-/-	A	800000	9.54	76320
	B	316667	10.2	32300
	C	800000	33.2	265600

Figure 3-4 Day 3 analysis of numbers of peripheral blood leukocytes and neutrophils in NTN

Blood samples were taken on day 3 of NTN. (i) Total leukocytes isolated from a set volume of peripheral blood were visualised using trypan blue and counted. (ii) Subsequent calculations of total neutrophil counts were determined by FACS. (iii) Raw data to illustrate the graphs derived for (i) and (ii).

FACS analysis of kidney single cell suspensions generated at day 3 suggested that the infiltrating macrophages in the Anx-/- kidney on day 3 were of a less inflammatory phenotype, based on their Ly6C MFI (median 270 versus 732) (Figure 3-5). There were no differences evident in the percentages of infiltrating neutrophils or macrophages compared to CD45+ events, or the CD11b expression of the macrophages, when comparing experimental groups.
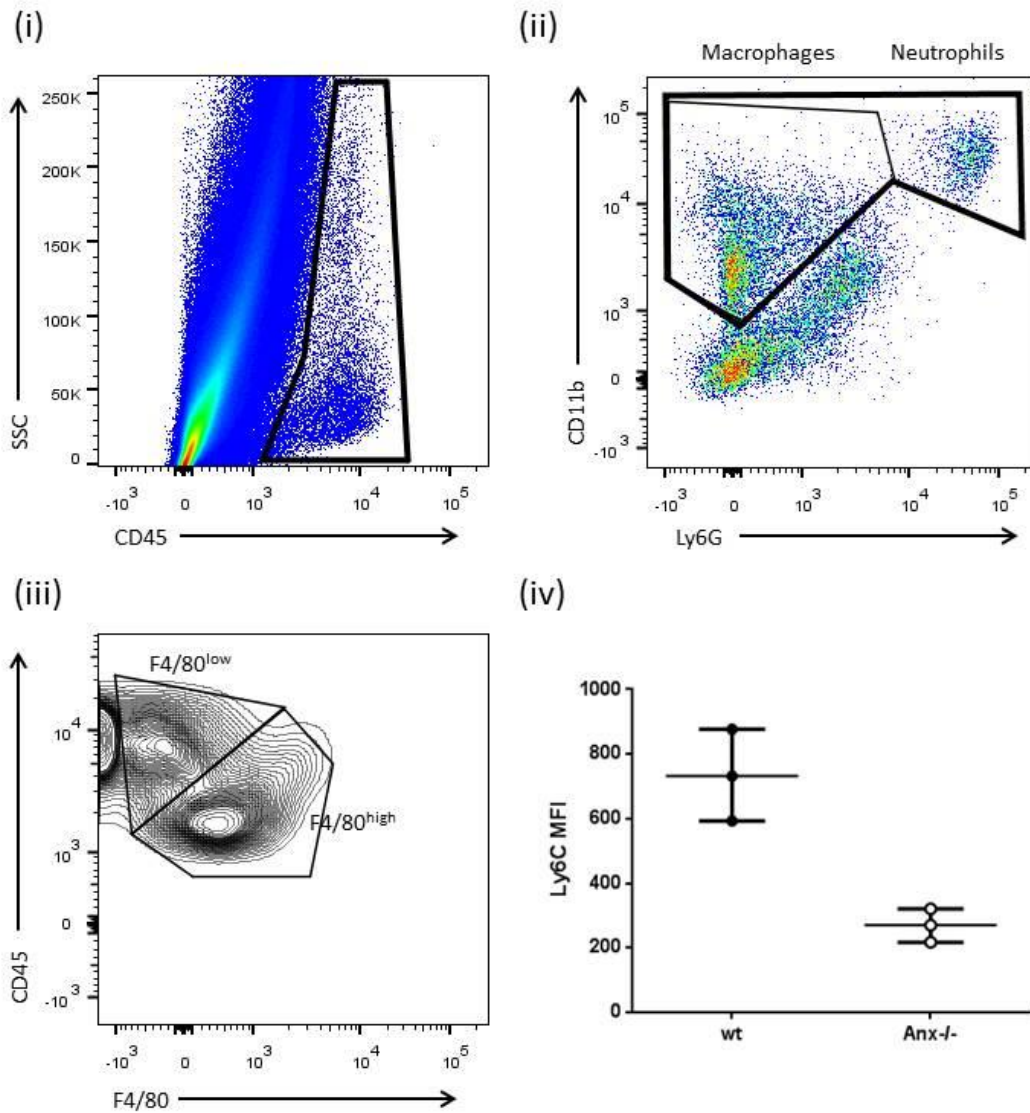
Figure 3-5 Day 3 analysis of Ly6C expression of infiltrating macrophages in NTN

FACS analysis of kidney single cell suspensions. (i) Leukocytes were determined by their positive expression of CD45. (ii) Macrophages and neutrophils were gated based on their expression of CD11b and Ly6G within the CD45+ gate. (iii) Infiltrating and resident macrophages were identified within the macrophage gate as F4/80low and F4/80high respectively. (iv) The median Ly6C MFI of the F4/80low macrophages was lower in Anx-/- mice compared to WT mice. Data are expressed as median with interquartile range.

Ly6G immunostaining of frozen kidney sections 3 days after LPS/NTS did not reveal a difference in the number of infiltrating neutrophils between the Anx-/- kidney and WT kidney (Figure 3-6).
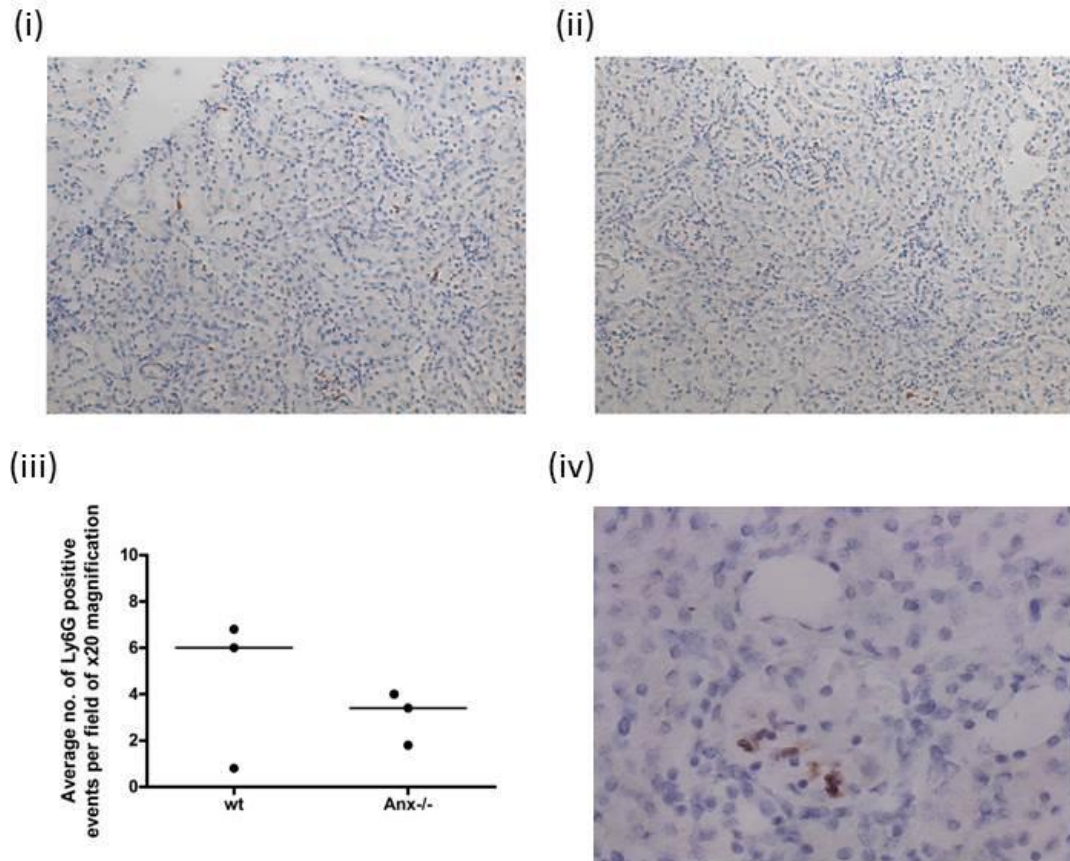
Figure 3-6 Ly6G staining of NTN day 3 frozen kidney

(i) Representative field of WT NTN day 3 kidney, x200 magnification. (ii) Representative field of Anx-/- NTN day 3 kidney, x200 magnification. (iii) Ly6G positive events were scored over 5x fields of x200 magnification to compare neutrophil infiltration between WT and Anx-/- mice on day 3 of NTN. (iv) High resolution image of Ly6G staining x630 magnification.

At day 7/8 after LPS/NTS Anx-/- mice exhibited a significantly elevated level of haematuria estimated by urinary dipstick (median 3 versus 1.5; p=0.03) and serum urea (89.8mmol/L versus 12.5mmol/L; p=0.029) compared to WT mice. The urine protein/creatinine ratio was not significantly different between experimental groups (0.3 mg/mmol versus 0.5mg/mmol) (Figure 3-7).
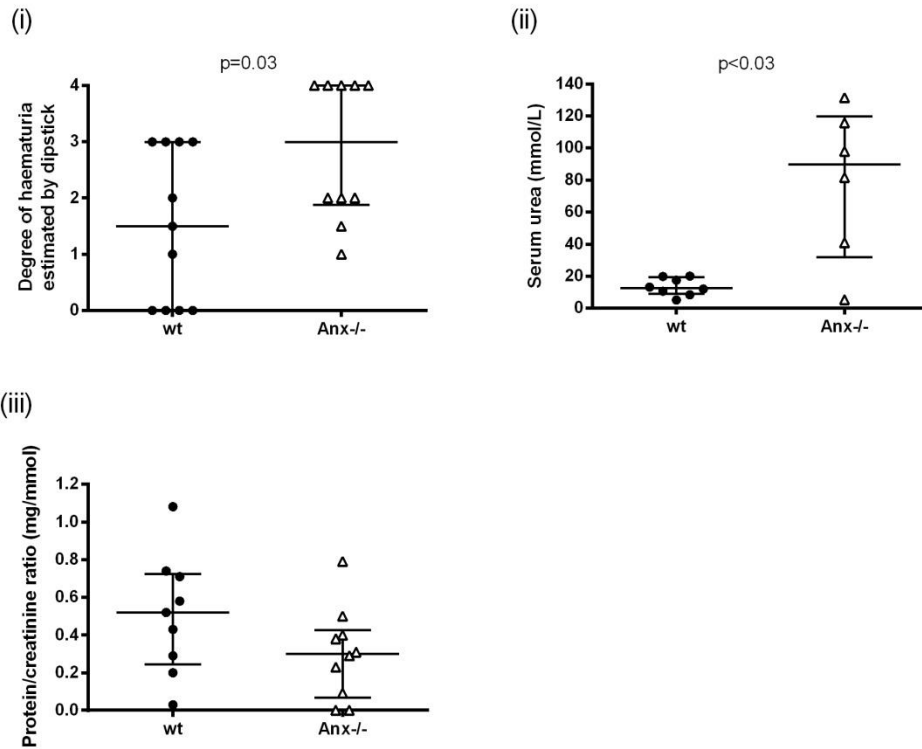
Figure 3-7 Annexin 1 deficient mice exhibit decreased renal function compared to wild-type mice in a mouse model of nephrotoxic nephritis

(i) Haematuria estimated by urinary dipstick was significantly elevated in the Anx-/- mice compared to WT mice (p=0.03). (ii) Serum urea was also significantly increased in the Anx-/- mice compared to WT mice (p=0.029). (ii) Urine protein/creatinine ratio was not significantly different between groups. All results were analysed using a Mann-Whitney t-test. Data are shown as median with interquartile range.

The degree of glomerular thrombosis, quantified on PAS-stained kidney sections, in the Anx-/- mice was significantly increased when compared to the WT mice at the experiment end (median 2.2 thrombosed quadrants versus 1.2 thrombosed quadrants; p=0.009) (Figure 3-8).
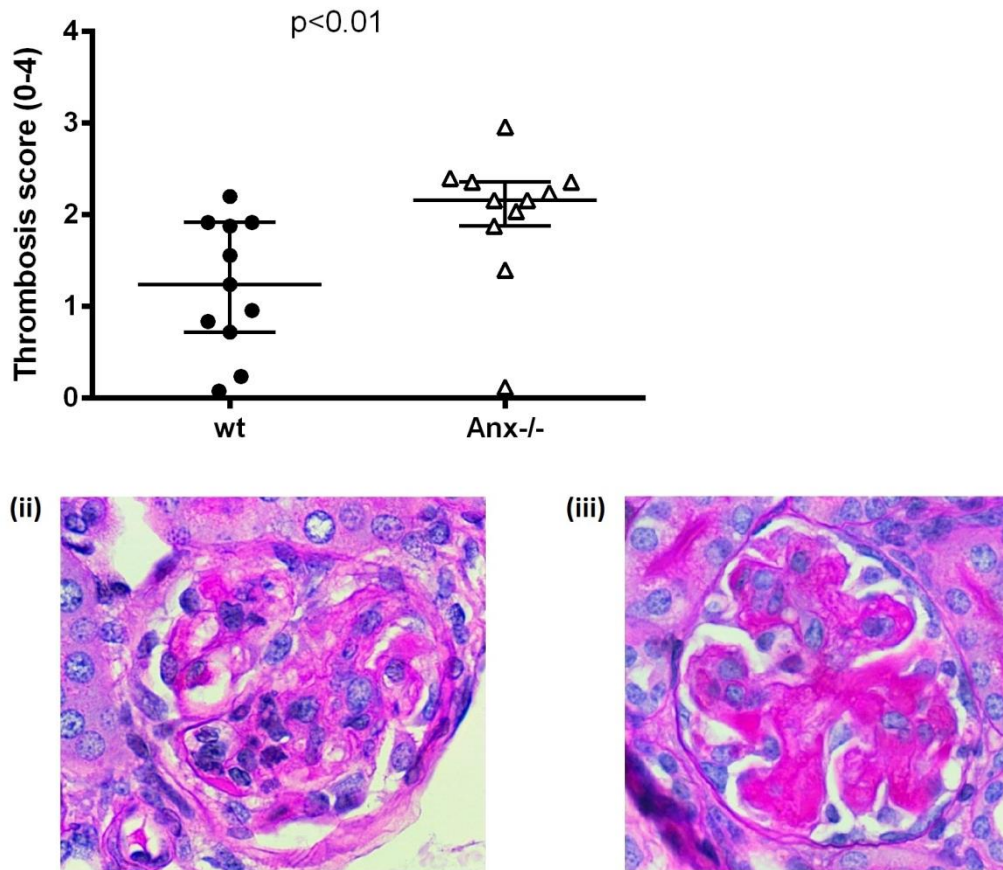
Figure 3-8 Glomerular thrombosis is significantly elevated in Annexin 1 deficient mice when compared to wild-type mice, in a model of nephrotoxic nephritis

(i) Glomerular thrombosis, scored on the number of quadrants involved, was significantly increased in the Anx-/- mice when compared to the WT mice, on day 7/8 of disease. Data are expressed as median with interquartile range. (ii) a typical PAS-stained glomerulus from a WT mouse, thrombosis score of 1. (iii) a typical PAS-stained glomerulus from an Anx-/- mouse, thrombosis score of 3.

There was no difference in the number of macrophages infiltrating into the glomerular space on day 7/8 between experimental groups when assessed by CD68 immunostaining of frozen kidney sections (Figure 3-9).
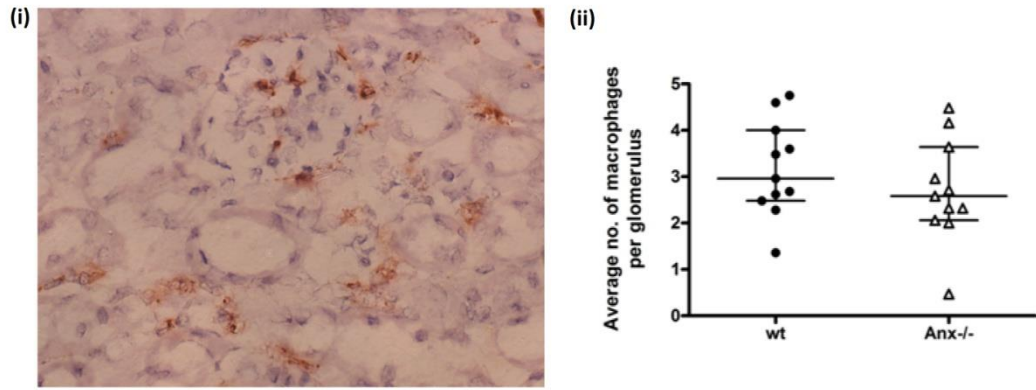
Figure 3-9 Infiltration of macrophages into the glomerular space remains unchanged when comparing wild-type mice and Annexin 1 deficient mice on day 7/8 of NTN

(i) CD68 immunostaining of frozen kidney section demonstrating infiltration of macrophages into the glomerular space on day 7 of NTN. (ii) When 25 glomeruli were scored, the average number of infiltrating macrophages into the glomerular space was no different between WT and Anx-/- mice on day 7/8 of NTN. Data are expressed as median with interquartile range.

The adaptive immune response, assessed by the response to Sheep IgG, between the Anx-/- mice and WT mice was comparable, when measured by ELISA.
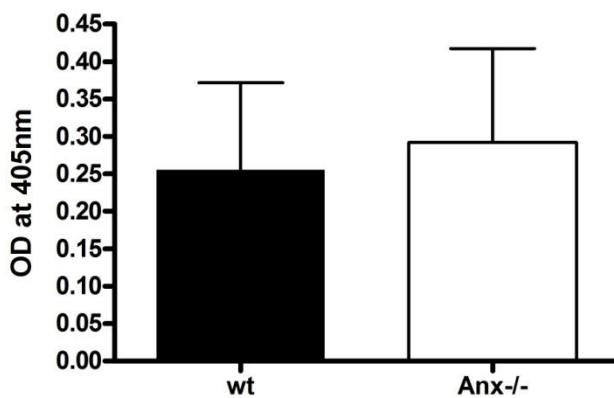
Figure 3-10 There is no difference in the adaptive immune response to Sheep IgG between wild-type mice and Annexin 1 deficient mice, when measured by ELISA

Systemic immune response to Sheep IgG was measured by ELISA using serum samples. Plates were coated with 0.1mg/ml sheep IgG overnight at 4°C. Plates were washed and blocked before incubating samples at a 1:1000 dilution for 1 hr at 37°C, followed by incubation with an alkaline phosphatase secondary antibody (1 in 1000 dilution) for 1 hr at 37°C. Plates were developed with *p*-nitrophenyl phosphate and read at 405nm.

FACS analysis of kidney single cell suspensions at day 7/8 did not reveal any differences between the percentages of infiltrating neutrophils or macrophages when comparing WT and Anx-/- mice. The CD11b and Ly6C phenotypes of the infiltrating macrophages were comparable between experimental groups.

3.4.2.1 *Microparticles in NTN*

Human neutrophil- and platelet-derived MPs (NMPs and PMPs) have pro-inflammatory capacity *in vitro* and correlate with disease activity in AAV.

I previously demonstrated that Annexin 1 deficient mice have aggravated disease, compared to wild-type mice in a murine model of NTN. In order to investigate whether MP numbers are altered in Annexin 1 deficient mice compared to wild-type mice in NTN, blood plasma was stained for FACS analysis with Annexin V and antibodies to Ly6G or CD41, to identify total MPs, NMPs and PMPs (staining and FACS performed by Dr Ying Hong, UCL). MP numbers were compared between wild-type and Annexin 1 deficient mice, n=7 per group. Mice pre-immunised with Sheep IgG were injected with 0.1µg LPS in combination with NTS IV on day 0. Mice were culled equally across experimental groups on day 7 or 8. One wild-type animal died between day 1 and day 3 of unknown causes.

In this NTN model total MP numbers and NMP numbers were significantly lower in the Anx-/- mice, compared to the WT mice (median total MPs 1.4×10^6 versus 3.5×10^6; p=0.035, NMPs 0.8×10^4 versus 2.6×10^4; p=0.0058, Figure 3-11). PMP numbers were comparable between the experimental groups.
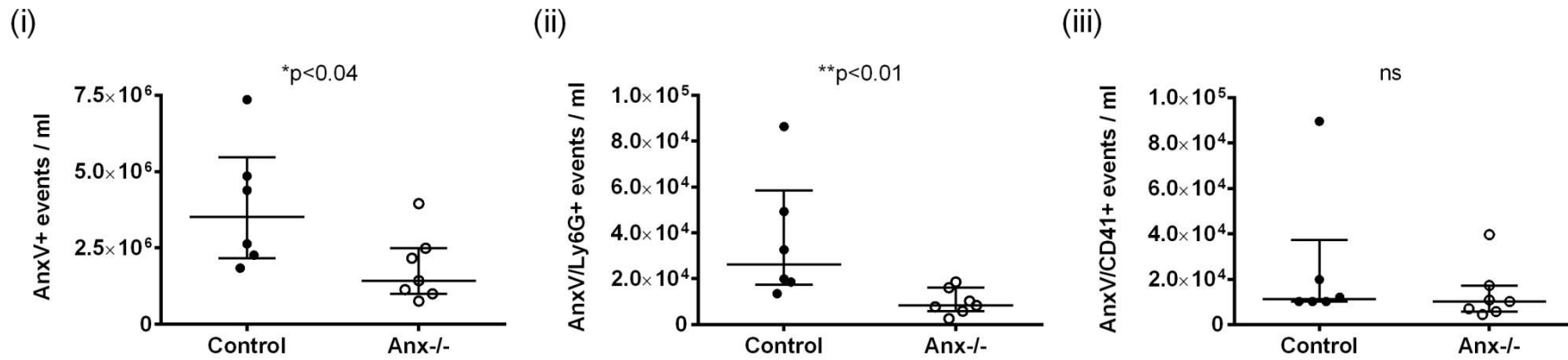
Figure 3-11 Total microparticle numbers and neutrophil-derived MPs are significantly less in Annexin 1 deficient mice in NTN

Microparticles were isolated from blood plasma at the experiment end. Cells were incubated with Annexin V and then stained with Ly6G or CD41 to identify total microparticles (i), and those derived from neutrophils (ii) or platelets (iii), respectively. MPs were analysed by FACS and latex beads (Sigma) were run concurrently with the samples to determine absolute MP numbers. Data are expressed as median with interquartile range.

3.4.3 Daily treatment of mice with 10µg dexamethasone does not reduce disease in this model of nephrotoxic nephritis

Annexin 1 has been demonstrated to mediate the anti-inflammatory effects of dexamethasone in two models of inflammatory arthritis (Patel et al., 2012, Yang et al., 2004).

To investigate whether the treatment of C57BL/6 mice with dexamethasone accelerates the resolution of nephrotoxic nephritis, animals were injected with 0.1µg LPS in combination with NTS IV on day 0 and 10µg dexamethasone (or vehicle) was administered by IP injection, daily, from 3 days post-NTS injection until mice were culled on day 7/8. To examine whether any effects of dexamethasone were mediated by Annexin 1, two groups of mice were used in the experiment; wild-type and Annexin 1 deficient mice (n=5 per group, dexamethasone or vehicle treated). One animal from the Anx-/- control group died due to unidentified causes 6 days post-NTS so no data was available to add to the following analysis.

The degree of haematuria estimated by urinary dipstick day 7/8 after NTS was no different when comparing WT control and WT dexamethasone-treated, or Anx-/- control and Anx-/- dexamethasone treated animals. It was unclear whether serum urea and urine protein/creatinine ratio were different between treated and untreated groups due to sample collection difficulties (Figure 3-12).
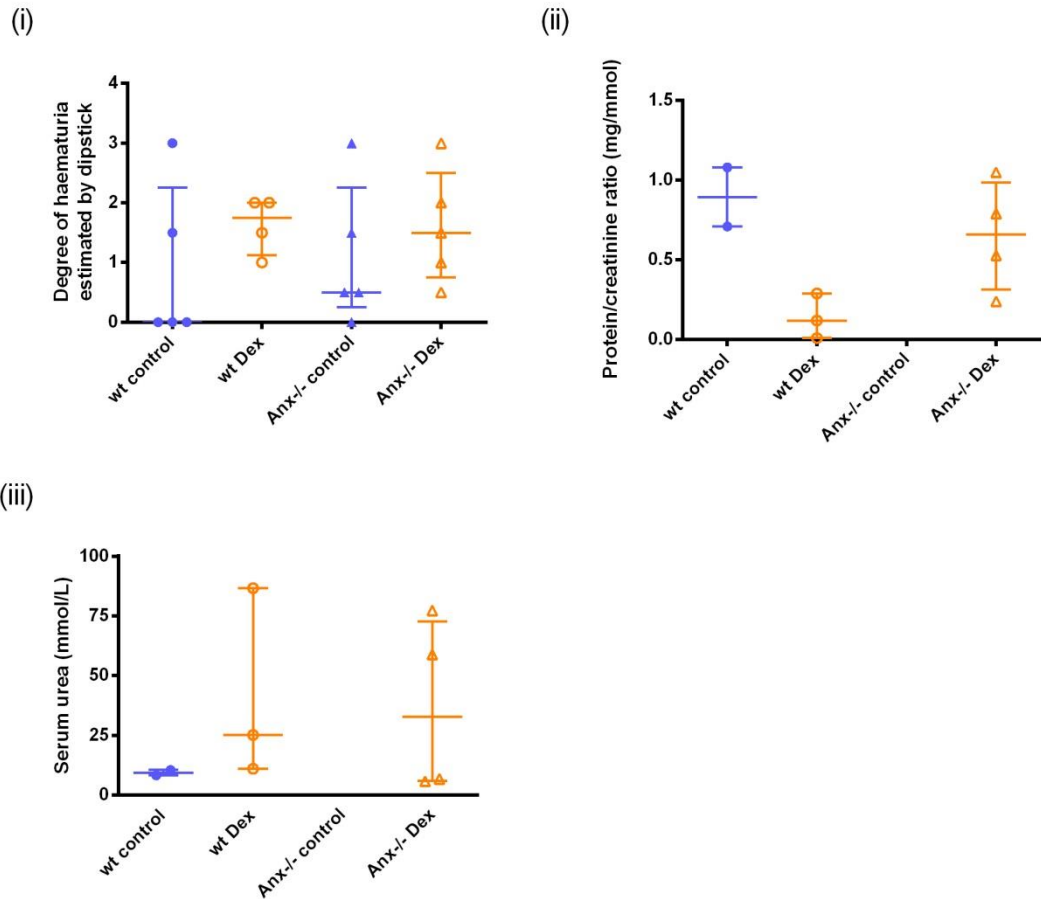
Figure 3-12 Treatment of wild-type and Annexin 1 deficient mice with 10μg dexamethasone has no effect on renal function, in this model of nephrotoxic nephritis.

(i) Haematuria estimated by urinary dipstick analysis was no different between WT or Anx-/- mice. Problems with serum isolation and measurement meant that it was unclear whether protein/creatinine ratio (ii) and serum urea (iii) were different between treated and untreated groups. Data are expressed as median with interquartile range.

When glomerular thrombosis was quantified on PAS-stained kidney sections, treatment of WT and Anx-/- mice with 10μg dexamethasone had no effect on renal injury (Figure 3-13).
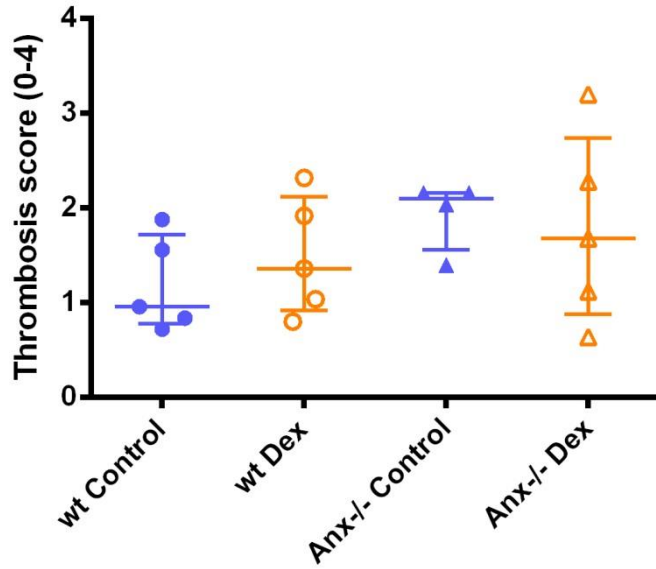
Figure 3-13 Treatment of wild-type and Annexin 1 deficient mice with 10µg dexamethasone has no effect on glomerular thrombosis, in this model of nephrotoxic nephritis.

When glomerular thrombosis was scored on PAS stained kidney sections, there was no difference evident between treated and untreated groups, in WT or Anx-/- mice. Data are expressed as median with interquartile range.

Glomerular macrophage infiltration on day 7/8 of NTN, determined by CD68 immunostaining of frozen kidney sections, was also similar when comparing treated and untreated groups (Figure 3-14).
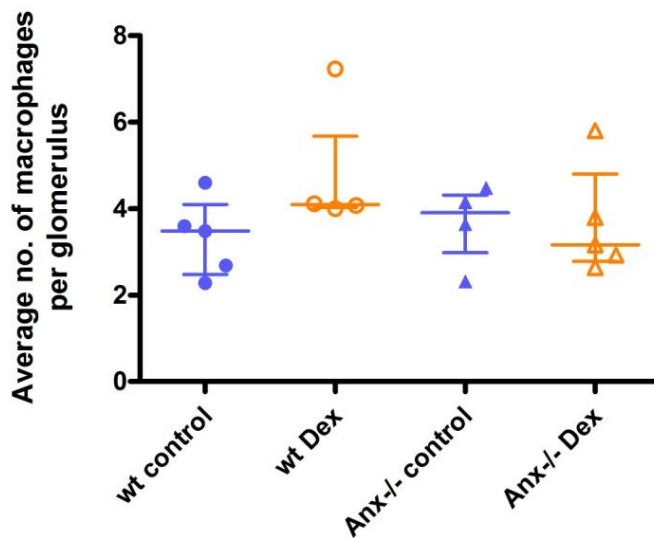
Figure 3-14 Treatment of wild-type and Annexin 1 deficient mice with 10μg dexamethasone has no effect on total macrophage infiltration into glomeruli, in this model of nephrotoxic nephritis.

CD68 immunostaining did not reveal a difference in macrophage infiltration into the glomerular space between treated and untreated groups, in WT or Anx-/- mice. Data are expressed as median with interquartile range.

3.4.4 Daily dosing of 1μg SuperAnxA1 has no effect on glomerular injury in this mouse model of accelerated nephrotoxic nephritis

Patel *et al.* demonstrated that treatment of mice with protease-resistant Annexin 1 (SuperAnxA1/SAnxA1) accelerates the resolution of inflammatory arthritis, when compared to the injection of human recombinant Annexin 1 or vehicle control *(Patel et al., 2012)*.

As I had previously demonstrated a protective role for Annexin 1 in disease I reasoned to test SAnxA1 as an effector of resolution in NTN. I utilised the same dosing strategy as Patel *et al.* and injected mice with 1μg SAnxA1, or vehicle control, from day -2 (before NTS) to day 7 or 8 at which point mice were culled equally across experimental groups (control n=7, SAnxA1 n=7) (see Figure 3-15). Following the establishment of the NTN model in my hands, I hypothesised that a less severe degree of disease should be utilised for the assessment of cleavage-resistant Annexin 1 peptides in order to observe any effect, so the dose of LPS was

reduced to 0.3μg/mouse, administered IV with the NTS. After performing an initial experiment with this dose of LPS, the experiment was repeated with a lower dose of LPS (0.1μg) to test whether a less severe disease phenotype would unmask any subtle effects of SAnxA1.
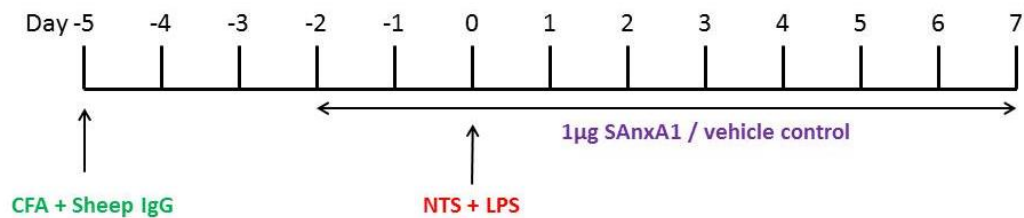
Figure 3-15 Dosing schedule for SuperAnnexinA1 in accelerated NTN

C57BL/6 mice were pre-immunised with 0.25mg sheep IgG in CFA on day -5. On day 0 mice were injected IV with NTS combined with LPS (0.1μg or 0.3μg per mouse). SAnxA1 was administered IP 1μg daily from day -2 until experiment end.

With this dosing strategy, when mice were given doses of 0.3μg or 0.1μg LPS in combination with NTS IV, haematuria and proteinuria estimated by urinary dipstick were no different between the control or SAnxA1 groups (Figure 3-16, i and ii). Serum urea and protein/creatinine ratio were also no different between the control and SAnxA1 groups (Figure 3-16, iii and iv). Glomerular thrombosis, scored on PAS stained kidney sections, exhibited a variable spread in both control and SAnxA1 groups (Figure 3-16, v). This dose of SAnxA1 did not have any effect on macrophage infiltration into the glomerulus on day 7/8, when mice were given doses of 0.3μg or 0.1μg in combination with NTS IV, although a lower dose of LPS was associated with a significantly lower number of infiltrating glomerular macrophages in the absence of SAnxA1 (1 versus 2 infiltrating macrophages; p=0.0157) (Figure 3-16, vi).
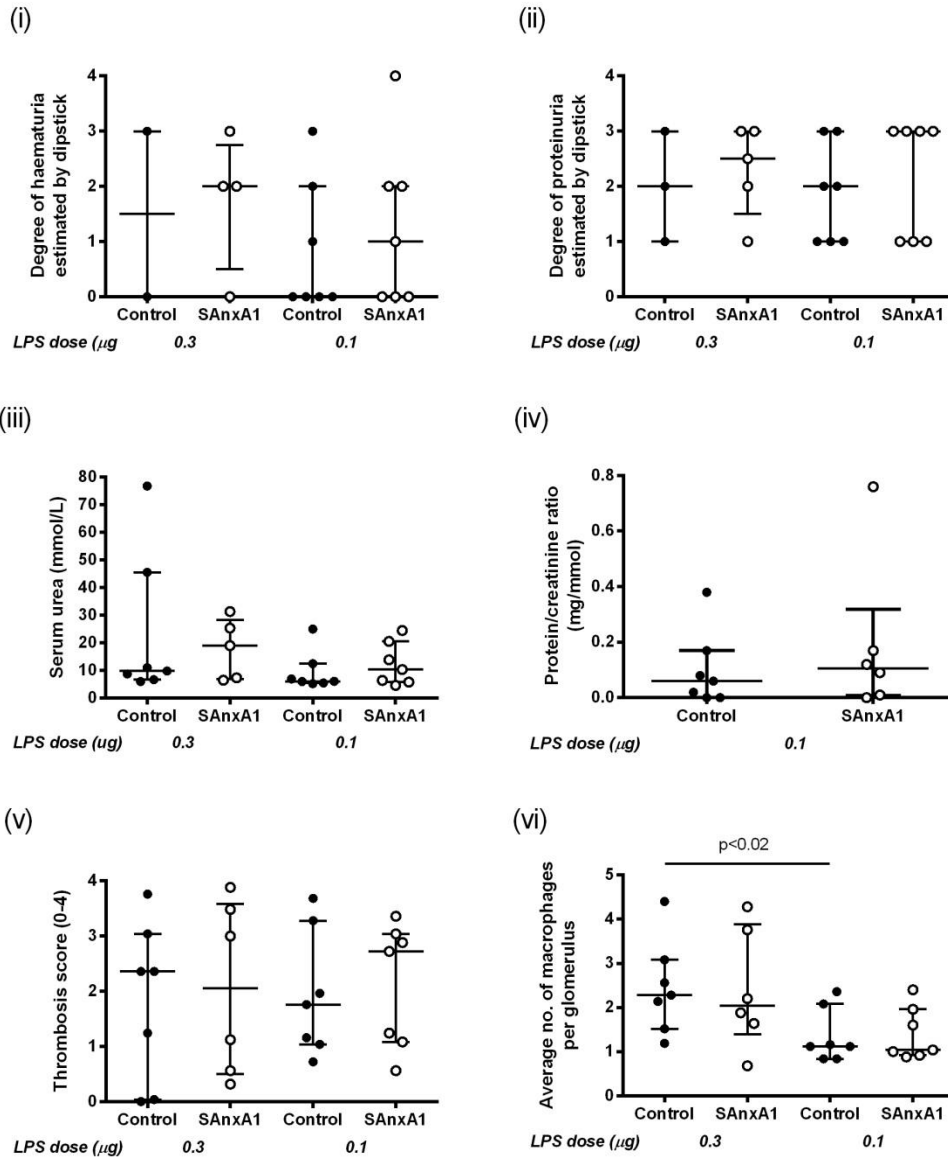
Figure 3-16 Daily dosing of 1µg SuperAnnexinA1 has no effect on renal injury or total macrophage infiltration in this mouse model of NTN

WT mice were administered 1µg SAnxA1 daily alongside the NTN injection schedule. Experiments with two different doses of LPS were performed (0.3µg or 0.1µg). Where urine was successfully collected, haematuria (i) and proteinuria (ii) estimated by urinary dipstick were no different between the control or SAnxA1 groups. Renal function, assessed by serum urea (iii) and protein/creatinine ratio (iv) was also no different between the control and SAnxA1 groups. Glomerular thrombosis (v) exhibited a variable spread in both control and SAnxA1 groups. (vi) The number of infiltrating macrophages into the glomerular space at day 7 was not different between control and SAnxA1 groups but was significantly reduced in

the control group when 0.1μg LPS was administered with the NTS, rather than 0.3μg (p=0.0157, Mann-Whitney t-test). Data are expressed as median with interquartile range.

3.4.4.1 Surface CD11b expression is reduced in infiltrating macrophages in mice treated with SuperAnnxinA1 in a model of NTN

To explore any subtle physiological effects of SAnxA1 in this model of NTN, cells were isolated from cardiac puncture blood and kidney single cell suspensions for FACS analysis at the experiment end. Cells were stained with antibodies to CD45, Ly6G, CD11b, Ly6C and F4/80 to enable the detection and phenotyping of neutrophils, monocytes and macrophages.

When NTS was given with low dose LPS (0.1μg) there were no differences in the relative percentages of neutrophils or monocytes, compared to total leukocyte events, between the control and SAnxA1 groups in the blood. When the phenotype of the blood monocyte population was examined by means of Ly6C expression, a bimodal population of Ly6C-expressing monocytes was revealed (Figure 3-17). These two populations, designated Ly6C^{mid} and Ly6C^{high} did not differ in their relative abundance, or as a fraction of the total leukocyte population when comparing the control and SAnxA1-treated animals. The MFIs of the Ly6C^{mid} and Ly6C^{high} populations were no different when comparing experimental groups.
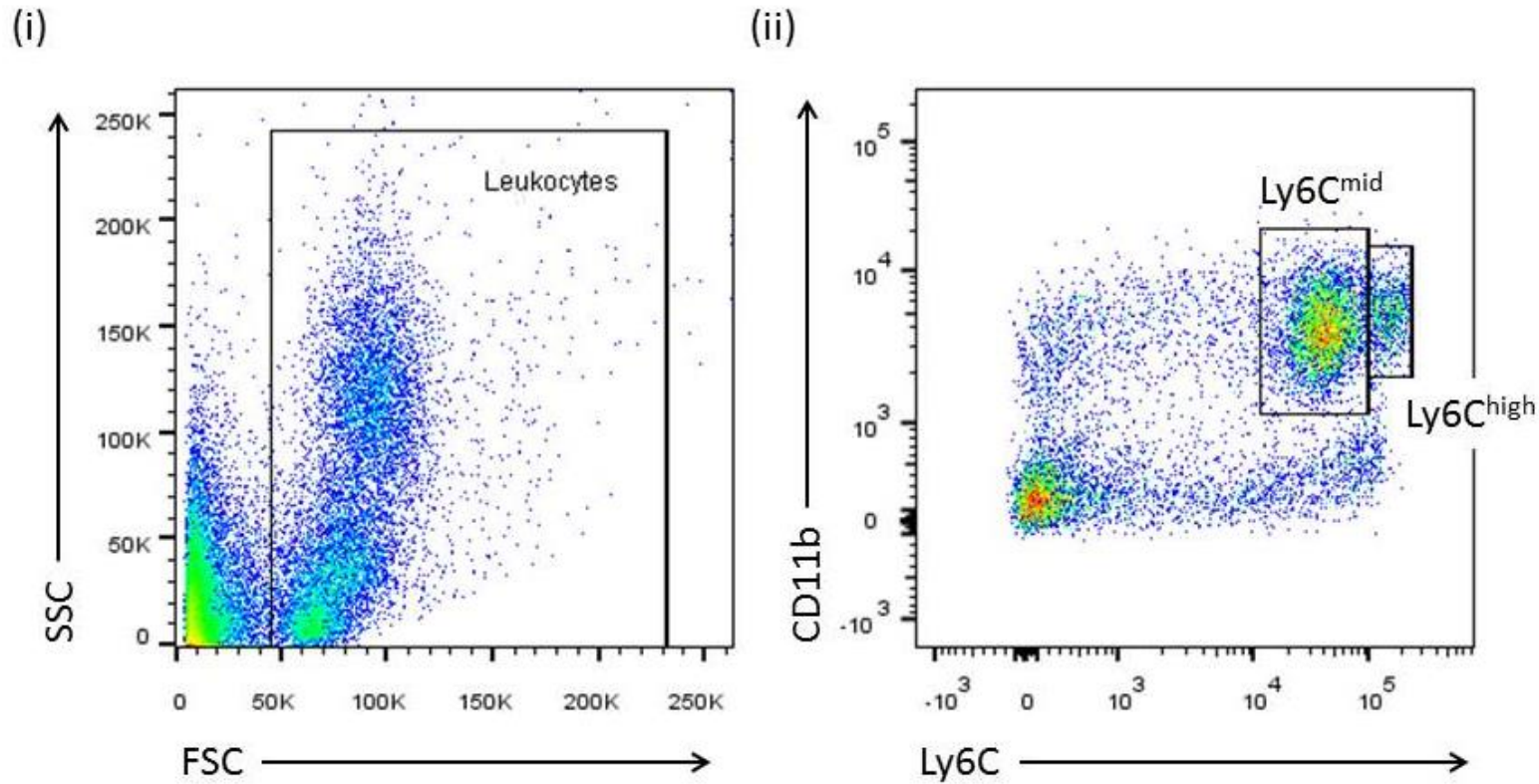
Figure 3-17 FACS analysis reveals two distinct populations of mouse monocytes in the blood based on surface expression of Ly6C

Anticoagulated blood from mouse cardiac puncture was red-cell lysed and stained with antibodies to CD11b and Ly6C. (i) Leukocyte events were gated based on their FSC/SSC profile to exclude cell debris. (ii) Monocytes were determined by their positive CD11b expression within the leukocyte gate and exhibited a bimodal pattern of Ly6C expression. These two populations were designated Ly6C^mid and Ly6C^high.

When NTS was given with low dose LPS (0.1µg) there was no difference in the percentage of infiltrating neutrophils detected by FACS in the kidney at day 7/8 when comparing the control and SAnxA1-treated groups. The percentages of infiltrating and resident macrophages, determined by their F4/80^{low} and F4/80^{high} phenotypes respectively, were comparable between experimental groups, when compared to total CD45^{+}CD11b^{+}Ly6G^{-} events and when considered relative to each other. The Ly6C expression of the infiltrating macrophages was no different when comparing the control and SAnxA1-treated groups. The CD11b expression of the infiltrating macrophages was significantly lower in the SAnxA1 group (1.0x10^{4} versus 1.1x10^{4}; p<0.04) when compared with the control group (Figure 3-18).
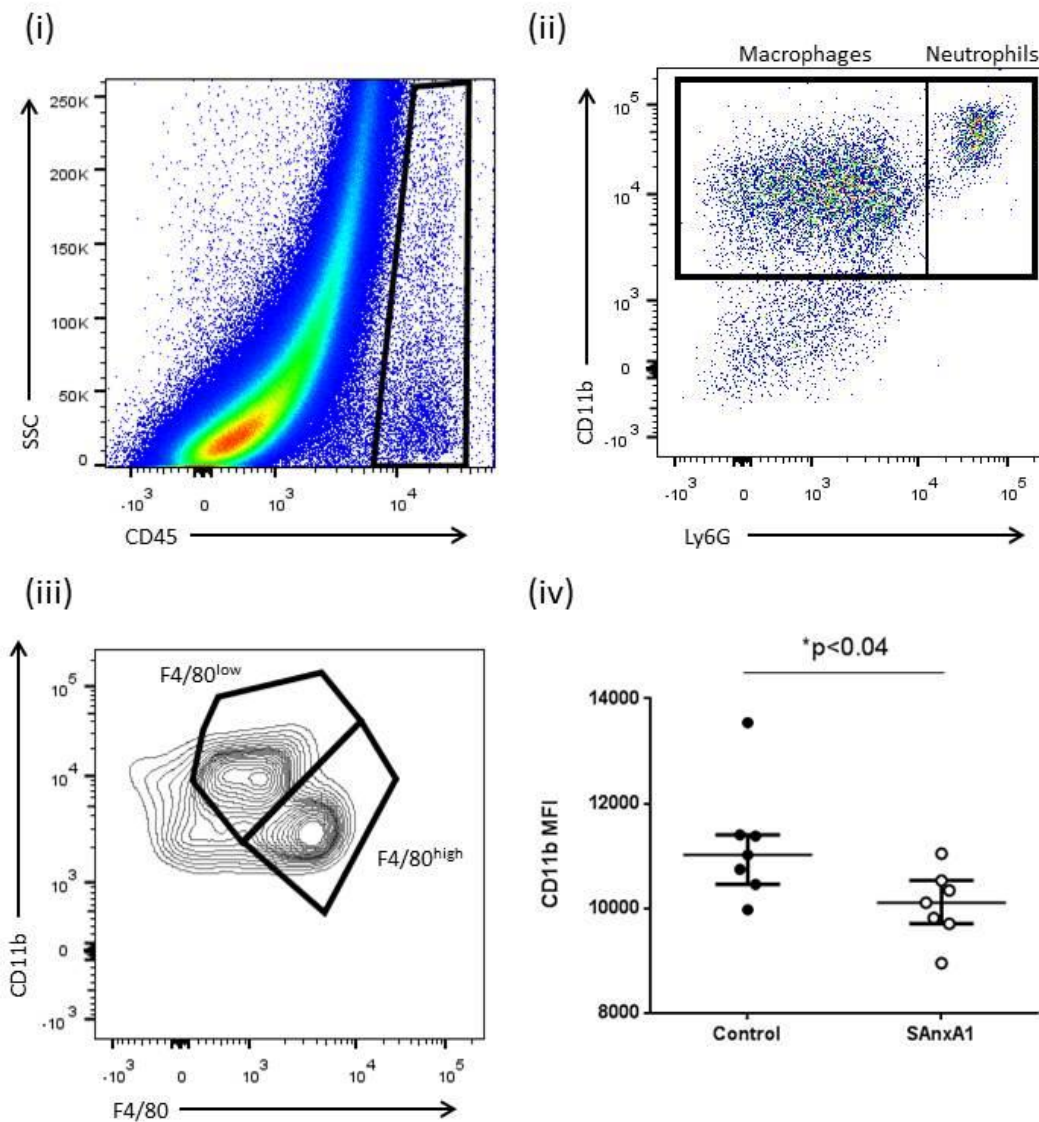
Figure 3-18 Surface CD11b expression is reduced in infiltrating macrophages in mice treated with SuperAnxA1 in a model of NTN

FACS analysis of kidney single cell suspensions. (i) Leukocytes were determined by their positive expression of CD45. (ii) Macrophages and neutrophils were gated based on their expression of CD11b and Ly6G within the CD45+ gate. (iii) Infiltrating and resident macrophages were identified within the macrophage gate as F4/80^{low} and F4/80^{high} respectively. (iv) The CD11b MFI of the F4/80^{low} macrophages was significantly lower in mice treated with SAnxA1 (p=0.037, Mann-Whitney t-test). Data are expressed as median with interquartile range.

3.5   Discussion

Success in generating the murine model of accelerated nephrotoxic nephritis in C57BL/6 mice was achieved when additional endotoxin was administered in the form of LPS IV combined with NTS solution. LPS has been used historically as an adjuvant to enhance the immune response to NTS and may reflect infectious stimuli found naturally in the environment, which might be absent in the sanitised surroundings of a Comparative Biology Unit.

The genetic absence of Annexin 1 resulted in significantly more severe disease in this model of NTN. Serum urea was elevated in Annexin 1 deficient mice and glomerular thrombosis was increased from an average of 1.2 to 2 (score 0-4 involved quadrants). FACS analysis of leukocyte activation markers early in disease suggested that infiltrating macrophages were of a less inflammatory phenotype in the Annexin 1 deficient animals, based on their Ly6C expression. The CD11b expression of the infiltrating macrophages was unchanged between experimental groups. Blood neutrophil counts have been shown to be significantly lower in Annexin 1 deficient mice 24hrs post LPS and Annexin 1 deficient leukocytes display an increased activation state (Damazo et al., 2005), however analysis of peripheral blood 3 days after NTS injection did not reveal any differences in the total numbers of circulating leukocytes or neutrophils between wild-type and Annexin 1 deficient animals. There were also no differences observed in the numbers of infiltrating neutrophils into the glomerulus early in disease when comparing experimental groups. At the disease end point, although renal impairment was more severe in Annexin 1 deficient mice, there was no apparent effect on total numbers of infiltrating glomerular macrophages. The absence of Annexin 1 did not appear to have a significant effect on the humoral immune response to pre-sensitization with Sheep IgG. I hypothesised that an Annexin 1 deficiency in NTN would lead to an increased early neutrophil influx, exacerbating the immune response to NTS, and resulting glomerular injury. It is possible that the early timepoint data examining neutrophil infiltration was lacking in experimental numbers. There were also not many neutrophils detected on the kidney immunostaining, indicating that neutrophil infiltration may have peaked prior to day 3. Alternatively, Annexin 1 has been shown to augment Th1 responses, and the Annexin 1 deficiency could be affecting the DTH-like response seen in this model, leading to an increased accumulation of Th1 cells. There was no TNF-α or IL-6 detectable by ELISA in the remaining serum

samples, so these cytokines could not be compared between the experimental groups.

There was a significant reduction in overall MP numbers and neutrophil-derived MPs in Annexin 1 deficient mice in this NTN model, compared to wild-type mice. Due to the significantly decreased renal function, and increased degree of glomerular thrombosis found in Annexin 1 deficient animals in this model, this would suggest that overall MP numbers are not a reliable reflection of vascular injury in mice, in contrast to what has been demonstrated in human AAV. Neutrophil-derived MPs have also been described to mediate anti-inflammatory effects (Dalli et al., 2008), so a reduction of these in NTN could be a mechanism by which disease is aggravated in Annexin 1 deficient mice, although there is no published evidence to suggest that Annexin 1 influences MP release directly.

Daily administration of 10µg dexamethasone did not appear to have any effect on renal injury or glomerular macrophage infiltration in this model of NTN; so the impact of this drug could not be compared between wild-type and Annexin 1 deficient mice. The dose of dexamethasone given here was based on previously published doses demonstrated to attenuate disease in accelerated NTN (see Table 5) and resolve inflammation in other disease models and was equivalent to an average of 4mg/kg according to the size of the mice. The administration of dexamethasone 3 days after NTS/LPS was intended to resolve inflammation after disease onset, reflecting how AAV would be treated in a clinical setting. It is possible that in this disease model, Annexin 1 is primarily important in the early neutrophil influx and the administration of dexamethasone after disease has initiated was too late to have an effect on resulting glomerular injury. In any future experiments, it would be important to establish an appropriate dose and timing schedule of dexamethasone administration before comparing experimental groups.

Table 5 Previously published dexamethasone doses for treatment of experimental inflammation

Author	Year	Experimental model	Dose of dexamethasone	Administration routine
Nagai *et al.*	1982	Accelerated NTN	1 or 5 mg/kg IP	Daily for 14 days post NTS

Mancuso *et al.*	1995	Zymosan peritonitis	1 mg/kg s/c	One dose 2hr prior to zymosan
Yang *et al.*	2004	Antigen-induced arthritis	0.5mg/kg IP	Daily from day 20 following disease onset
Patel *et al.*	2012	Serum-induced arthritis	10μg/animal IP	Daily from day 2 following disease onset

s/c: subcutaneous

When mice were injected with 1μg SAnxA1 prophylactically and during the disease timecourse there was a significant reduction observed in the CD11b expression of infiltrating macrophages. It was unexpected to find CD11b expression reduced in the SAnxA1-treated group as CD11b expression is reduced in Annexin 1 deficient monocytes and macrophages (Hannon et al., 2003, Yona et al., 2004, Yona et al., 2006). This has been postulated to account for an impaired phagocytic mechanism in Annexin 1 deficient cells. However, CD11b also plays an important role in adherence of monocytes to activated endothelium, so a reduction of macrophage CD11b expression could play an anti-inflammatory role through reduced monocyte trafficking. GCs reduce CD11b expression and as a regulator of Annexin 1, this could agree with these SAnxA1 data (Burton et al., 1995, Das et al., 1997, Lim et al., 2000). There was no observed effect of this dosing of SAnxA1 on renal injury or glomerular macrophage infiltration at the experiment end, when mice were given doses of 0.3μg or 0.1μg LPS in combination with NTS IV. Although, lower doses of LPS did not lead to reduced disease severity as assessed by urinary dipstick findings or glomerular thrombosis score. While SAnxA1 has been shown to reduce leukocyte infiltration *in vivo*, in an experimental model of resolving arthritis, SAnxA1 administration did not affect the initial neutrophil and mononuclear cell-driven stage of disease but did accelerate the resolution-phase. The disease manifested this model of accelerated NTN may be too robust to allow any pro-resolving effects of this dose of SAnxA1.

Summary:

- The genetic absence of Annexin 1 results in significantly more severe disease in a murine model of immune complex-mediated crescentic glomerulonephritis, nephrotoxic nephritis (NTN).

- Total microparticle and neutrophil-derived microparticle numbers are reduced in Annexin 1 deficient mice, compared to wild-type mice in this NTN model.

- Daily administration of 10µg dexamethasone did not have any impact on disease in this NTN model, so the impact of this drug in Annexin 1 deficient mice could not be studied.

- There was no effect on disease severity when SuperAnnexinA1 was administered according to this dosing schedule in this NTN model, however there was a significant reduction in the CD11b expression of infiltrating macrophages.

Chapter 4 The effect of Annexin 1 in murine models of MPO-related glomerulonephritis

4.1 Introduction

The data in Chapter 3 demonstrate that Annexin 1 plays a protective role in a murine model of crescentic glomerulonephritis (NTN). To investigate further what role Annexin 1 might have in AAV, the effect of the protein was examined in murine models of MPO-related glomerulonephritis.

Murine models of myeloperoxidase anti-neutrophil cytoplasm antibody disease have been essential in determining key pathogenic pathways involved in the development of AAV. The passive transfer model of anti-myeloperoxidase associated glomerulonephritis developed by Xiao *et al.* is dependent on the infiltration of neutrophils for the development of crescentic GN which is apparent 3 days post-IgG. Recombinase-activating gene-2–deficient (Rag2–/–) mice, lacking functional T and B lymphocytes are equally susceptible to disease development (Xiao et al., 2005). The disease penetrance in this model can be exacerbated by the injection of LPS and granulocyte-colony stimulating factor (GCSF), which in combination leads to an increased number of circulating neutrophils early in disease, followed by an exacerbated infiltration of glomerular macrophages and a higher incidence of glomerular crescents (Freeley et al., 2013, Huugen et al., 2005). An alternative model of anti-myeloperoxidase associated glomerulonephritis developed by the group of Holdsworth and Kitching is generated by delivering a subnephritogenic dose of NTS to mice sensitised with mouse MPO in adjuvant. This model is unique in replicating the DTH-like response to planted MPO, seen in AAV and is useful in examining both the humoral and cellular response to anti-MPO as both neutrophils and CD4+ T cells play a key role in disease (Gan et al., 2010, Ruth et al., 2006). The same group has recently identified an immunodominant MPO T-cell epitope that when injected in place of native mouse MPO produces an equivalent immune response and focal crescentic glomerulonephritis (FCGN) (Ooi et al., 2012).

Annexin 1 regulates neutrophil and monocyte accumulation *in vivo* and has a particular role in moderating leukocyte/endothelial interactions in the microvasculature. The genetic absence of Annexin 1 in murine models of acute and chronic inflammation results in augmented neutrophil and monocyte accumulation (Getting et al., 1997, Lim et al., 1998, Damazo et al., 2005, Yang et al., 1997). In models of chronic inflammation, Annexin 1 deficient mice display enhanced

macrophage recruitment, extracellular matrix deposition and fibrosis (Damazo et al., 2011, Locatelli et al., 2014). Annexin 1 can also play a protective role within a DTH response, limiting T cell proliferation and the production of IFN-γ and IL-17A (Yang et al., 2013).

Microparticles (MPs) may play a role in vascular injury and heightened levels of neutrophil- and platelet-derived MPs correlate with disease activity in AAV. I demonstrated in the previous chapter that total MP numbers and NMP numbers are significantly reduced in Annexin 1 deficient mice in model of NTN, but their abundance in other models of crescentic glomerulonephritis is yet to be addressed.

4.2 Aim

This chapter investigates the role of Annexin 1 in murine models of MPO-related glomerulonephritis. The primary aim was to establish a model of anti-myeloperoxidase glomerulonephritis, with which the absence of Annexin 1 could be examined by comparing disease in wild-type and Annexin 1 deficient mice. Attempts were made to induce anti-MPO IgG in MPO-deficient mice using two different antigens; murine MPO (mMPO) alone or with the additional injection of immunodominant MPO CD4+ T-cell epitope ($MPO_{409-428}$). Anti-MPO IgG was isolated from pooled MPO-deficient serum and assessed for purity and titre against purified anti-MPO IgG with previously established pathogenicity. Initially steps were taken to establish a model of anti-myeloperoxidase glomerulonephritis based on the original passive transfer approach described by Xiao *et al.* (2002). Once successfully set up, this model was used to compare disease between wild-type and Annexin 1 deficient mice. A further attempt at modelling MPO-associated glomerulonephritis was intended to reproduce the model described by Holdsworth and Kitching (Ruth et al., 2006) with the accompaniment of additional doses of immunodominant MPO peptide.

4.3 Experimental Design

Wild-type and Annexin 1 deficient mice used were sex- and age-matched and between 8-20 weeks of age. Initial optimisation of the anti-myeloperoxidase glomerulonephritis model was carried out trialling the passive transfer of varying doses of anti-MPO IgG isolated from MPO deficient mice immunised with purified mMPO. Accompanying injections of LPS and GCSF were used to exacerbate disease. An additional attempt to model MPO-associated glomerulonephritis

involved the injection of two doses of mMPO combined with MPO immunodominant peptide in adjuvant followed by the transfer of subnephritogenic doses of nephrotoxic serum (NTS). MPO antibody titres were determined by ELISA on serum or plasma isolated from peripheral or cardiac blood. Disease severity was assessed by renal function; haematuria and proteinuria estimated by urinary dipstick of 24 hour urine collection, proteinuria quantified by sulphosalicylic acid method and serum urea measured by Clinical Chemistry, MRC, Harwell. Glomerular crescent formation was scored on PAS stained kidney sections. Macrophage infiltration into the intra-glomerular space was quantified by CD68 immunostaining of frozen kidney sections. Plasma samples were stored for microparticle analysis.

4.4　Results

Circulating anti-MPO IgG was induced in MPO-deficient mice by the injection of mMPO in adjuvant. Anti-MPO IgG was isolated from MPO-deficient serum but was relatively low in titre compared to anti-MPO IgG with previously established pathogenicity. A murine model of anti-myeloperoxidase associated glomerulonephritis was ultimately established through the IV injection of 100μg/g bodyweight anti-MPO IgG followed by an IP injection of 0.1μg LPS/animal 1hr after IgG transfer. IP injections of 26μg GCSF were given in addition; 4 days prior to IgG, alongside the IgG injection and 4 days after IgG. An alternative model of anti-myeloperoxidase associated glomerulonephritis based on the injection of mMPO and MPO immunodominant peptide followed by NTS failed to induce an immune response and any associated renal injury. Comparison of the passive-transfer model of anti-myeloperoxidase associated glomerulonephritis between wild-type and Annexin 1 deficient mice did not reveal any significant differences in renal function or glomerular disease at the experiment end.

4.4.1　Generation of anti-MPO IgG in MPO-deficient mice

Xiao *et al.* (2002) originally described the generation of circulating anti-MPO specific antibodies in MPO-deficient mice through the injection of mouse MPO in adjuvant over a course of 36 days. Purified IgG from these mice is then injected into recipient mice to induce a pauci-immune crescentic glomerulonephritis that develops over a course of 6 days.

To generate a source of anti-MPO IgG, MPO-deficient (B6.129X1-Mpo^{tm1Lus}/J, MPO-/-) mice were initially injected IP with 20μg mMPO on day 0 in a Complete Freund's

Adjuvant (CFA) emulsion, followed by an IP injection of 10μg mMPO on day 21 and day 36 in an Incomplete Freund's Adjuvant (IF) emulsion. Control age-matched MPO-/- mice were injected with an eqimolar concentration of bovine serum albumin (BSA) in CFA (or IF) emulsion over the course of 36 days. Anti-MPO IgG titres were determined before sacrifice from peripheral blood samples by ELISA. Animals were sacrificed on day 42 for serum collection and subsequent IgG purification. Table 6 summarises the experiments to induce circulating anti-MPO IgG in MPO-/- mice.

Table 6 Table detailing the induction of anti-MPO IgG in MPO deficient mice

Experiment ID	Experimental groups	Number of mice in experimental group	Average age at injection start date (wks)	Gender (M/F)	Source of mMPO	Additional immunodominant peptide (Y/N)	Normalised mean antibody titre
A	Mpo-/-_mMPO	8	11	7/1	P.Heeringa	N	0.02
	Mpo-/-_BSA	7	8	4/3	-	N	0.00
B	Mpo-/-_mMPO	13	12	5/8	P.Heeringa	N	0.77
C	Mpo-/-_mMPO	8	7	8/0	P.Heeringa	N	0.27
D	Mpo-/-_mMPO	17	13	9/8	Mine	N	0.76
E	Mpo-/-_mMPO	8	11	3/5	Mine	Y	0.12

All mice were injected with a total of 40μg mMPO or BSA over a course of 36 days. Additional 50μg immunodominant peptide $MPO_{409-428}$ was combined with mMPO on day 0 and day 21 in experiment E. Anti-MPO IgG titres were determined by ELISA from MPO-/- serum on day 40.

Titres were normalised to the Ec50 of purified anti-MPO IgG provided by P.Heeringa run alongside as a way to control for inter-experimental variation (see Figure 4-1).

The first injections of mMPO were carried out using protein kindly donated by P.Heeringa (University of Groningen, Netherlands) that had previously been proven to induce circulating anti-MPO IgG in MPO-deficient mice. When circulating antibody titres were ascertained by ELISA on day 40, the anti-MPO IgG levels were very low when compared to purified anti-MPO IgG (also donated by P.Heeringa) (Figure 4-1, experiment A). Repeats of the experimental protocol described above, injecting either mMPO from P.Heeringa or isolated by myself (from a mouse promyelocytic cell line) produced varying circulating anti-MPO IgG titres (Figure 4-1, experiment B-D). The addition of 50µg immunodominant peptide MPO$_{409-428}$ combined with the mMPO injection at day 0 and day 21 did not appear to increase antibody titres by the experiment end (Figure 4-1, experiment E).

Source of mMPO	P.Heeringa	P.Heeringa	P.Heeringa	Mine	Mine
Additional MPO$_{409-428}$	No	No	No	No	Yes
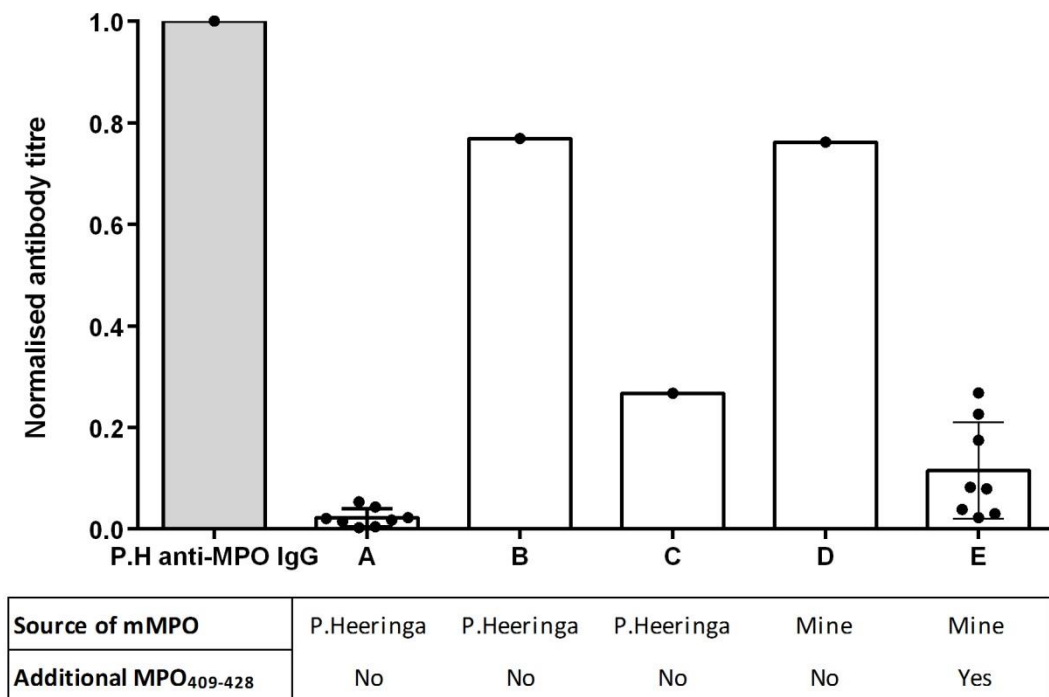
Figure 4-1 Circulating antibody titres from MPO-deficient mice injected with mMPO with or without MPO immunodominant peptide MPO$_{409-428}$

Graph shows anti-MPO IgG titres from consecutive experiments A-E. All mice were injected with a total of 40µg mMPO over a course of 36 days. Additional 50µg immunodominant peptide MPO$_{409-428}$ was combined with mMPO on day 0 and day 21 in experiment E. Anti-

MPO IgG titres were determined by ELISA from MPO-/- serum on day 40. Titres were normalised to the Ec50 of purified anti-MPO IgG provided by P.Heeringa run alongside as a way to control for inter-experimental variation. Anti-MPO IgG titres were variable when either mMPO from P.Heeringa or our lab was used to inject MPO-/- mice and did not rise when immunodominant peptide $MPO_{409-428}$ was combined with the mMPO. Single data points represent pooled serum values.

Serum isolated from the first MPO-/-s injected (experiment A) and the subsequent experiments with the highest anti-MPO IgG titres (experiments B and D) were selected for IgG purification. Purified anti-MPO IgG titres ranged from 7-30% of the concentration of purified anti-MPO IgG supplied by P.Heeringa (Figure 4-2). The titre of the anti-MPO IgG isolated from the batch of MPO-/- serum with the highest concentration (experiment D) was 10 times less that the purified anti-MPO IgG supplied by P.Heeringa.
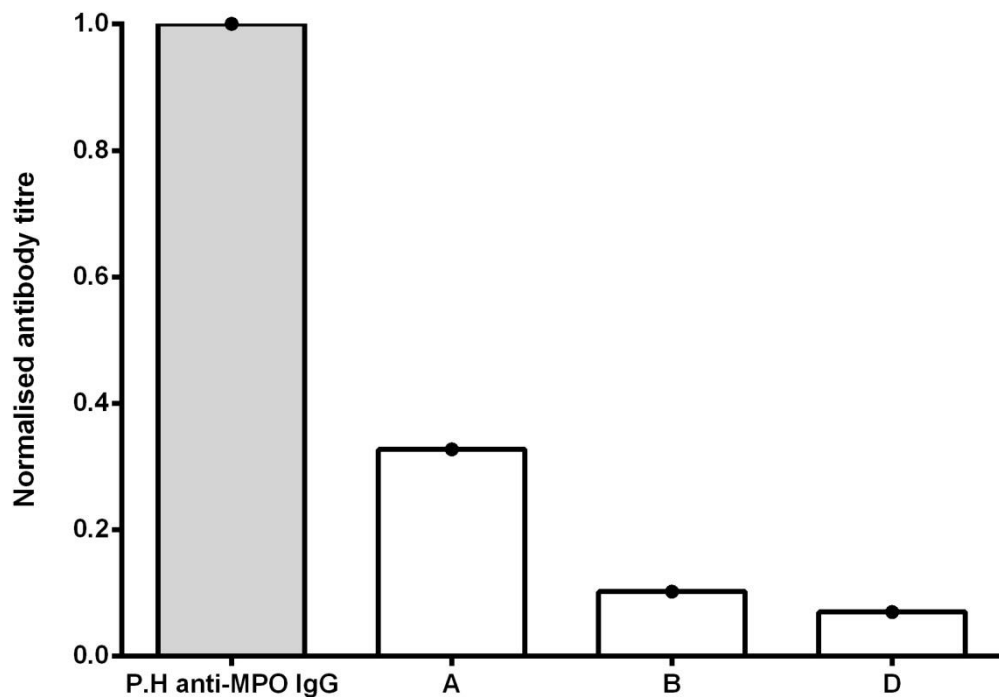
Figure 4-2 Anti-MPO IgG titres of purified IgG from serum

Anti-MPO IgG titres were determined by ELISA from purified IgG (pooled from previous experiments A, B & D (see Figure 4-1). Titres were normalised to the Ec50 of purified anti-MPO IgG provided by P.Heeringa run alongside as a way to control for inter-experimental

variation. Anti-MPO IgG titres were very low in my purified IgG product compared to purified anti-MPO IgG supplied by P.Heeringa.

To assess the purity of my isolated anti-MPO IgG an aliquot was run on a polyacrylamide gel alongside commercially-sourced purified sheep IgG and the unbound fraction from the purification (Figure 4-3). Coomassie blue-staining of the gel revealed that my isolated anti-MPO IgG product was largely free from other contaminating proteins and was the expected size corresponding to ~50kDa. The unbound fraction from the purification process did not appear to contain any IgG and the main fragment was likely albumin (~67kDa).
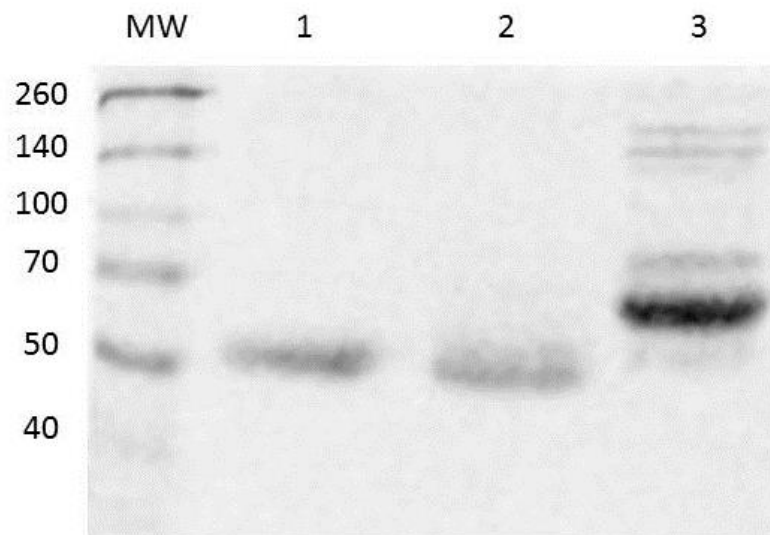
Figure 4-3 Coomassie blue stained SDS-PAGE reveals that purified anti-MPO IgG is largely free from other contaminating proteins

5μg total protein was loaded per lane. Lane 1: Purified sheep IgG, lane 2: isolated anti-MPO IgG, lane 3: unbound fraction from anti-MPO IgG purification.

As my isolated anti-MPO IgG was determined to be largely pure it was used in the following passive transfer model of anti-myeloperoxidase associated glomerulonephritis. Due to the titre of my purified anti-MPO IgG being very low compared to the purified anti-MPO IgG supplied by P.Heeringa, any of my anti-MPO IgG used in the following experiments was adjusted according to the titre compared

to the purified anti-MPO IgG supplied by P.Heeringa which had been successfully used to induce disease.

4.4.2 Establishing the passive transfer model of anti-myeloperoxidase associated glomerulonephritis

Experimental use of the passive transfer model of anti-myeloperoxidase associated glomerulonephritis developed by Xiao *et al.* has been crucial in identifying the non-redundant role of neutrophils in the development of crescentic GN and the importance of the alternative complement pathway in disease pathogenesis (Xiao et al., 2005, Xiao et al., 2007). The model has also exposed the potential for disease to be aggravated in the presence of bacterial LPS and GCSF (Huugen et al., 2005, Freeley et al., 2013). I intended to use this model to investigate the role of Annexin 1 in the anti-MPO mediated renal injury present in anti-myeloperoxidase associated glomerulonephritis.

The aim was to establish a model of anti-myeloperoxidase associated glomerulonephritis in order to test the effect of the absence of Annexin 1 on disease. I attempted to replicate the passive transfer model developed by Xiao *et al.* (Xiao et al., 2002) in which mice are intravenously injected with antibody isolated from MPO-deficient mice immunised with purified murine MPO. I used additional injections of LPS and GCSF as described by Freeley *et al.* (Freeley et al., 2013) to exacerbate the disease phenotype. Experiments to generate the passive transfer model of anti-myeloperoxidase associated glomerulonephritis are summarised in Table 7.

Table 7 Table detailing the generation of the passive transfer model of anti-myeloperoxidase associated glomerulonephritis

Experiment ID	I	II	III	IV	V
Number of mice in experimental group	5	3	3	3	3
Gender	M	F	M	M	F
Average age at injection start date (wks)	8	8	8	20	8

Dose of anti-MPO IgG (µg/g bodyweight)		50	50	50	100	100
Source of anti-MPO IgG		P.Heeringa	P.Heeringa	P.Heeringa	Mine	P.Heeringa
LPS dose		150 EU/g	0.1µg	0.1µg	0.1µg	0.1µg
Route of LPS injection		IP	IV	IP	IP	IP
Time of LPS injection according to IgG		t=1hr	t=0hr	t=1hr	t=1hr	t=1hr
GCSF injection		No	Day -4, 0, +4	Day -4, 0, +4	Day -8, -4, 0, +4	Day -4, 0, +4
Haematuria	day 1	N/A [1]	0, 0, 2	0, 0, 0	0, 0, 0	3, 3, 2
	day 7	0, 0 [2]	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, N/A [3]
Proteinuria day 7 (mg/ml)		not measured			2.3, 0.6, 2.1	0.1, 1.0, N/A [3]
Mean antibody titre (Ec50)	day 1	N/A [4]	61.07	171.3	N/A [4]	133.2
	day 7	53.70	41.67	239.1	57.2	151.0
Glomerular crescents (%)		0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	4.2 23.4 5.7

Haematuria was estimated by urine dipstick. [1] Urine was not available for day 1 analysis in experiment I. [2] 3/5 mice died of LPS toxicity between 1 and 3 days post LPS/IgG in experiment I. [3] Urine was not available for day 7 analysis from one animal in experiment V. [4] Blood not available for antibody titre determination.

A preliminary attempt to trial the passive-transfer model of anti-myeloperoxidase associated glomerulonephritis in mice encompassed the IV injection of 50µg/g bodyweight anti-MPO IgG (donated by P.Heeringa) accompanied by 150 EU/g LPS injected IP 1hr after IgG (experiment I). Of a total of five 8 week old male C57BL/6

mice, three of five animals succumbed to LPS-toxicity between 1 and 3 days post IgG/LPS. Of the two mice that survived, 7 days post IgG there was no evidence of renal injury estimated by urinary dipstick analysis or observed on PAS-stained kidney sections and MPO antibody titres were very low (Figure 4-4).

Figure 4-4 Anti-MPO antibody ELISA titres from anti-myeloperoxidase associated glomerulonephritis trial experiments

Anti-MPO antibody titres were measured by ELISA in serum collected 1 or 7 days after anti-MPO IgG injection. Data are shown as mean + SEM.

To avoid any further mortalities due to LPS-related toxicity, a subsequent attempt at establishing disease was made using a dose of LPS established in the NTN model; the IV injection of 50µg/g bodyweight anti-MPO IgG was combined with 0.1µg LPS/animal (experiment II). Of a total of three 8 week old female C57BL/6 mice, one animal exhibited raised haematuria estimated by urinary dipstick 1 day post IgG but renal injury estimated by urinary dipstick on day 7 was not apparent in any of the three animals. There was no kidney injury observed on histology and antibody titres were relatively low on day 1 and day 7 (Figure 4-4).

To replicate the protocol described by Freeley *et al.* (Freeley et al., 2013) the next attempt to induce anti-myeloperoxidase associated glomerulonephritis involved the IV injection of 50µg/g bodyweight anti-MPO IgG followed by an IP injection of 0.1µg LPS/animal 1hr after IgG (experiment III). IP injections of 26µg GCSF were also given 4 days prior to IgG, at the time of the IgG injection and 4 days after IgG. None of the three male 8 week old C57BL/6 mice demonstrated any renal abnormalities estimated by urinary dipstick although antibody titres were significantly elevated on day 1 and day 7 after IgG compared to previous experiments (Figure 4-4). There no significant glomerular abnormalities evident on PAS-stained kidney sections in any of the three mice injected with anti-MPO IgG (Figure 4-5).
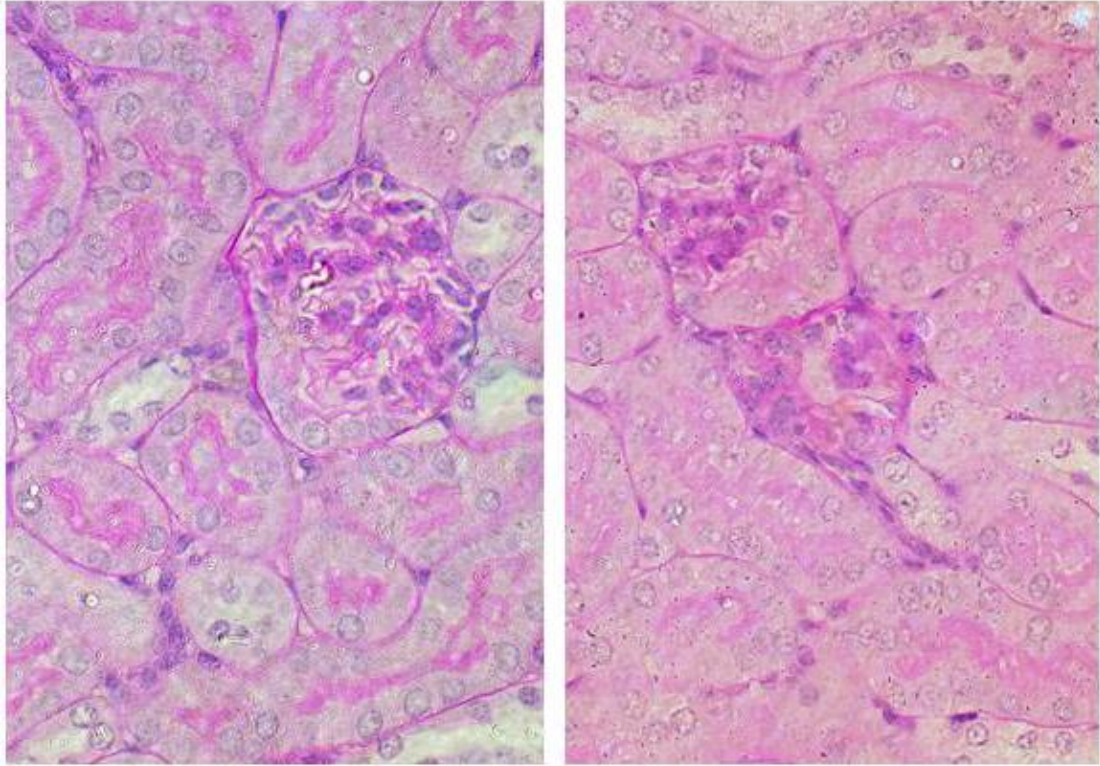
Figure 4-5 No significant glomerular abnormalities observed in an attempt to establish the passive-transfer model of anti-myeloperoxidase associated glomerulonephritis

C57BL/6 mice were injected IV with 50μg/g bodyweight anti-MPO IgG followed by an IP injection of 0.1μg LPS/animal 1hr after IgG. IP injections of 26μg GCSF were also given 4 days prior to IgG, at the time of the IgG injection and 4 days after IgG (experiment III). No significant renal abnormalities were observed on PAS-stained kidney sections in any of the three mice 7 days after receiving anti-MPO IgG. Magnification x400.

As the antibody titres in the animals in the experiments thus far were relatively low and there was no significant renal injury observed, the following experiment used 100μg/g bodyweight anti-MPO IgG (my stock) followed by an IP injection of 0.1μg LPS/animal 1hr after IgG (experiment IV). IP injections of 26μg GCSF were also given 8 and 4 days prior to IgG, at the time of the IgG injection and 4 days after IgG. There was no evidence of renal injury estimated by urinary dipstick analysis or observed on histology in any three of the 20 week old male C57BL/6 mice injected. Proteinuria was low in all three animals with a mean of 1.7mg/ml and antibody titres were still consistently low on day 7 (Figure 4-4).

Success in inducing anti-myeloperoxidase associated glomerulonephritis was ultimately achieved through the IV injection of 100µg/g bodyweight anti-MPO IgG (from P.Heeringa) followed by an IP injection of 0.1µg LPS/animal 1hr after IgG (experiment V). IP injections of 26µg GCSF were also given 4 days prior to IgG, alongside the IgG injection and 4 days after IgG. All three female 8 week old mice injected exhibited enhanced haematuria estimated by urinary dipstick analysis on day 1, indicating acute kidney injury, which resolved by day 7. Proteinuria was low in all three animals with a mean of 0.6mg/ml. Anti-MPO antibody titres averaged 133 (EC_{50}) on day 1 and were maintained to day 7; averaging 151 (Figure 4-4). Glomerular crescents were visible on PAS stained kidney sections in all three animals 7 days after receiving anti-MPO IgG; ranging through 4.2%, 5.7% and 23.4%. There was also some focal fibrinoid necrosis and a degree of peri-glomerular leukocyte accumulation observed (Figure 4-6).
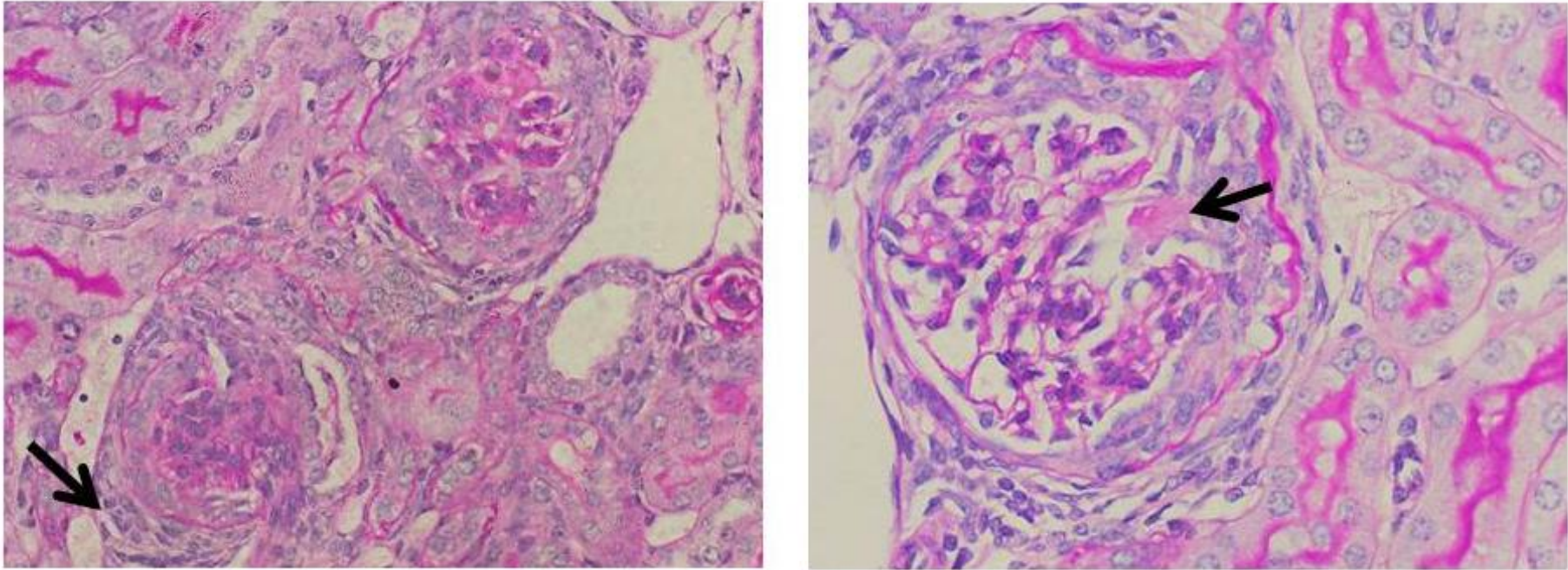
Figure 4-6 Significant renal injury observed in mouse model of anti-myeloperoxidase associated glomerulonephritis

C57BL/6 mice were injected IV with 100μg/g bodyweight anti-MPO IgG (from P.Heeringa) followed by an IP injection of 0.1μg LPS/animal 1hr after IgG. IP injections of 26μg GCSF were also given 4 days prior to IgG, alongside the IgG injection and 4 days after IgG. Glomerular crescents were observed on PAS-stained kidney sections in all 3 animals 7 days after receiving anti-MPO IgG. There was also some focal fibrinoid necrosis (right arrow) and a degree of peri-glomerular leukocyte accumulation (left arrow). Magnification x400 (left image) x630 (right image).

4.4.3 Effect of the absence of Annexin 1 in anti-myeloperoxidase associated glomerulonephritis

With a model of anti-myeloperoxidase associated glomerulonephritis set up in my hands, an experiment was carried out to examine any differences in disease penetrance between wild-type and Annexin 1 deficient mice. Annexin 1 -/- mice have shown increased susceptibility to murine models of acute and chronic inflammation, demonstrating increased neutrophil and monocyte infiltration and excessive macrophage recruitment, extracellular matrix deposition and fibrosis (Damazo et al., 2011, Locatelli et al., 2014).

To investigate whether the absence of Annexin 1 in C57BL/6 mice confers a greater susceptibility to the development and severity of glomerular inflammation in a mouse model of anti-myeloperoxidase associated glomerulonephritis, I induced disease in wild-type and Annexin 1 -/- mice. 8 week old female wild-type and Annexin 1 -/- mice (n=4 per group) were injected IV with 100μg/g bodyweight anti-MPO IgG (from P.Heeringa) or a saline control (wild-type n=4) on day 0. In conjunction with this, all mice received an IP injection of 0.1μg LPS one hour after IgG or saline administration and IP injections of 26μg GCSF on day -4, 0 and +4. 24 hour urine collections were carried out in metabolic cages on day 0 and day 6 and tail bleeds were performed on day 1 for determination of anti-MPO antibody titres. Mice were sacrificed on day 7.

In this passive transfer model of anti-myeloperoxidase associated glomerulonephritis, antibody titres on day 1 and day 7 from the experiment comparing disease in wild-type and Annexin 1 -/- mice were comparable to those in the pilot experiment trialling the same disease induction protocol in wild-type mice (n=3) (Figure 4-7). Therefore additional readouts relating to disease were combined across experiments.
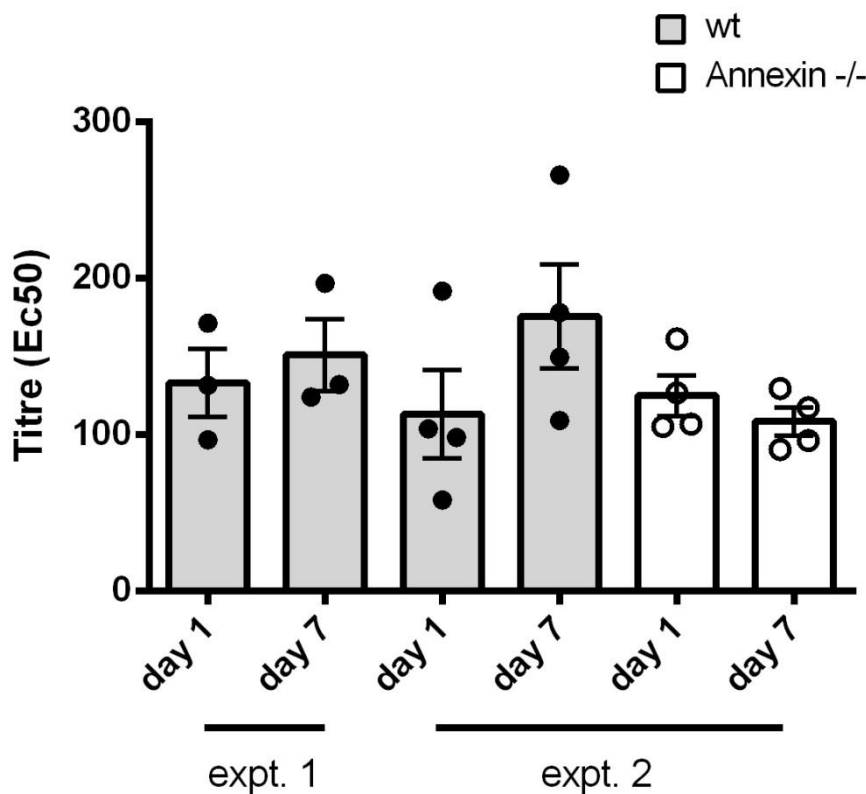
Figure 4-7 Administration of anti-MPO IgG in combination with intraperitoneal injections of LPS and GCSF, into wild-type and Annexin 1 deficient mice results in sustained anti-MPO IgG titres from day 1 to day 7 after injection of IgG

Plasma or serum was isolated from blood collected on day 1 or day 7 to determine anti-MPO titre by ELISA. Anti-MPO IgG titres were sustained between day 1 and day 7 in both wild-type and Annexin 1 -/- mice. The titres across experiment 1 (wild-type n=3) and experiment 2 (wild-type n=4, Annexin 1 -/- n=4) did not show significant variation. Data are shown as mean with standard error of the mean.

Haematuria estimated by urinary dipstick was negative for all animals on day 7. When combining the data across experiments, at day 7 proteinuria and serum urea levels were very low in all animals; less than 1mg/ml, and less than 10mmol/l respectively. There was no difference in serum urea or protein/creatinine ratio between wild-type and Annexin 1 -/- mice (Figure 4-8, i and ii).

On examination of PAS stained kidney sections on day 7 there were between 2-23% crescentic glomeruli found in 5 of 7 wild-type mice but there was no glomerular

crescent formation observed in the Annexin 1 -/- mice. Due to the small experimental group numbers, this difference did not reach statistical significance. There was no difference in the number of infiltrating macrophages into the glomerulus between experimental groups (Figure 4-8, iii and iv).
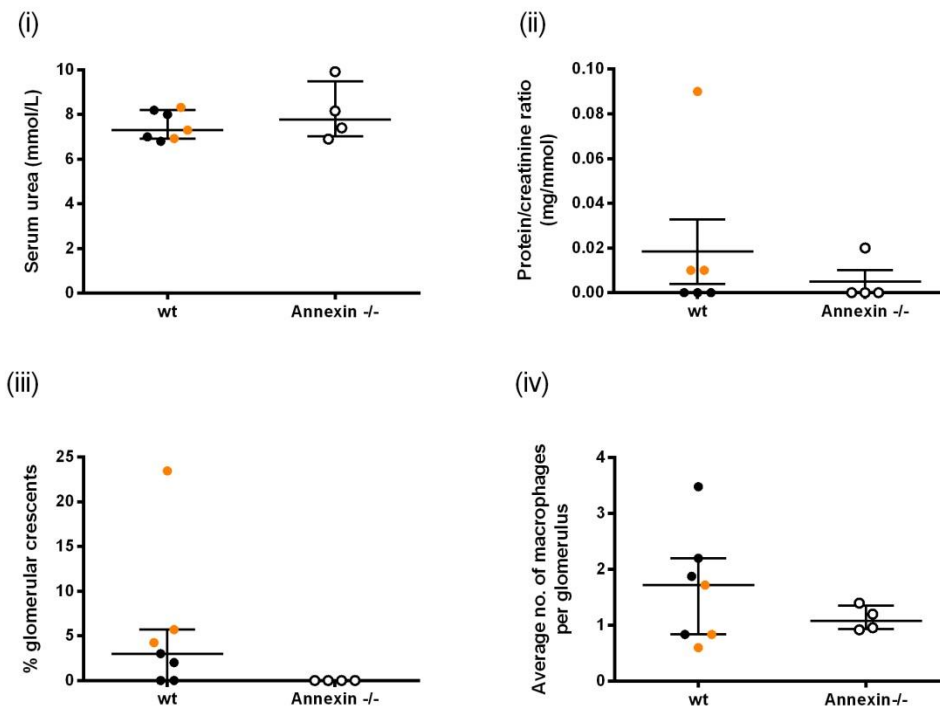
Figure 4-8 Renal injury is no different between wild-type and Annexin 1 deficient mice in this murine model of anti-myeloperoxidase associated glomerulonephritis

7 days after anti-MPO IgG injection, serum urea levels were low (i) and no different between the wild-type and Annexin 1 -/- mice. (ii) Protein/creatinine ratio was also no different between the wild-type and Annexin 1 -/- mice. Quantification of PAS-stained kidney sections (iii) revealed that compared to an average of 3% crescentic glomeruli in wild-type mice, Annexin 1 -/- mice did not develop any glomerular crescents. This difference was not statistically significant when analysed with a Mann-Whitney test. The number of infiltrating macrophages into the glomerular space at day 7 was no different between the experimental groups (iv). N.B. Data from two experiments were combined due to comparable antibody titres as demonstrated in Figure 4-7. Wild-type animals from the pilot experiment are indicated in orange. Data are shown as median with interquartile range.

4.4.3.1 *Microparticles in MEV*

Microparticles have a potential role in vascular injury and I demonstrated in the previous chapter that total MP numbers and NMP numbers are significantly diminished in Annexin 1 deficient animals in a murine model of NTN.

In order to investigate MP numbers in MEV and whether these are affected by the absence of Annexin 1, blood plasma was stained for FACS analysis with Annexin V and antibodies to Ly6G or CD41, to identify total MPs, NMPs and PMPs (staining and FACS performed by Dr Ying Hong, UCL). 8 week old female wild-type and Annexin 1 -/- mice (n=4 per group) were injected intravenously with 100µg/g bodyweight anti-MPO IgG (from P.Heeringa) or a saline control (wild-type n=4) on day 0. In conjunction with this, all mice received an IP injection of 0.1µg LPS one hour after IgG or saline administration and IP injections of 26µg GCSF on day -4, 0 and +4. Mice were sacrificed on day 7.

In this MEV model total MP numbers were significantly elevated in the Annexin 1 deficient mice, compared to the wild-type mice (p=0.0286) but did not differ from the LPS control group (Figure 4-9). Numbers of NMPs and PMPs did not differ between experimental groups.
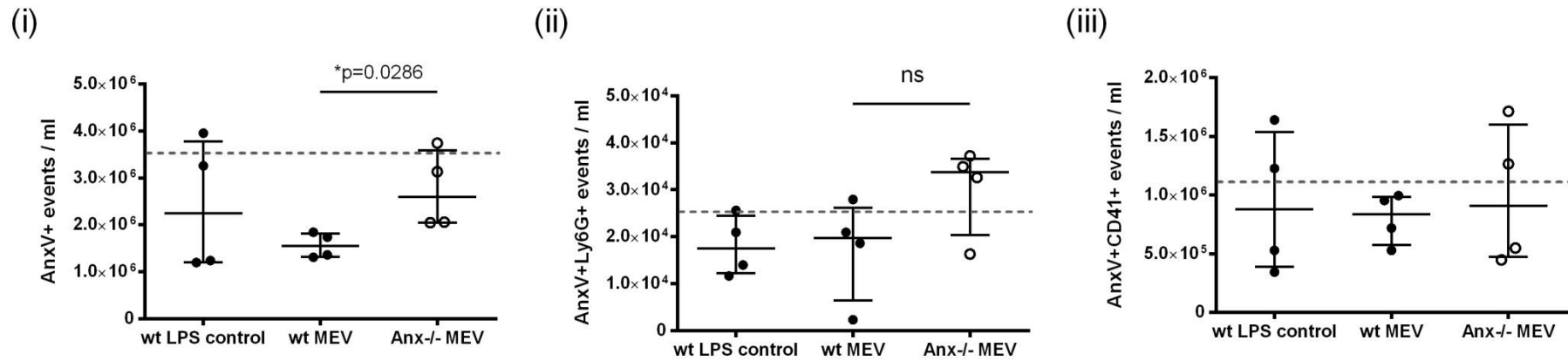
Figure 4-9 Total microparticle numbers are significantly elevated in Annexin 1 deficient mice a model of MEV

Microparticles were isolated from blood plasma at the experiment end. Cells were incubated with Annexin V before staining with Ly6G or CD41 to identify total microparticles (i), and those derived from neutrophils (ii) or platelets (iii), respectively. MPs were analysed by FACS and latex beads (Sigma) were run concurrently with the samples to determine absolute MP numbers. 8 week old female wild-type and Annexin 1 -/- mice (n=4 per group) were injected IV with 100μg/g bodyweight anti-MPO IgG (from P.Heeringa) [MEV] or a saline control (wild-type n=4) on day 0 [wt LPS control]. In conjunction with this, all mice received an IP injection of 0.1μg LPS one hour after IgG or saline administration and IP injections of 26μg GCSF on day -4, 0 and +4. Data are expressed as median with interquartile range. Dotted line represents equivalent median value for NTN control (see Figure 3-11).

4.4.4 Attempt to model anti-myeloperoxidase associated glomerulonephritis using mMPO in adjuvant and nephrotoxic serum with additional MPO immunodominant peptide

A further attempt was made to induce anti-myeloperoxidase associated glomerulonephritis using a protocol based on the experimental model described by Holdsworth and Kitching (Ruth et al., 2006). This model is based on the hypothesis that MPO acts as an autoantigen in the kidney and cell-mediated immunity plays a key role in disease pathogenesis. It has been has been successfully used in demonstrating the importance of CD4+ effector T cells and IL-17 in the development of crescentic GN. A specific immunodominant MPO CD4+ T-cell epitope ($MPO_{409-428}$) was recently identified by the same group and has been shown to induce disease in a similar capacity to that of the native protein, when low dose anti-GBM antibody is used to direct the immune response to the glomerulus (Ooi et al., 2012). I used this immunodominant peptide in conjunction with injection of whole murine MPO in an effort to accentuate the immune response to the deposited whole MPO. I intended to use this experimental model to investigate the role of Annexin 1 in both the humoral and cellular arms of the immune response within the development of crescentic glomerulonephritis.

9 week old female C57BL/6 mice (n=6) and Annexin 1 -/- mice (n=6) were injected subcutaneously in the left flank with 40µg murine MPO + 50µg MPO immunodominant peptide in CFA. This injection was repeated in the right flank 7 days later in IFA. Control animals (C57BL/6 n=6) were injected with saline in CFA or IFA. After a further 7 days, all mice were injected IV with 100µl NTS diluted 1:1 in saline, 200µl total volume. This injection was repeated the following day. After 4 days, mice were culled after collecting urine overnight in metabolic cages.

No renal injury was evident when estimated by urine dipstick analysis 4 days after the final NTS injection and anti-MPO antibody titres assessed by ELISA from cardiac puncture serum were negative in all animals. There was no renal injury apparent on examination of PAS-stained kidney sections, with the exception of one Annexin 1 -/- animal that exhibited some localised tubular damage.

4.4.5 Discussion

Injection of MPO deficient mice with murine MPO in adjuvant as previously described by Xiao *et al.* (2002) produced varying antibody titres. Additional administration of immunodominant peptide $MPO_{409-428}$, designed to encourage loss

of tolerance to the native MPO, did not appear to increase the immune response as reflected in the anti-MPO IgG titres. This lack of, and variation in, immune response to MPO may have resulted from low purity of the injected protein as subsequent mMPO immunisations in our lab have been more successful and required less protein to produce an immune response in MPO deficient mice.

Total IgG was isolated from serum from the MPO deficient mice with the highest anti-MPO IgG titres. Anti-MPO IgG titres of purified IgG were very low when compared with IgG with previously established pathogenicity, although the protein product was largely uncontaminated. There were technical issues with the purification of IgG from serum resulting from fibrin clotting, disrupting the isolation which may have resulted in the loss of antibody.

I attempted to replicate the passive transfer model of anti-myeloperoxidase associated glomerulonephritis developed by Xiao *et al.* (2002) with additional injections of LPS and GCSF. LPS has historically been used to exacerbate the disease phenotype in this model and leads to an early increase in neutrophil influx and a late increase in glomerular macrophage infiltration (Huugen et al., 2005). Recently GCSF has also been used in conjunction with LPS to increase disease penetrance as this glycoprotein is thought to prime neutrophils for MPO-ANCA induced activation (Freeley et al., 2013). Although purified IgG from MPO deficient mice was adjusted according to titre against IgG with previously established pathogenicity, it failed to produce a sustained circulating antibody response in wild-type mice. FCGN was achieved when mice were injected IV with 100µg anti-MPO IgG (from P.Heeringa) followed by an IP injection of 0.1µg LPS/animal 1hr after IgG. IP injections of 26µg GCSF were also given 4 days prior to IgG, alongside the IgG injection and 4 days after IgG. The degree of glomerular crescent formation averaged 5% (range 0-23%), there was also some focal fibrinoid necrosis visible and a degree of peri-glomerular leukocyte inflammation. Using this passive transfer model of anti-myeloperoxidase associated glomerulonephritis Xiao *et al.* have reported a variable penetrance of disease with the average number of crescentic glomeruli ranging from 3-11.5% in the absence of additional endotoxin or inflammatory agents. Although LPS and GCSF injections were given in this model, the disease severity fell short of 29% crescentic glomeruli reported by Freeley *et al.* where both of these agents were used.

In this passage transfer model of anti-myeloperoxidase associated glomerulonephritis Annexin 1 deficient mice maintained an equivalent antibody titre to wild-type mice over the experimental time course, however they did not develop any glomerular crescents compared to an average of 5% glomerular crescents in wild-type mice. Glomerular macrophage infiltration was comparable in Annexin 1 deficient and wild-type mice as were serum urea readings and protein/creatinine ratios at the experiment end. However, 1 day following anti-MPO IgG injection, haematuria estimated by urinary dipstick was elevated in the wild-type mice in the pilot experiment, compared with all wild-type and Annexin 1 deficient mice in the second experiment, which exhibited low or negative levels of haematuria. This is a crude measurement but could indicate lower disease penetrance in the second experiment when compared to the pilot experiment as antibody titres are not always reflected in disease severity. Annexin 1 plays a protective role in a number of inflammatory disease models and regulates neutrophil/endothelial interactions in the microcirculation (Damazo et al., 2005, Locatelli et al., 2014). As glomerular injury in the passive-transfer model is primarily mediated by the influx and degranulation of neutrophils, accompanied by the infiltration of mononuclear cells, I hypothesised that Annexin 1 deficiency would exacerbate disease. It is likely that the difference observed in glomerular histology between the wild-type and Annexin 1 deficient animals is due to experimental variation, and the disease penetrance was not substantial enough across the experimental groups to demonstrate a protective effect of Annexin 1. In experimental autoimmune encephalomyelitis (EAE) Annexin 1 deficient mice have impaired capacity to develop disease, although unlike the murine model of anti-myeloperoxidase associated glomerulonephritis, this disease is T-cell mediated (Paschalidis et al., 2009).

There was a significant increase in overall MP numbers in Annexin 1 deficient mice, compared to wild-type mice, in this MEV model. As there was no significant difference detected in disease between Annexin 1 deficient and wild-type mice in MEV, it is difficult to comment on the relationship of these MPs to vascular injury in mice. However, an increase in overall MP numbers in Annexin 1 deficient mice is opposite to what was observed in NTN, where there was exacerbation of disease, suggesting that MPs could be protective in MEV. Much of the research on neutrophil MPs has indicated that they are of an anti-inflammatory nature, however there is also research to suggest that PMN-derived MPs can be pro-inflammatory and activate endothelial cells (Mesri and Altieri, 1998). The nature of MPs may influenced by their activation stimulus. The average total MP numbers in the LPS

control were higher than in the wild-type MEV group. This was unexpected and could be a reflection of a lack of disease penetrance in the wild-type animals and general variation in MP numbers in the experimental groups after LPS treatment. It is possible that the higher MP numbers in the Annexin 1 deficient animals are due to a lack of TNF-α inhibition, exacerbating the inflammatory effects of LPS and release of pro-inflammatory MPs. Inclusion of an Annexin 1 deficient LPS control group would assess this hypothesis.

I attempted to replicate the anti-myeloperoxidase associated glomerulonephritis model developed by the group of Holdsworth and Kitching (Ruth et al., 2006). Although additional MPO immunodominant peptide was combined with the murine MPO, designed to enhance the immune response to the deposited protein, no evidence of disease was found in wild-type mice and circulating anti-MPO antibody was undetectable. There was limited success inducing anti-MPO IgG in MPO deficient mice with my purified mMPO and this failure to induce an immune response could also be reflective of a deficiency in mMPO purity.

Summary:

- In this instance, the passive transfer model of anti-myeloperoxidase associated glomerulonephritis required additional injections of LPS and GCSF to generate a pauci-immune crescentic glomerulonephritis in C57BL/6 mice.
- The disease penetrance across experimental groups in this MEV model prevented the conclusion to whether the absence of Annexin 1 had an effect on disease.
- An attempt to replicate the DTH-like response to planted antigen in the mouse kidney as described by Ruth *et al.* (2006) was unsuccessful, despite the fact that additional immunodominant MPO CD4+ T-cell epitope was injected alongside the native protein.

Chapter 5 The effect of Annexin 1 on human neutrophil responses to ANCA

5.1   Introduction

Chapters 3 and 4 describe potentially opposing roles for Annexin 1 in two alternative models of crescentic glomerulonephritis. To investigate the cellular mechanisms that might account for either a protective or injurious role for Annexin 1 in AAV, the following chapter investigates Annexin 1 in the context of patient and healthy control neutrophils *in vitro.*

There is previous evidence that a higher neutrophil membrane expression of proteinase 3 (PR3) is associated with AAV and correlates with risk of disease relapse (Rarok et al., 2002, Witko-Sarsat et al., 1999). Also transcriptional expression of PR3 has been shown to relate to disease activity and higher levels can be an indicator of subsequent poor response to treatment (Cheadle et al., 2010, Grayson et al., 2015). PR3 and Annexin 1 co-localize on the neutrophil surface on activation, allowing the cleavage of Annexin 1 by PR3, putatively preventing the proteins' pro-resolution functions. Annexin 1 cleavage products can be detected in the BALF of patients with abnormal lungs and in experimental-colitis in rats and increased Annexin 1 cleavage has been associated particularly with a heightened degree of neutrophil infiltration (Muller Kobold et al., 1998, Vong et al., 2007).

Seminal work by Falk *et al.* (1990) revealed that neutrophils are activated by ANCA, which results in the release of free radicals and primary granule contents causing direct damage to the endothelium as well as depositing MPO in the kidney as a planted autoantigen. This binding of ANCA to neutrophils is understood to occur through FcRγ engagement (Mulder et al., 1994). Annexin 1 has been shown to inhibit binding of IgG to Fcγ receptors on peripheral blood monocytes and neutrophils *in vitro* and neutrophil FcγR-mediated respiratory burst can be inhibited by treatment with the Annexin 1 N-terminal peptide Ac2-26 (Goulding and Guyre, 1993, Goulding et al., 1998). A PR3 cleavage-resistant form of Annexin 1, SAnxA1, has a longer sequence and a higher affinity for FPR2/ALXR than the peptide Ac2-26. This peptide can inhibit PMN accumulation *in vivo* and accelerate the resolution of inflammation within the arthritic joint, but its' direct actions on neutrophil activation have not been investigated (Pederzoli-Ribeil et al., 2010, Patel et al., 2012, Perretti and Dalli, 2009).

5.2 Aim

The initial aim of this chapter is to investigate whether the expression of PR3 on the neutrophil surface is altered in this PR3-positive GPA patient group when compared to healthy controls. The second objective is to observe any associations between neutrophil surface PR3 expression and Annexin 1 cleavage. Finally, the ability of cleavage-resistant forms of Annexin 1 to inhibit ANCA-induced activation of primed neutrophils is explored.

5.3 Experimental Design

Neutrophils were isolated from whole blood anti-coagulated with EDTA from patients and healthy controls by layering on Histopaque 1119 followed by a discontinuous Percoll gradient. For the investigation of membrane PR3 expression and Annexin 1 cleavage, neutrophils were stimulated with/without TNF-α (10ng/ml) for 30 minutes at 37°C before separating into cells for FACS and whole cell lysates for Western blotting. Cells for FACS analysis were stained with anti-PR3 FITC antibody or isotype control. Whole cell lysates were generated by resuspending cells at 10×10^6 cells/ml in a non-denaturing lysis buffer (20mM Tris HCl pH 8.0, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA) with protease inhibitors and incubating on ice for 30 minutes before centrifuging ≥10,000g at 4°C for 10 minutes. The ability of cleavage-resistant forms of Annexin 1 to inhibit ANCA-induced activation of primed neutrophils was trialled using varying concentrations of SAnxA1 and CR-AnxA1$_{2-50}$. Neutrophils were primed with cytochalasin b and TNF-α alongside the relevant peptide or control before activation with ANCA or fMLP was assessed by the detection of MPO release.

Table 8 Experimental groups

	Healthy controls	**PR3-positive GPA patients**
Number	10	12
Gender (M/F)	3/7	7/5
Mean age (yr)	44 (25-70)	53 (20-78)
Median ANCA titre (U/ml)	N/A	26 (<1 – >177)

Steriod treatment (percentage of patients receiving prednisolone)	N/A	75% (Mean dose 14mg/d; range 3mg/d – 60mg/d)

Age, median ANCA titre and steroid dose are all reflective of the date at which the blood sample was taken.

5.4   Results

5.4.1   Isolation of neutrophils from peripheral whole blood

To enable the detection of surface PR3 expression and Annexin 1 cleavage products, neutrophils were isolated as described. Neutrophils were assessed for purity by generating a cytospin and averaged greater than 95% (Figure 5-1).
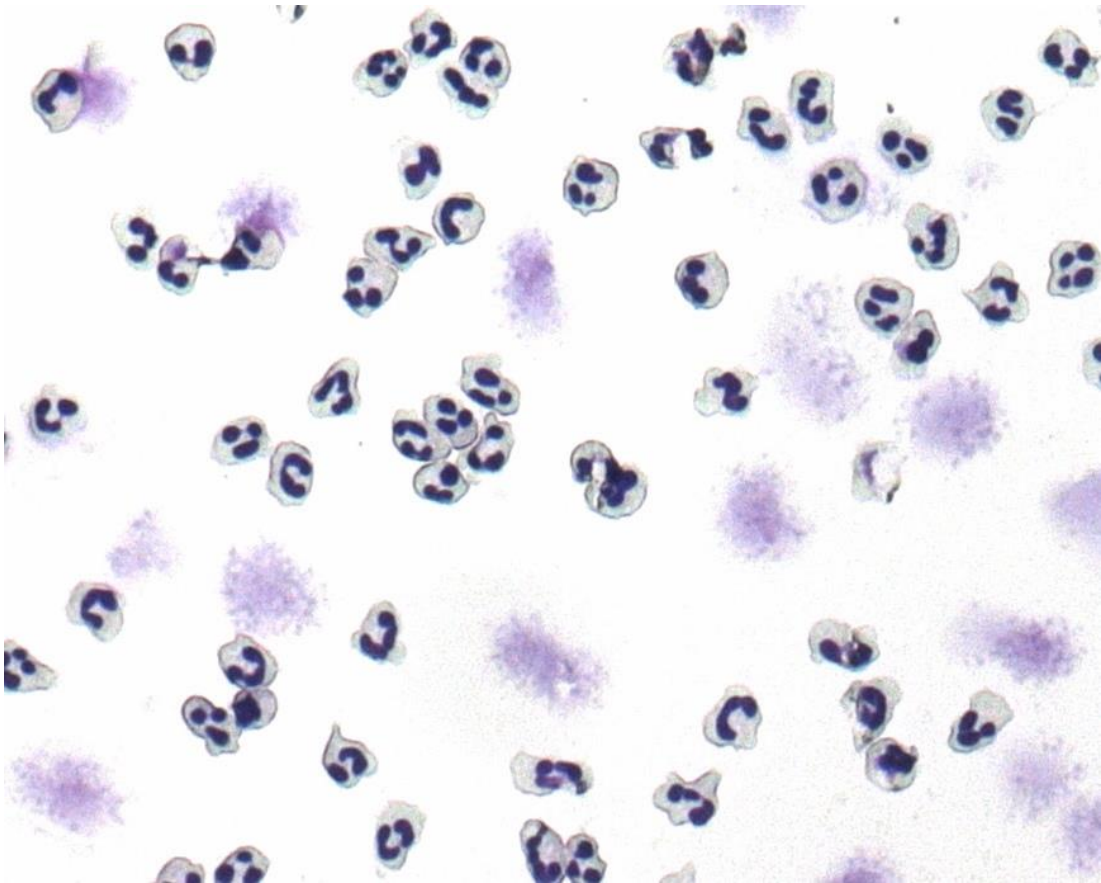
Figure 5-1 Diff-Quick staining of a cytospin reveals that isolated neutrophil purity is greater than 95%

Neutrophils isolated from peripheral blood were assessed for purity by loading 150μl of a 0.75×10^6 cells/ml cell suspension into a cuvette and spinning at 450rpm for 6 minutes in a

cytocentrifuge. Cells were immediately fixed and stained with Diff-Quik before visualisation. Magnification is x100.

5.4.2 Neutrophil surface expression of PR3 is bimodal

To determine neutrophil surface expression of PR3 (mPR3), purified cells were stained with anti-PR3 FITC antibody. FACS analysis revealed a bimodal expression pattern of PR3 on the neutrophil surface across all healthy controls and GPA patient cells (Figure 5-2). Hereafter, these two distinct neutrophil populations will be referred to as mPR3^{low} and mPR3^{high}, based on their surface PR3 expression.
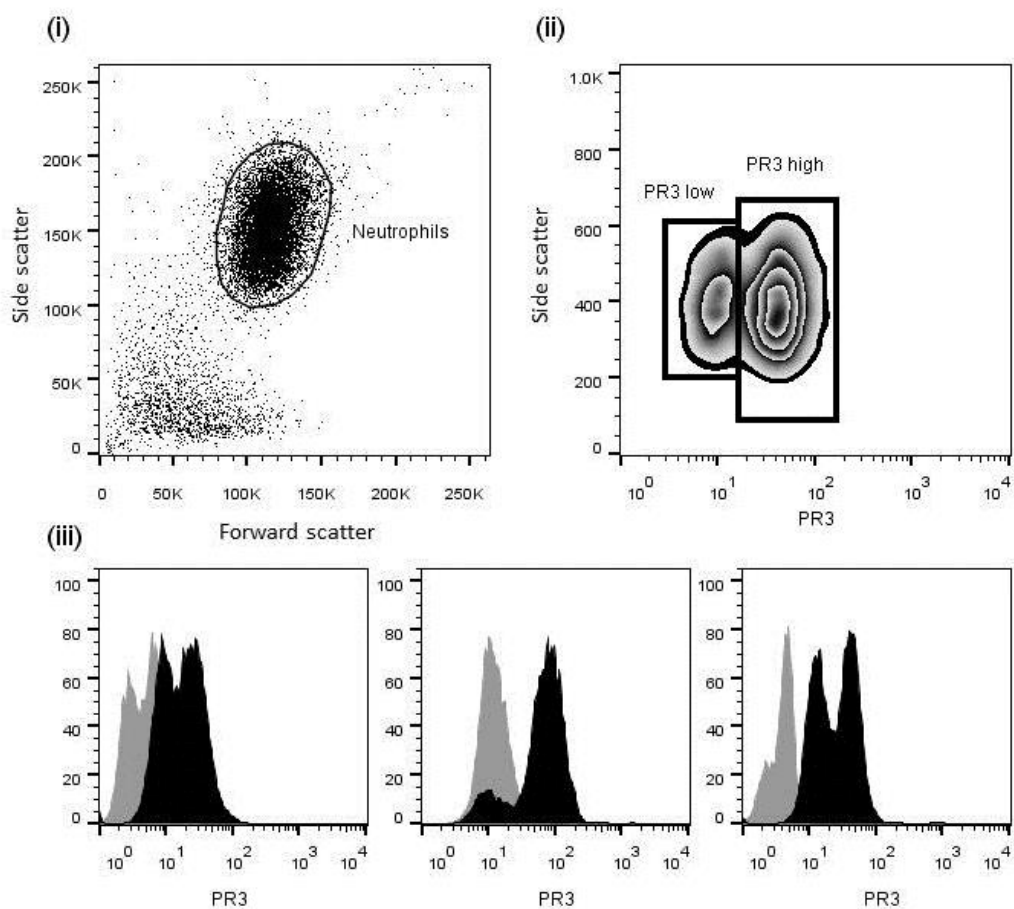
Figure 5-2 Neutrophil surface expression of PR3 is bimodal

Neutrophils isolated from peripheral blood were incubated with anti-PR3 FITC antibody for 30 minutes on ice in the dark before washing and fixing with 1% PFA. One-colour flow cytometry was performed on a BD FACSCalibur and 10,000 neutrophil events were collected based on their forward- and side-scatter (FSC/SSC) profile. Subsequent analysis revealed a discrete population of neutrophils based on their FSC/SSC (i). These cells

exhibited a bimodal pattern of PR3 expression (ii) which I have designated mPR3^{low} and mPR3^{high} based on their SSC vs PR3 MFI profile. (iii) Representative histograms of the range of neutrophil surface PR3 expression displayed across healthy controls and GPA patients (anti-PR3 stained samples in black, isotype control in grey).

5.4.3 Surface PR3 expression is higher in neutrophils from GPA patients when compared with healthy controls

To validate whether neutrophil surface PR3 expression was inherently different between my healthy control and GPA patient groups, isolated neutrophils were stained for FACS analysis with and without *in vitro* stimulation by TNF-α (10ng/ml). The mPR3 expression of the neutrophil population as a whole was significantly higher (p=0.03) in the patients when compared with healthy controls within the TNF-α treatment condition. There was also a trend (that did not reach statistical significance) toward the mPR3 expression of the neutrophil population being elevated in the GPA patients when compared with healthy controls in the absence of TNF-α stimulation (Figure 5-3). There was no difference between the mPR3 expression of the neutrophil population with/without TNF-α treatment within the healthy control group, but there was a suggestion that the mPR3 expression of the neutrophil population may increase within the GPA patient group with *in vitro* stimulation; however this value did not reach statistical significance.
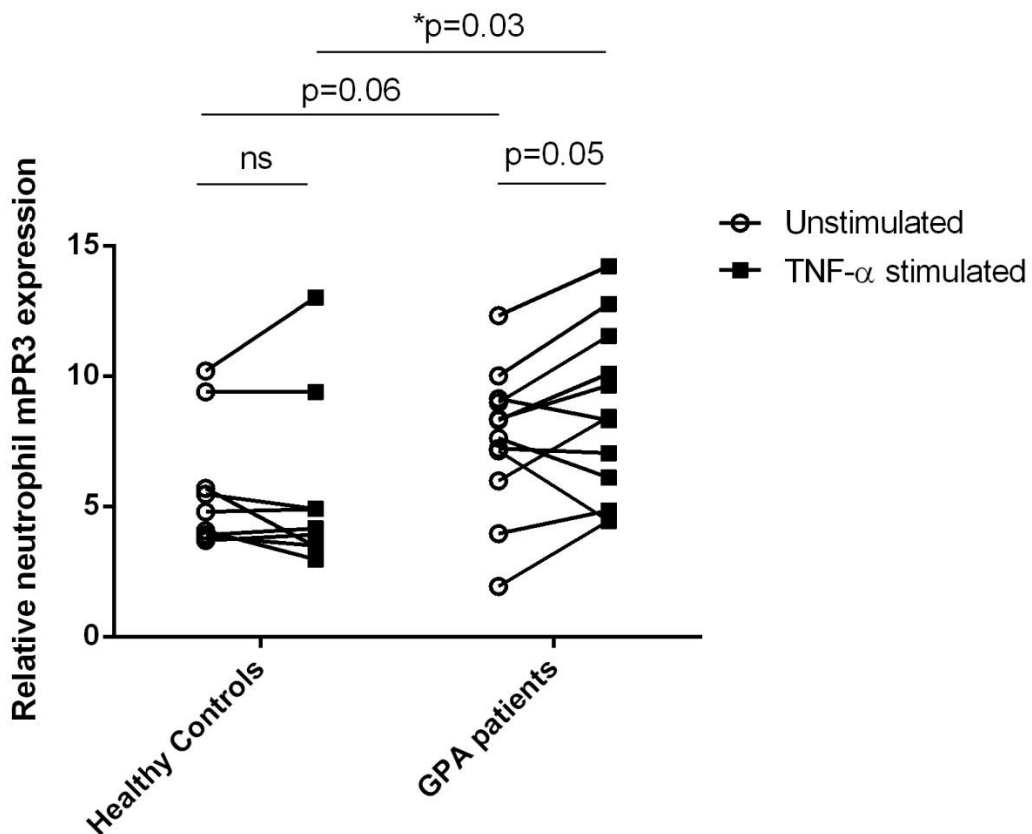
Figure 5-3 Neutrophil surface expression of PR3 is elevated in GPA patients when compared with healthy controls after *in vitro* neutrophil activation

Neutrophils stimulated with/without TNF-α (10ng/ml) for 30 minutes at 37°C were incubated with anti-PR3 FITC antibody or isotype control. The MFI of the PR3-stained neutrophil population as a whole, was divided by the MFI of the equivalent population stained with the isotype control antibody to produce a value for relative mPR3 expression. The neutrophil surface expression of PR3 (mPR3) was significantly higher in GPA patients than healthy controls when treated with TNF-α (10ng/ml) (p=0.03, unpaired t-test).

There have been differences reported in the PR3 expression of the mPR3[high] subset when comparing neutrophils from healthy controls and GPA patients (Rarok et al., 2002). When neutrophils were split into mPR3[high] and mPR3[low] subsets based on their PR3 MFI and SSC profile (see Figure 5-2) the surface expression of PR3 in the mPR3[high] subset significantly increased (p=0.005) with TNF-α stimulation in healthy control neutrophils when compared to unstimulated neutrophils (Figure 5-4). This effect was not observed in neutrophils from GPA patients. When comparing the surface expression of PR3 in the mPR3[high] subset from neutrophils from healthy controls and GPA patients there was a trend towards the MFI being higher in the GPA patients in unstimulated cells, although the *p* value fell short of statistical significance. This trend was not seen in the presence of TNF-α stimulation.
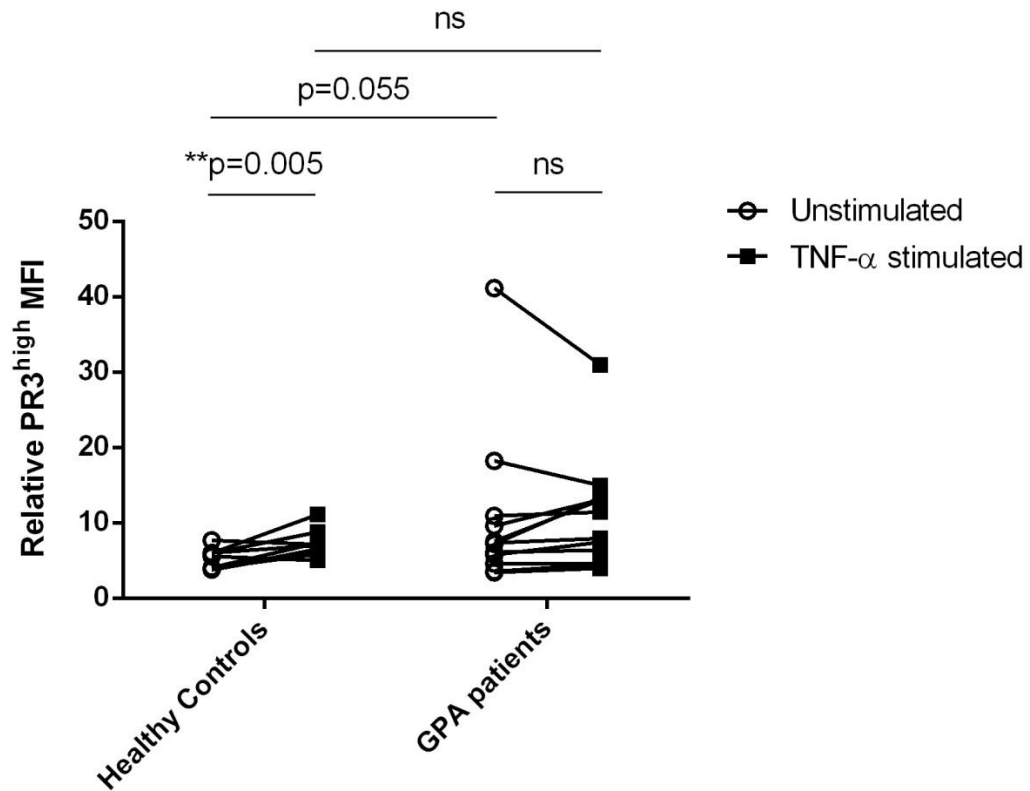
Figure 5-4 Surface PR3 expression of mPR3^{high} neutrophil subset is increased with *in vitro* neutrophil activation in healthy controls

Neutrophils were stimulated and stained as Figure 5-3. The MFI of the mPR3^{high} neutrophil subset, stained with anti-PR3, was divided by the MFI of the equivalent population stained with the isotype control antibody to produce a value for relative mPR3 expression. Gates to distinguish mPR3^{high} and mPR3^{low} neutrophil populations were set on unstimulated conditions for anti-PR3 stained or isotype stained samples and transferred to the TNF-α treatment condition. The surface expression of PR3 was significantly higher in the mPR3^{high} neutrophil subset in healthy controls when treated with TNF-α (10ng/ml), compared to untreated cells (p=0.005, paired t-test).

The surface PR3 expression of the mPR3^{low} subset was significantly higher (p=0.03) in the GPA patients with TNF-α stimulation when compared to untreated neutrophils. There was a trend towards this phenomenon in the healthy control neutrophils but the *p* value did not reach significance. There was no difference between the surface PR3 expression of the mPR3^{low} subset when comparing unstimulated or TNF-α stimulated cells between healthy controls and GPA patients (Figure 5-5).
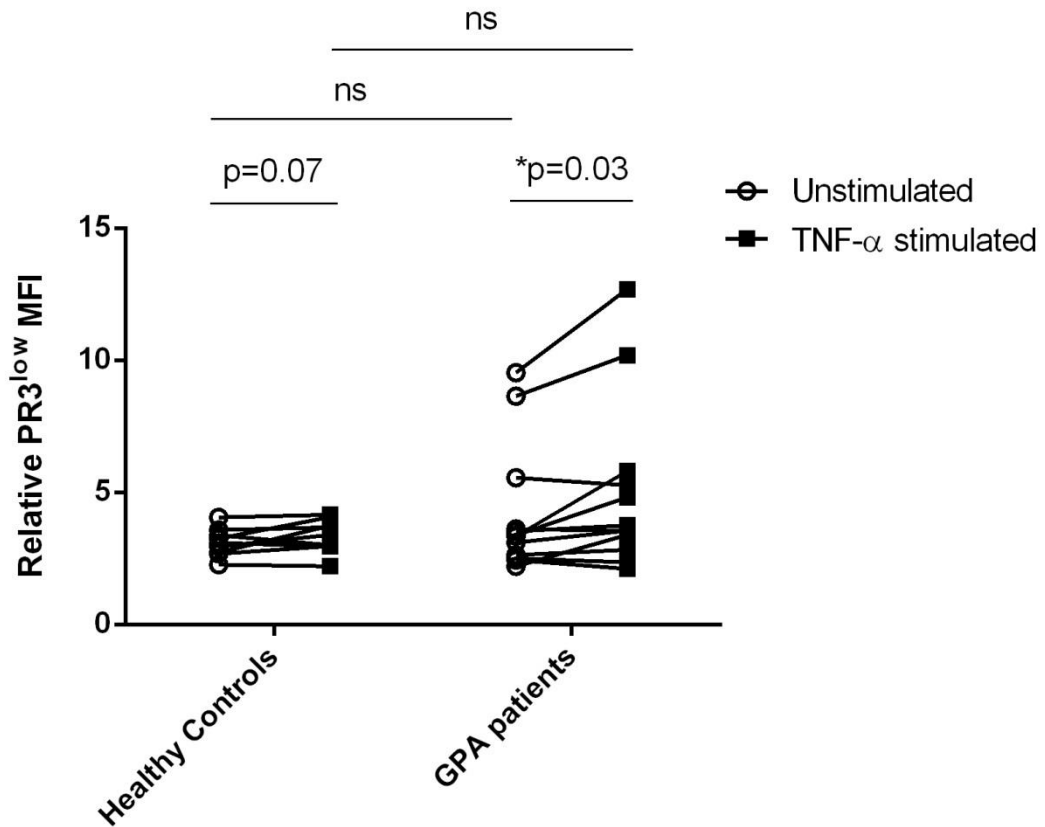
Figure 5-5 Surface PR3 expression of mPR3^{low} neutrophil subset is increased with *in vitro* neutrophil activation in GPA patients

Neutrophils were stimulated and stained as Figure 5-3. The MFI of the mPR3^{low} neutrophil subset, stained with anti-PR3, was divided by the MFI of the equivalent population stained with the isotype control antibody to produce a value for relative mPR3 expression. Gates to distinguish mPR3^{high} and mPR3^{low} neutrophil populations were set on unstimulated conditions for anti-PR3 stained or isotype stained samples and transferred to the TNF-α treatment condition. The surface expression of PR3 was significantly higher in the mPR3^{low} neutrophil subset in GPA patients when treated with TNF-α (10ng/ml), compared to untreated cells (p=0.03, paired t-test).

When examining the relative abundance of the mPR3^{high} neutrophil subset compared to the mPR3^{low} neutrophil subset, TNF-α-stimulated neutrophils from the healthy controls had significantly less mPR3^{high} neutrophils (mean= 62.0%) compared to unstimulated cells (mean= 67.8%, p=0.01). The abundance of the mPR3^{high} subset was unchanged in GPA patient neutrophils when comparing

stimulated and unstimulated cells and when comparing healthy control and GPA patient neutrophils under either experimental condition (Figure 5-6).
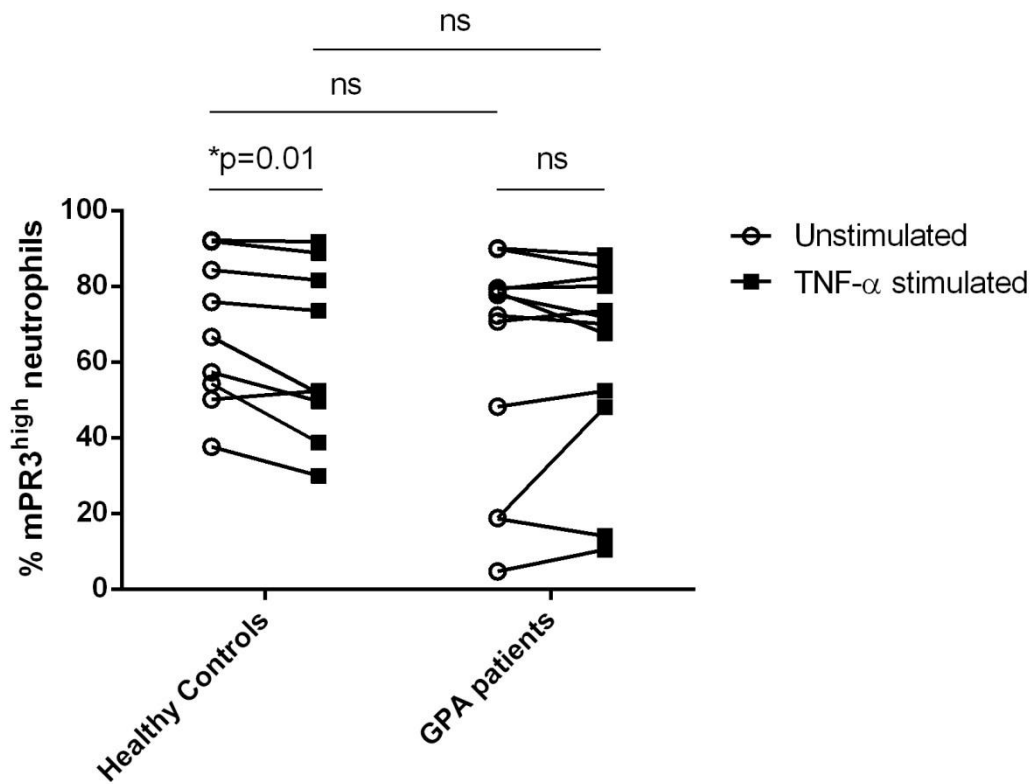
Figure 5-6 Abundance of mPR3^{high} subset decreases with *in vitro* neutrophil activation in healthy controls

Neutrophils were stimulated and stained as Figure 5-3. Neutrophils were determined as mPR3^{high} or mPR3^{low} as depicted in Figure 5-2. The percentage of the mPR3^{high} subset decreased in healthy controls when treated with TNF-α (10ng/ml), compared to untreated cells (p=0.01, paired t-test).

5.4.4 Annexin cleavage products can be detected by Western blotting in whole cell lysates of neutrophils treated with/without TNF-α *in vitro*

In order to investigate Annexin 1 cleavage in neutrophils and any differences between healthy controls and patients, whole cell lysates were generated from unstimulated neutrophils and neutrophils stimulated with TNF-α (10ng/ml). Protein from unstimulated or stimulated cells was detected by Western blotting using a monoclonal mouse anti-human Annexin 1 antibody.

Annexin 1 protein was detected in neutrophil whole cell lysates from healthy controls and GPA patients by Western blotting. There was no protein detectable in the cell supernatants recovered from the experiments when determined by BCA assay. Within the whole cell lysates a ~37kDa band corresponding to intact Annexin 1 was revealed alongside two smaller bands corresponding to ~30kDa and ~28kDa (Figure 5-7).
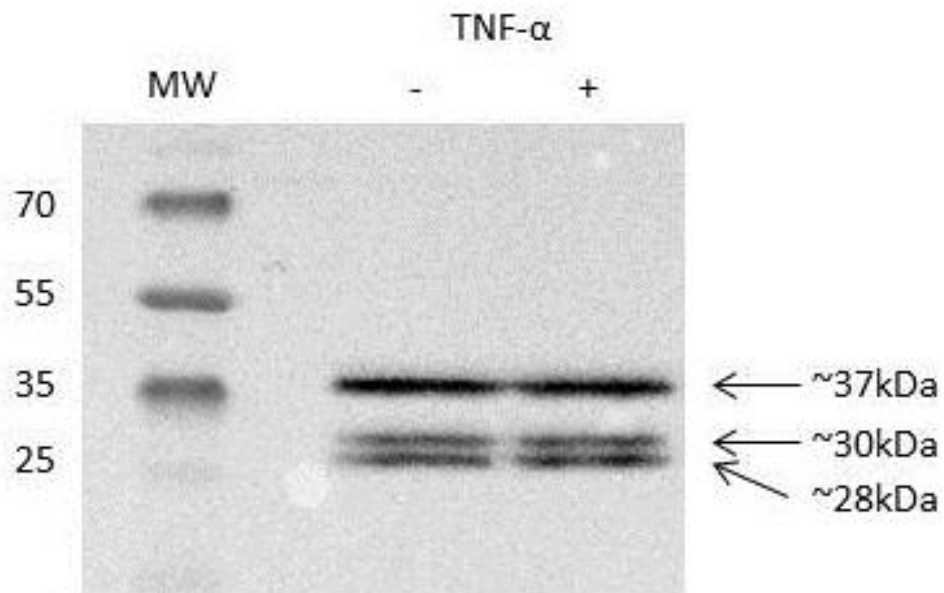
Figure 5-7 Annexin 1 cleavage products can be detected by Western blotting in whole cell lysates of neutrophils treated with/without TNF-α *in vitro*

Neutrophil whole cell lysates were generated by resuspending cells at 10×10^6 cells/ml in a non-denaturing lysis buffer (20mM Tris HCl pH 8.0, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA) with protease inhibitors and incubating on ice for 30 minutes before centrifuging ≥10,000g at 4°C for 10 minutes. 10µg protein (determined by BCA assay) was loaded from each sample onto a reducing SDS-polyacrylamide gel. Protein was detected by Western blotting using mouse anti-human Annexin 1 antibody. An expected band of ~37kDa was observed for intact Annexin 1 in addition to two smaller cleavage products corresponding to ~30kDa and ~28kDa.

Image J software was used to quantify the proportions of intact and cleaved Annexin 1 products detected by Western blotting in each sample (Figure 5-8, i). When examining the amount of total cleaved Annexin 1 in whole neutrophil lysates

compared to total Annexin 1 protein detected in each sample, there was no significant difference observed between healthy control and GPA patient cells under control or TNF-α treatment conditions, although the median percentage of cleaved Annexin 1 was lower in GPA patient neutrophils (Figure 5-8, ii, iv). The proportion of cleaved Annexin 1 was unchanged when comparing unstimulated cells to cells stimulated with TNF-α (10ng/ml). When investigating the 30kDa and 28kDa cleavage products in relation to each other, there was no significant difference between the abundance of the 30kDa peptide when comparing healthy control and GPA patient neutrophils in both control and treatment conditions, although the median percentage of the 30kDa peptide was higher in GPA patient neutrophils overall (2-way ANOVA: healthy control/patient group accounting for 15% of variation $p=0.08$).
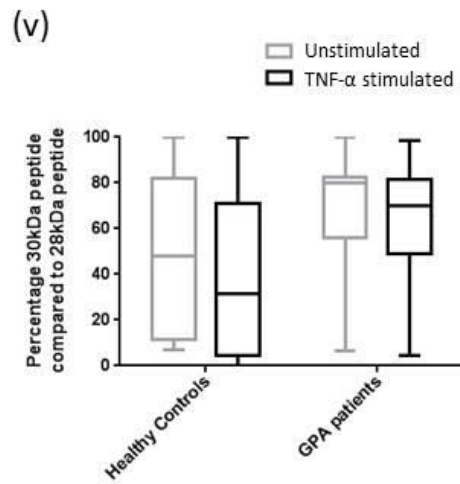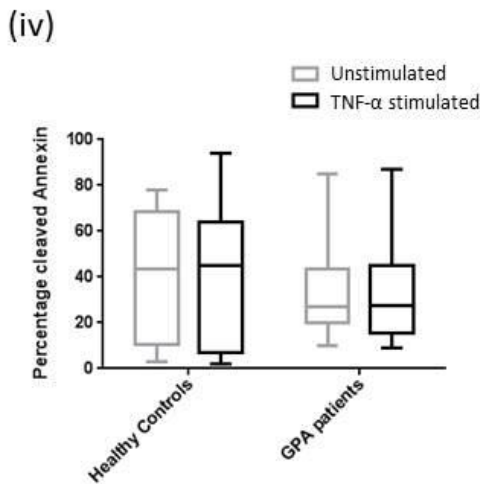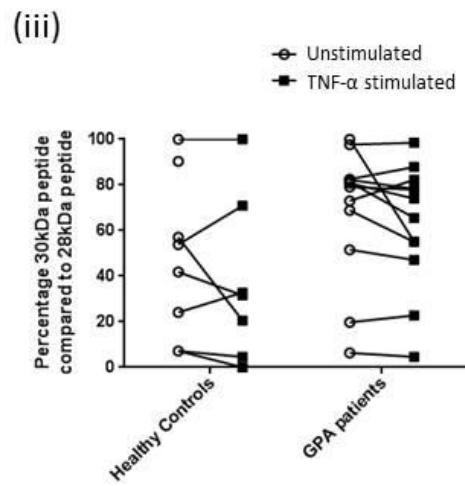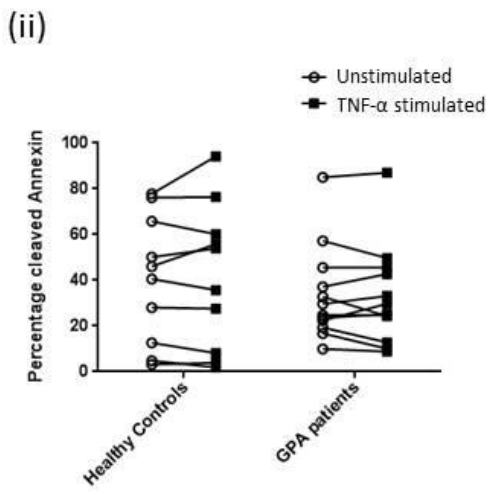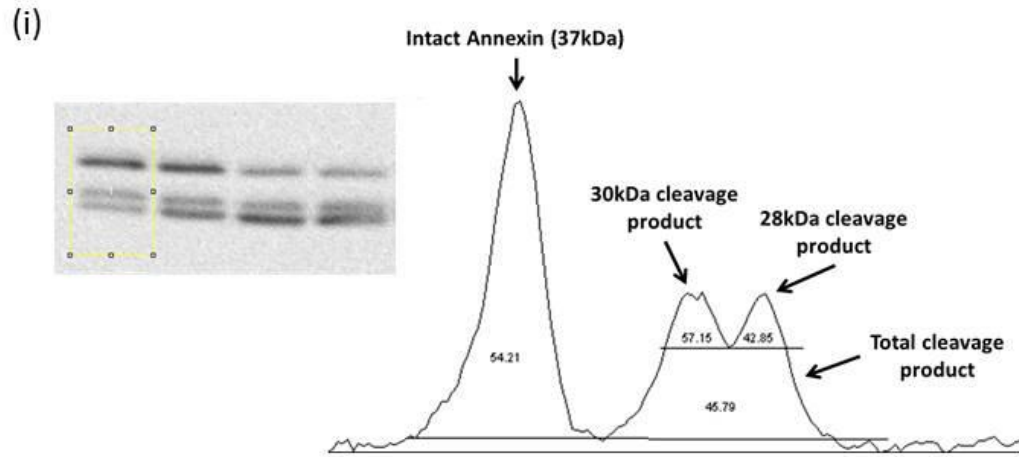
Figure 5-8 There are no significant differences in the abundance of Annexin 1 cleavage products detected in neutrophil whole cell lysates when comparing GPA patient and healthy control cells

(i) Image J software was used to quantify the proportion of intact and cleaved Annexin 1 detected by Western blotting in each sample (ii) total cleaved Annexin 1 was expressed as a percentage of total Annexin 1 detected in each sample (iii) the proportion of 30kDa cleavage product was expressed as a percentage of 30kDa and 28kDa areas combined (iv,v) interleaved box and whisker plots indicating the minimum and maximum as an alternative representation of graphs (ii) and (iii).

There was not enough neutrophil whole cell lysate available to probe for a loading control (β-actin, GAPDH etc.) to determine total Annexin 1 content across every sample. As a standard amount of whole cell lysate was loaded per well (determined by BCA assay), a ratio was determined within each sample to compare the abundance of intact Annexin 1 with and without TNF-α stimulation. The ratio was created by expressing each area of the intact Annexin 1 band determined by ImageJ as a percentage of the bands combined. i.e. if the TNF-α band was twice as strong as the control band, the TNF-α condition would read as 0.7 and the control band 0.3. This ratio allowed the investigation of whether the total amount of intact Annexin 1 varied with TNF-α stimulation within each sample, and whether this variation differed between healthy controls and GPA patients. When a ratio of intact Annexin 1 between the two conditions was expressed, there was no significant difference observed in the total amount of intact Annexin 1 detected with and without TNF-α treatment in either healthy controls or GPA patients (Figure 5-9).
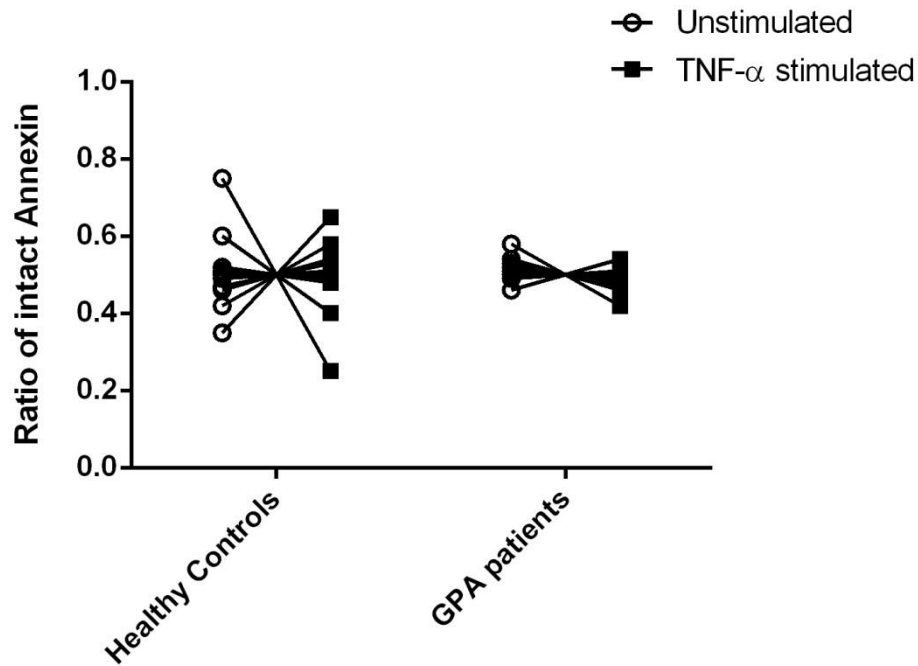
Figure 5-9 There is no observed effect on total amounts of intact Annexin 1 with TNF-α stimulation in healthy controls or GPA patient neutrophils, when detected in whole cell lysates by Western blotting

Image J software was used to quantify the amount of intact Annexin 1 detected by Western blotting and data were expressed as a ratio of intact Annexin 1 detected in each sample with and without TNF-α stimulation.

5.4.5 Neutrophil surface expression of PR3 is associated with Annexin 1 cleavage

To explore any relationship between neutrophil surface PR3 expression and Annexin 1 cleavage, correlations were investigated between neutrophil surface PR3 expression determined by FACS analysis and the abundance of Annexin 1 cleavage products in proportion to the intact 37kDa protein, established by Western blotting. Correlations were considered between unstimulated cells and cells stimulated *in vitro* with TNF-α, as described previously.

Neutrophil surface PR3 expression (a relative value determined by FACS analysis, as previously described) showed a trend towards a positive correlation with the abundance of total cleaved Annexin 1 in healthy control cells treated with TNF-α (10ng/ml), although this correlation did not meet statistical significance (Spearman

correlation coefficient = 0.65, p=0.067) (Figure 5-10). This trend was not observed for GPA patient neutrophils (Spearman correlation coefficient = 0.32, p=0.30) or when cells were untreated.

The chart displays a scatter plot with "Percentage total cleaved Annexin" on the y-axis (0 to 100) and "Relative PR3 expression" on the x-axis (0 to 15). Text in the plot reads: Healthy controls r=0.65, p=0.067; GPA patients r=0.32, p=0.30.
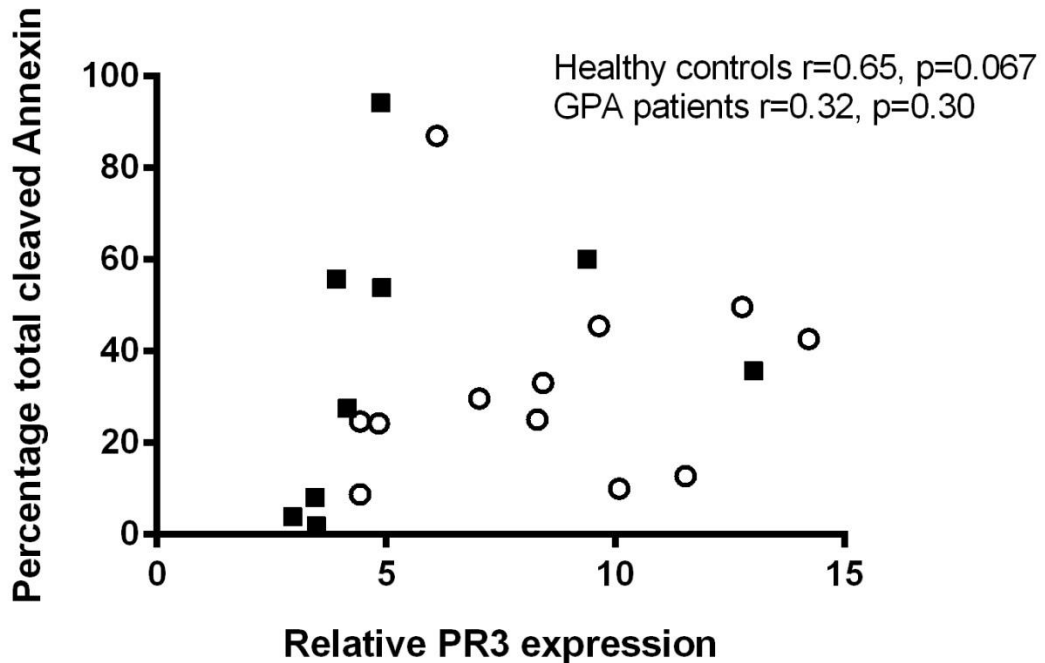
Figure 5-10 Association between neutrophil surface PR3 expression and quantity of Annexin 1 cleavage products detected in whole cell lysates after *in vitro* activation

Neutrophils were stimulated and stained as Figure 5-3. A higher surface expression of PR3 determined by FACS analysis loosely correlated with a higher proportion of total cleaved Annexin 1 detected by Western blotting in whole cell lysates from healthy control neutrophils when treated with TNF-α (10ng/ml) (Spearman correlation coefficient = 0.65, p=0.067). There was no correlation between surface expression of PR3 and the proportion of total cleaved Annexin 1 in GPA patients when treated with TNF-α (Spearman correlation coefficient = 0.32, p=0.30). ■ Healthy controls ○ GPA patients.

The relative abundance of the mPR3^{high} subset compared to the mPR3^{low} subset within the total neutrophil population showed a trend towards a positive correlation with the amount of total cleaved Annexin 1 found in neutrophil whole cell lysates from healthy controls with and without TNF-α stimulation (unstimulated: Spearman correlation coefficient = 0.68, p=0.0503; stimulated: Spearman correlation coefficient

= 0.62, p=0.0857) (Figure 5-11, i and ii). This correlation became significant in stimulated cells when both healthy control and GPA patient groups were analysed in combination (unstimulated combined groups: Spearman correlation coefficient = 0.41, p=0.06; stimulated combined groups: Spearman correlation coefficient = 0.475, p<0.03), but was not observed in GPA patient neutrophils alone.

The percentage of the 28kDa Annexin 1 cleavage product in relation to the 30kDa peptide significantly positively correlated with the percentage of mPR3^{high} neutrophils in unstimulated cells when whole cell lysates from healthy controls and GPA patients were analysed together (Spearman correlation coefficient = 0.47, *p=0.04) (Figure 5-11, iii). There was no correlation observed in healthy controls or GPA patients when cells were stimulated with TNF-α (Figure 5-11, iv).
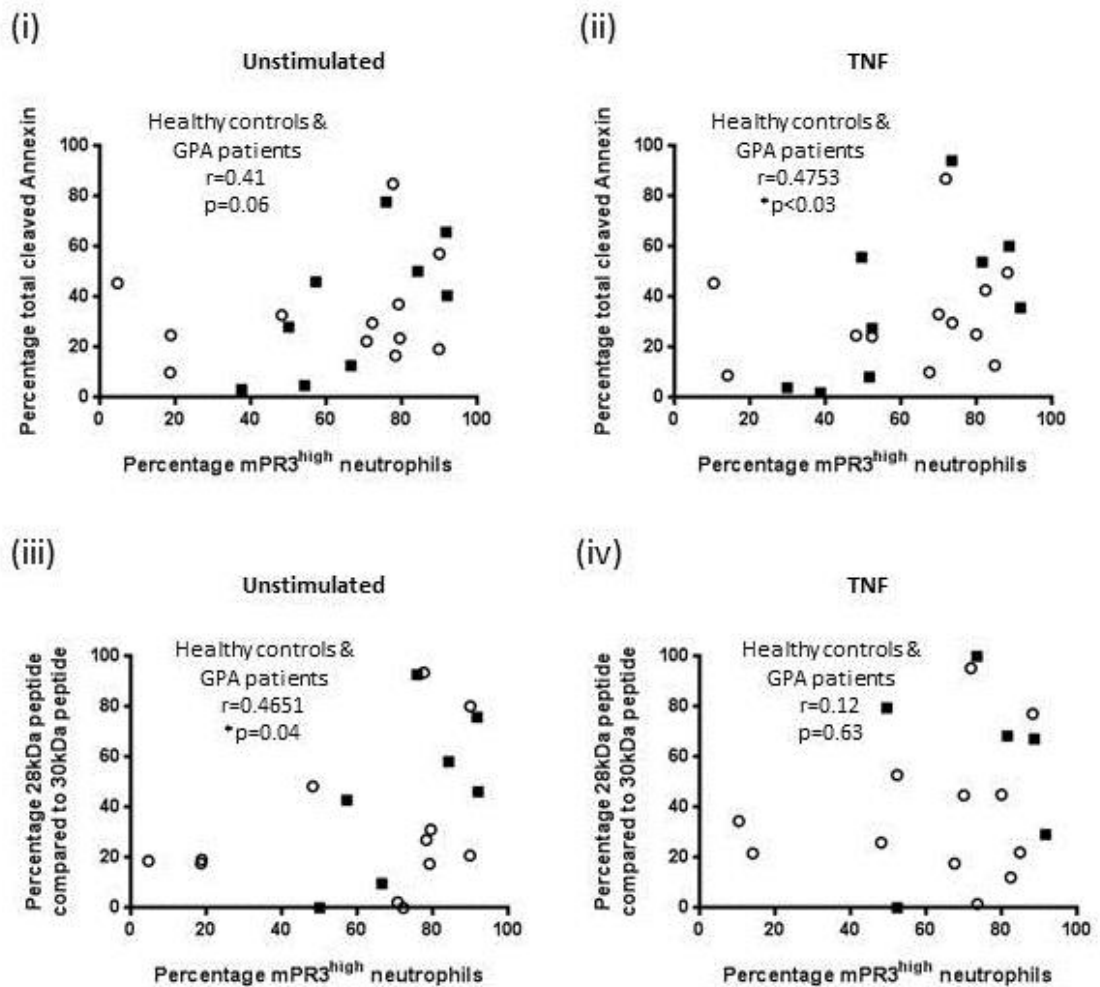
Figure 5-11 The abundance of the mPR3^{high} neutrophil subset positively correlates with the detection of Annexin 1 cleavage products detected in whole

cell lysates from healthy control cells and GPA patients with and without *in vitro* activation

Neutrophils were stimulated and stained as Figure 5-3. (i) The relative abundance of the mPR3^{high} subset within the total neutrophil population, determined by FACS analysis, showed a trend towards a positive correlation with a higher proportion of total cleaved Annexin 1 detected by Western blotting in whole cell lysates from unstimulated healthy control neutrophils (Spearman correlation coefficient = 0.68, p=0.0503). This trend was also observed when healthy control and GPA patient groups were analysed together (Spearman correlation coefficient = 0.41, p=0.06). (ii) When cells were stimulated with TNF-α (10ng/ml), there was a trend towards this same correlation in the healthy control neutrophils (Spearman correlation coefficient = 0.6167, p=0.0857). When healthy control and GPA patient neutrophils were analysed in combination, the correlation reached statistical significance (Spearman correlation coefficient = 0.4753, p<0.03). (iii) There was a significant positive correlation between the abundance of the 28kDa Annexin 1 cleavage product, compared to the 30kDa peptide, and the relative abundance of the mPR3^{high} subset in unstimulated cells when whole cell lysates from healthy controls and GPA patients were analysed together (Spearman correlation coefficient = 0.47, p=0.04). (iv) There was no correlation between the abundance of the 28kDa Annexin 1 cleavage product, and the relative abundance of the mPR3^{high} subset when cells were stimulated with TNF-α. ■ Healthy controls ○ GPA patients.

There were no correlations between the MFI of the mPR3^{high} population and the amount of total cleaved Annexin 1 found in neutrophil whole cell lysates from healthy control or GPA patient neutrophils, with and without TNF-α stimulation. The MFI of the mPR3^{high} population exhibited a significant negative correlation with the abundance of the 28kDa Annexin 1 cleavage product when compared with the 30kDa cleavage product, in unstimulated neutrophils from healthy controls (Spearman correlation coefficient = -0.86, p=0.02) (Figure 5-12, iii). There were no correlations between these two factors in GPA patients, or when cells were stimulated with TNF-α.

There were no correlations observed between the MFI of the mPR3^{low} population and the abundance of Annexin 1 cleavage products.
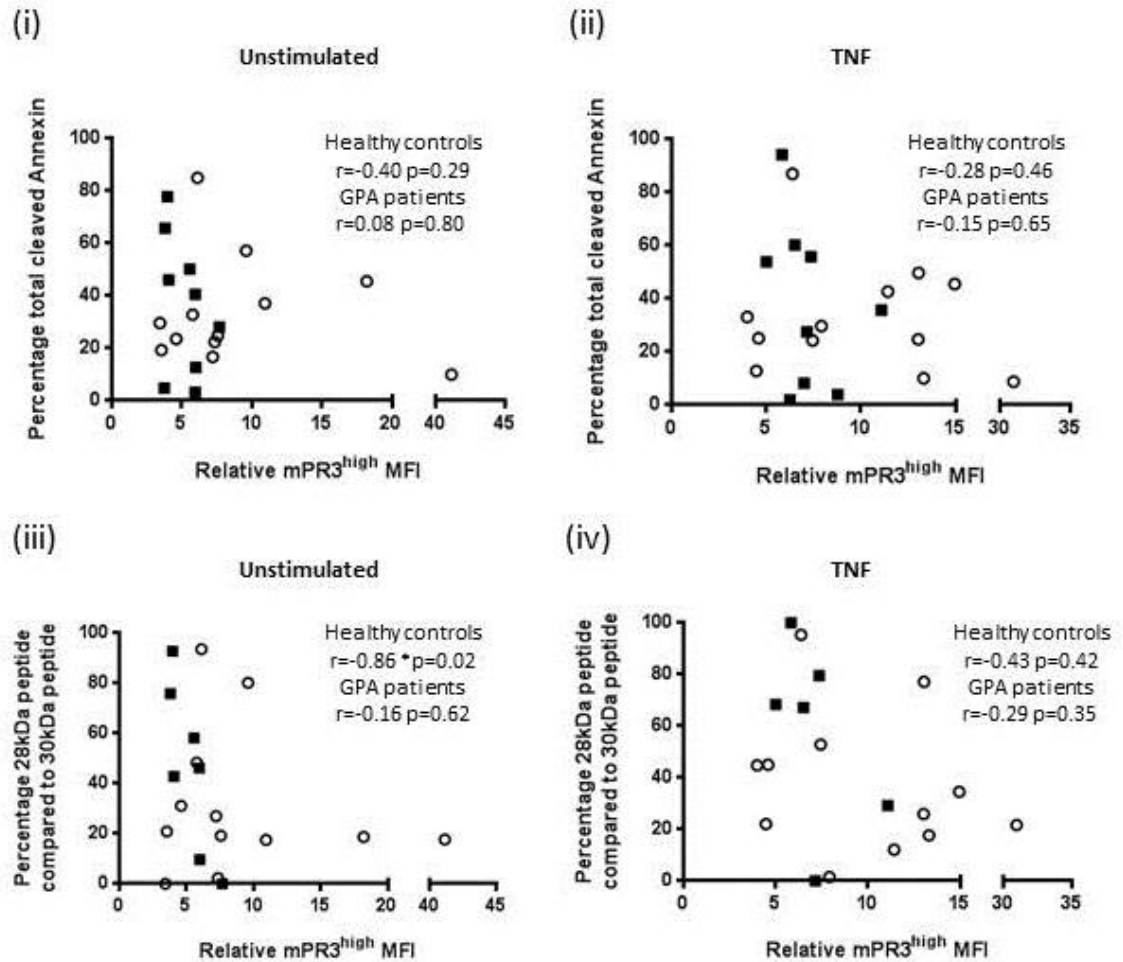
Figure 5-12 The MFI of the mPR3^{high} neutrophil subset negatively correlates with the detection of the 28kDa Annexin 1 cleavage product detected in whole cell lysates from healthy control cells in the absence of *in vitro* activation

Neutrophils were stimulated and stained as Figure 5-3. The MFI of the mPR3^{high} subset within the total neutrophil population, determined by FACS analysis, was compared with cleaved Annexin 1 detected by Western blotting in whole cell lysates. There were no correlations between the mPR3^{high} MFI and total cleaved Annexin 1 in healthy control or GPA patient neutrophils when cells were untreated (i) or stimulated with TNF-α (ii). There was a significant negative correlation between the mPR3^{high} MFI and the percentage of 28kDa peptide in unstimulated healthy control neutrophils (Spearman correlation coefficient = -0.86, p=0.02) (iii), but not in unstimulated GPA patient neutrophils. There was no correlation between the mPR3^{high} MFI and the percentage of 28kDa peptide in TNF-α stimulated neutrophils. ■ Healthy controls ○ GPA patients.

The previous analyses in this section compared neutrophil surface PR3 expression with the abundance of Annexin 1 cleavage products, in relation to total Annexin 1 detected in each individual sample. In order to determine whether neutrophil surface PR3 expression relates to total Annexin 1 expression, the above analyses were repeated with the data for total intact Annexin 1 generated in Figure 5-9. These data are a ratio of intact Annexin 1 with and without TNF-α stimulation within each sample. When this ratio was compared to the abundance of mPR3[high] neutrophils in relation to mPR3[low] neutrophils detected by FACS, in the relevant condition, there was a trend towards a correlation between an increased abundance of mPR3[high] neutrophils and decreased total amount of intact Annexin detected in whole cell lysates from GPA patient cells in the presence of *in vitro* activation (Spearman correlation coefficient = -0.51, p=0.0507) (Figure 5-9). There was no correlation between these two factors in healthy control cells, or when cells were unstimulated.
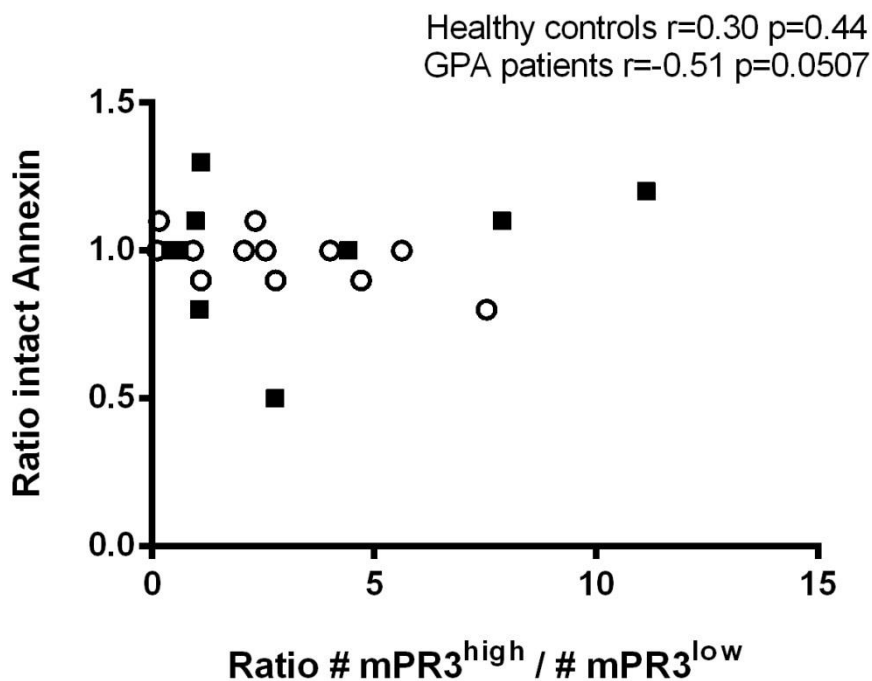
Figure 5-13 Association between the abundance of mPR3[high] neutrophils and total amount of intact Annexin detected in whole cell lysates in the presence of *in vitro* activation

Image J analysis of amounts of intact Annexin 1 was performed as Figure 5-9. The ratio of mPR3[high] to mPR3[low] neutrophils was compared to the ratio of intact Annexin detected in neutrophil lysates with or without TNF-α stimulation. In GPA patient cells, there was a trend

towards a negative correlation between the two factors (Spearman correlation coefficient = -0.5052, p=0.0507). There was no correlation in healthy control cells. ■ Healthy controls ○ GPA patients.

5.4.6   Activation of primed human neutrophils by patient ANCA is modulated by a cleavage-resistant form of Annexin 1 *in vitro*

To investigate whether cleavage-resistant forms of Annexin 1 might moderate the ANCA-induced activation of human neutrophils, neutrophils were isolated from an individual healthy control and primed with cytochalasin b and TNF-α, with or without SAnxA1, before incubating with either IgG isolated from a healthy control, or a PR3- or MPO-positive AAV patient. fMLP was used as a positive control and individual experiments were normalised to PBS controls, allowing data to be combined. MPO-release was measured as an indicator of neutrophil degranulation.

Incubation of neutrophils with SAnxA1 appeared to inhibit the activation of human neutrophils by patient MPO- and PR3-ANCA in a dose-dependent manner when measured by MPO-release (Figure 5-14), although experimental replicates were not high enough to reach statistical significance. Increasing concentrations of SAnxA1 also inhibited MPO-release in fMLP stimulated and control IgG conditions, indicating that this inhibition was not ANCA-specific.

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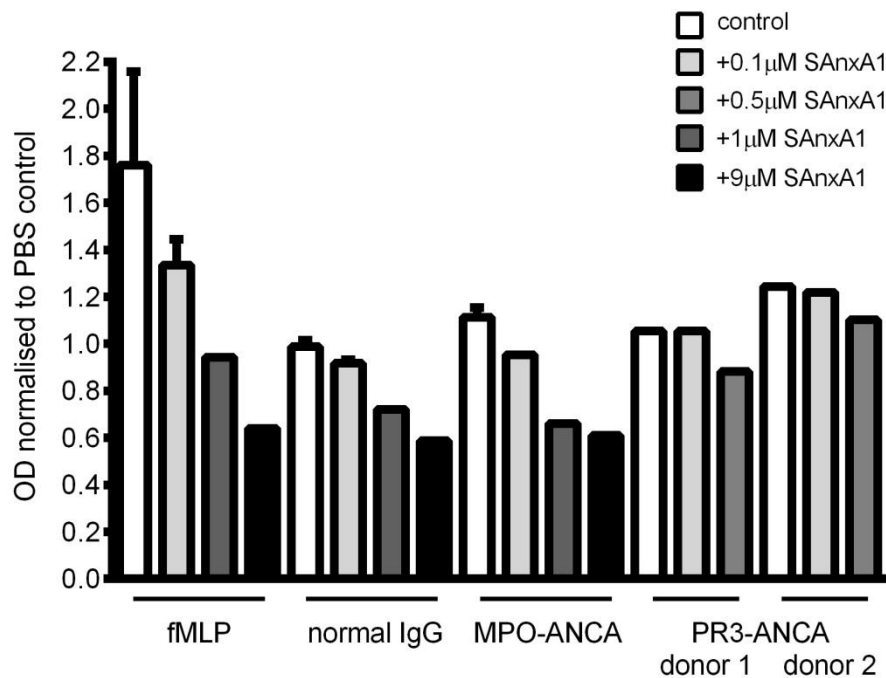
Figure 5-14 The activation of human neutrophils is inhibited in a dose-dependent manner by increasing concentrations of SuperAnnexinA1

Neutrophils were primed with 5µg/ml cytochalasin b and 2ng/ml TNF-α at 2.5 x 10^6 cells/ml, with or without SuperAnnexinA1 (SAnxA1) before stimulation with endotoxin-depleted patient or normal human IgG (0.2mg/ml). fMLP (1µM) was used as a positive control for activation. Degranulation was quantified by MPO production using a colorimetric assay and normalised to PBS controls; n=3 experiments.

5.5 Discussion

FACS analysis of neutrophil surface PR3 expression revealed a bimodal population of neutrophils in both healthy controls and PR3-positive GPA patients in remission. This was in line with previous reports of neutrophil surface PR3 expression consisting of a PR3^{high} and PR3^{low} subset (Schreiber et al., 2003, Witko-Sarsat et al., 1999). When comparing the surface PR3 expression of isolated peripheral blood neutrophils between patients and healthy controls, when neutrophils were stimulated with TNF-α, the overall neutrophil surface expression of PR3 was significantly higher in patient cells compared to healthy controls. When healthy control cells were stimulated with TNF-α, the neutrophil surface PR3 expression of the PR3^{high} subset significantly increased, although the fraction of this population decreased, leaving

the overall neutrophil surface PR3 expression unchanged. In patient cells, the neutrophil surface PR3 expression of the PR3^{low} subset significantly increased with TNF-α stimulation, leading to a trend towards an increase in overall PR3 expression that did not reach statistical significance. The ratios of PR3^{high} and PR3^{low} subsets were unchanged with TNF-α stimulation in patient neutrophils. Under control conditions there was no statistically significant difference in neutrophil surface PR3 expression between healthy controls' and patients' cells, although there was a trend towards a higher overall expression of PR3 in patient neutrophils. When examining the PR3^{high} and PR3^{low} subsets individually, this phenomenon was revealed to originate from a trend towards a higher PR3 expression in the PR3^{high} patient neutrophils. The observations reported here agree with previous accounts of a higher overall PR3 expression in GPA patients (Rarok et al., 2002, Schreiber et al., 2003) although this was only found to be significant with TNF-α stimulation in this data set. The results described here elaborate on the specific contribution of the PR3^{high} and PR3^{low} subsets to the overall neutrophil surface PR3 expression. Previous reports of neutrophil surface PR3 expression have been of the opinion that the proportions of PR3^{high} and PR3^{low} subsets are consistent over time in healthy controls and patients and do not change with *in vitro* neutrophil activation or disease activity, although an overall increase in MFI is observed when cells are activated with a combination of cytochalasin b and fMLP (Halbwachs-Mecarelli et al., 1995, Witko-Sarsat et al., 1999). The data here agree with the notion of an overall increase in neutrophil surface PR3 expression with cell stimulation, although this was only observed in patient cells and not in healthy control cells. These results also challenge the concept that the proportions of PR3^{high} and PR3^{low} subsets are predetermined. The results described here suggest that there could be a novel discrepancy between the reponse of healthy control and patient neutrophils to *ex vivo* stimulation. Monocyte expression of TNF-α is elevated in active AAV (Deguchi et al., 1990) and this could provide a physiological basis for augmented surface PR3 expression in patient neutrophils; not only providing increased antigen for ANCA-interaction but potentially supplying increased enzymatic capacity for Annexin 1 cleavage. Three quarters of the patients in this data set were receiving steroid treatment with a mean dose of 14mg/d. There is anacedotal evidence that neutrophil surface PR3 expression is not influenced by steroid treatment but may be raised in other inflammatory diseases (Schreiber et al., 2003, Witko-Sarsat et al., 1999). There was no correlation between steroid dose and total neutrophil surface PR3 expression either at baseline or after TNF-α treatment in this patient group (Appendix Figure 6-2).

The detection of Annexin 1 in neutrophil whole cell lysates by Western blotting revealed the intact protein (~37kDa) alongside two smaller cleavage products corresponding to ~30kDa and ~28kDa. Comparison of Annexin 1 cleavage between neutrophils isolated from PR3-positive GPA patients and healthy controls did not reveal any significant differences in the abundance of intact Annexin 1 or either of the smaller cleavage products, in the presence or absence of TNF-α. This is in contrast to previous data published by Vong *et al.* (2007) where an increase in Annexin 1 cleavage was detected in neutrophils with TNF-α stimulation (Vong et al., 2007). It is possible that the neutrophil isolation process and subsequent steps leading to the creation of the whole cell lysate resulted in a level of Annexin 1 cleavage that was unaffected by further TNF-α stimulation in my experiments. Neutrophils are short-lived and are ideally separated and used for *in vitro* activation assays within a 2-4hr window following blood collection. The nature of collecting and processing multiple patient samples from the clinic for FACS analysis alongside the generation of whole cell lysates resulted in some samples being processed after a delay of up to 1hr 30min, which could have led to the deterioration of the cells and potentially cleavage of Annexin 1 before *in vitro* stimulation.

The initial analysis of Annexin 1 cleavage was a ratio of intact Annexin 1 to cleaved product within each neutrophil whole cell lysate. With the total amount of neutrophil whole cell lysate avaliable for Western blotting there was insufficient protein to detect a loading control (GAPDH, β-actin etc.) for calculating total protein abundance. Therefore as each lane was loaded with the same amount of total neutrophil whole cell lysate (determined by BCA assay), the density of each intact Annexin 1 band was compared with and without TNF-α within each patient/healthy control sample to produce a ratio of intact Annexin 1. There was no difference observed between the total amounts of intact Annexin 1 when comparing unstimulated and stimulated cells, in either healthy control or patient neutrophils. There may have been a degree of variation in the amount of protein loaded in each sample, which would have been accounted for if a standard was available, which may have masked any subtle changes in total intact Annexin 1 expressed in each condition. Glucocorticoids upregulate Annexin 1 expression and so I would expect to find increased levels of total Annexin 1 protein in the steroid-treated patient group, compared to the healthy controls. Whether this increased expression of Annexin 1 would result in a disparity of cleavage between the patient and control group, or how neutrophils respond to TNF-α activation is unclear.

When associations were examined between surface PR3 expression and Annexin 1 cleavage in neutrophil whole cell lysates, there was a significant correlation between an increased abundance of PR3^{high} neutrophils and an increased proportion of cleaved Annexin 1, in TNF-α stimulated conditions, when both healthy control cells and patient cells were analysed in combination. There was also a significant correlation between an increased proportion of PR3^{high} neutrophils and a higher percentage of the smaller 28kDa peptide, when compared to the total Annexin 1 cleavage pool. This was observed in unstimulated cells when healthy control and patient cells were analysed together. Although the increased abundance of the 28kDa peptide exhibited a positive correlation with the percentage of PR3^{high} neutrophils, there was a significant negative correlation with the PR3 expression of the PR3^{high} subset, in unstimulated neutrophils from healthy controls. Taken together, the correlations observed in this data set indicate that an increased proportion of PR3^{high} neutrophils is associated with an increase in Annexin 1 cleavage *in vitro*. These results suggest that the overall abundance of the PR3^{high} subset might be of more importance than the PR3 MFI of each individual neutrophil subset in terms of association with Annexin 1 cleavage, although limited experimental group numbers may have prevented any observation of a correlation between overall neutrophil surface PR3 expression and Annexin 1 cleavage.

In vitro experiments investigating the effect of increasing concentrations of a PR3 cleavage-resistant peptide SuperAnnexinA1 (SAnxA1) in a neutrophil degranulation assay suggest that the peptide may be effective in inhibiting the activation of human neutrophils by MPO- and PR3-ANCA. This effect was also seen in the control IgG and fMLP conditions, suggesting that SAnxA1 has a general inhibitory effect on neutrophil activation, irrespective of stimulus. An inhibitory effect of SAnxA1 on neutrophil activation is consistent with reports of Annexin 1 and it's N-terminal peptide Ac2-26 impeding FcγR-mediated neutrophil and monocyte activation (Euzger et al., 1999, Goulding et al., 1998, Goulding and Guyre, 1993). Annexin 1 has been shown to have effects on the MAPK pathway and SAnxA1 may be inhibiting neutrophil degranulation through inhibition of MAPK activation (van der Veen et al., 2011, Yang et al., 2006). Unfortunately lack of avaliability of SAnxA1 prevented the generation of further experimental replicates which may have pushed the data towards statistical significance. It would be also useful to establish whether this inhibitory effect on MPO-release here was related to cell toxicity/death. Experiments were attempted to replicate the data with proteolysis-resistant N-terminal Annexin 1 peptide CR-AnxA1$_{2-50}$ but healthy control responses to ANCA

failed to exceed control IgG degranulation levels, preventing the investigation of any inhibitory effect on ANCA-induced neutrophil activation.

Taken together, these results suggest that the neutrophil surface PR3 expression is higher in PR3-positive GPA patient cells compared to healthy controls, and an increase in the PR3^{high} neutrophil fraction correlates with an increased detection of Annexin 1 cleavage products in whole cell lysates. *In vitro* experiments suggest a potential role for the PR3 cleavage-resistant peptide SAnxA1 in the general inhibition of neutrophil activation.

Summary:

- Neutrophil surface PR3 expression is significantly higher in PR3-positive GPA patient cells compared to healthy control cells when stimulated with TNF-α *in vitro*.
- There is a significant correlation between an increased PR3^{high} neutrophil fraction and an increased proportion of cleaved Annexin 1.
- It is unclear whether there are different amounts of cleaved Annexin 1 in patient neutrophils compared to healthy controls.
- The activation of human neutrophils is inhibited in a dose-dependent manner by increasing concentrations of SuperAnnexinA1 *in vitro*.

Chapter 6 Discussion

6.1 Summary of results

Chapter 3 investigated the role of Annexin 1 in a murine model of immune complex-mediated crescentic glomerulonephritis (NTN). The effect of SuperAnnexinA1 on disease was examined and attempts were initiated to establish whether any effects of glucocorticoid treatment on disease were mediated by Annexin 1. The results in this chapter demonstrated that the genetic absence of Annexin 1 resulted in significantly more severe disease in the murine model of NTN, quantified by the degree of glomerular thrombosis. There were significantly less microparticles and neutrophil-derived microparticles found in the Annexin 1 deficient mice, compared to the wild-type mice. There was no observed effect on disease severity when 1μg SuperAnnexinA1 was administered daily in the NTN model; however the infiltrating macrophages in the treatment group had significantly reduced CD11b expression. The injection of 10μg dexamethasone did not have any impact on disease in this NTN model, preventing the exploration of glucocorticoid treatment in Annexin 1 deficient mice.

Chapter 4 went on to explore the role of Annexin 1 in murine models of anti-MPO associated glomerulonephritis. The passive transfer model of anti-myeloperoxidase associated glomerulonephritis required additional injections of LPS and GCSF to generate a pauci-immune crescentic glomerulonephritis in C57BL/6 mice. When comparing wild-type and Annexin 1 deficient mice, a lack of disease penetrance across experimental groups prevented the deduction as to whether the absence of Annexin 1 had an effect on disease. Attempts to reproduce the murine model of anti-MPO associated glomerulonephritis developed by Ruth *et al.* (2006) were unsuccessful, despite the fact that additional immunodominant MPO CD4+ T-cell epitope was injected alongside the native protein to encourage the breaking of immune tolerance.

Chapter 5 addressed the relationship between human neutrophil surface expression of PR3 and the degree of Annexin 1 cleavage; in neutrophils isolated from the peripheral blood of healthy controls and PR3-positive GPA patients in remission. In addition, the effect of a PR3 cleavage-resistant peptide (SuperAnnexinA1) on ANCA-induced neutrophil activation was investigated. The results in this chapter demonstrated that neutrophil surface PR3 expression is significantly higher in patient cells compared to healthy control cells when stimulated with TNF-α *in vitro*

and there is a significant correlation between an increased abundance of PR3^{high} neutrophils and the detection of cleaved Annexin 1, in healthy control and patient cells, in TNF-α stimulated conditions. It was unclear from the data whether there are intrinsic differences in the amount of Annexin 1 cleavage in healthy control and patient neutrophils. *In vitro* experiments examining the activation of healthy human neutrophils by patient ANCA suggested a dose-dependent inhibition of degranulation responses by SuperAnnexinA1.

6.2 Hypothesis for the role of Annexin 1 in human and experimental glomerulonephritis

Annexin 1 is an endogenous inhibitor of inflammation, preventing inappropriate neutrophil extravasation into the tissues. When inflammatory stimuli are present Annexin 1 levels increase under glucocorticoid hormone control, allowing the protein to function as a pro-resolution mediator; promoting the apoptosis and efferocytosis of neutrophils, accelerating tissue repair and allowing the tissue to return to a homeostatic state.

Annexin 1 plays a protective role in a number of murine models of acute and chronic inflammation, through the inhibition of innate and adaptive immune responses. The data in this thesis indicate that Annexin 1 deficient mice are more susceptible to disease in a mouse model of immune complex-mediated crescentic glomerulonephritis (NTN) and the PR3-cleavage resistant Annexin 1 peptide SuperAnnexinA1 has an anti-inflammatory effect on the phenotype of infiltrating macrophages in this model. Experiments addressing the role of Annexin 1 deficiency in a murine model of anti-MPO associated glomerulonephritis were inconclusive, and the lack of disease penetrance may have masked the effect of Annexin 1. The pathogenesis of AAV involves important contributions from both the innate and adaptive immune responses. The two murine models used here address separate aspects of the immune response in the development of crescentic glomerulonephritis; the NTN model is Th1-driven whereas this passive-transfer model of anti-MPO associated glomerulonephritis relies primarily on the innate immune response to initiate an inflammatory response.

PR3 cleaves Annexin 1, preventing interaction with its receptor FPR2/ALXR. The results in this thesis demonstrate that PR3 expression is significantly higher in neutrophils from PR3-positive GPA patients in remission compared to healthy controls when stimulated with TNF-α. Also, a higher neutrophil PR3 expression

correlates with increased Annexin 1 cleavage in healthy control and patient cells, in resting and TNF-α stimulated conditions. These results agree with previously published data indicating a higher neutrophil expression of PR3 in patient neutrophils and reveal a novel association between neutrophil PR3 expression and Annexin 1 cleavage in an *ex vivo* environment. Neutrophil infiltration is a fundamental component of disease pathogenesis in AAV and a high expression of PR3 on the neutrophil surface may not only act as an auto-antigen perpetuating the immune response to ANCA, but could lead to excessive cleavage of Annexin 1, preventing the proteins' anti-inflammatory actions. The finding that neutrophil PR3 expression is heightened in remission patients compared to healthy controls suggests a propensity for an inflammatory phenotype in these individuals which could contribute to the relapsing disease course often seen in PR3-positive GPA patients. The results in this thesis suggest that patient neutrophil PR3 expression might increase with cell activation. AAV patients with active disease display increased levels of TNF-α in peripheral blood mononuclear cells and anti-TNF inhibitors have shown some limited success in clinical trials (Deguchi et al., 1990, Morgan et al., 2011, Laurino et al., 2010). This heightened cytokine response in patients with active disease could contribute to an increase in neutrophil PR3 expression and associated Annexin 1 cleavage, contributing to the chronic, non-resolving phenotype characteristic of AAV.

Annexin 1 peptidomimetics have been shown to exert a number of anti-inflammatory effects *in vitro* and in *in vivo* models of inflammation. The results presented in this thesis demonstrate that SuperAnnexinA1 may be effective in inhibiting the activation of human neutrophils by MPO- and PR3-ANCA. This hypothesis would agree with reports that Annexin 1 and its N-terminal peptide Ac2-26 impede FcγR-mediated neutrophil and monocyte activation (Euzger et al., 1999, Goulding et al., 1998, Goulding and Guyre, 1993) and provides insight into the ways in which Annexin 1 peptidomimetics or FPR2/ALXR agonists could act as potential therapeutics in AAV.

There are number of ways in which Annexin 1 deficiencies could potentially contribute to the pathology of AAV (see Figure 6-1).
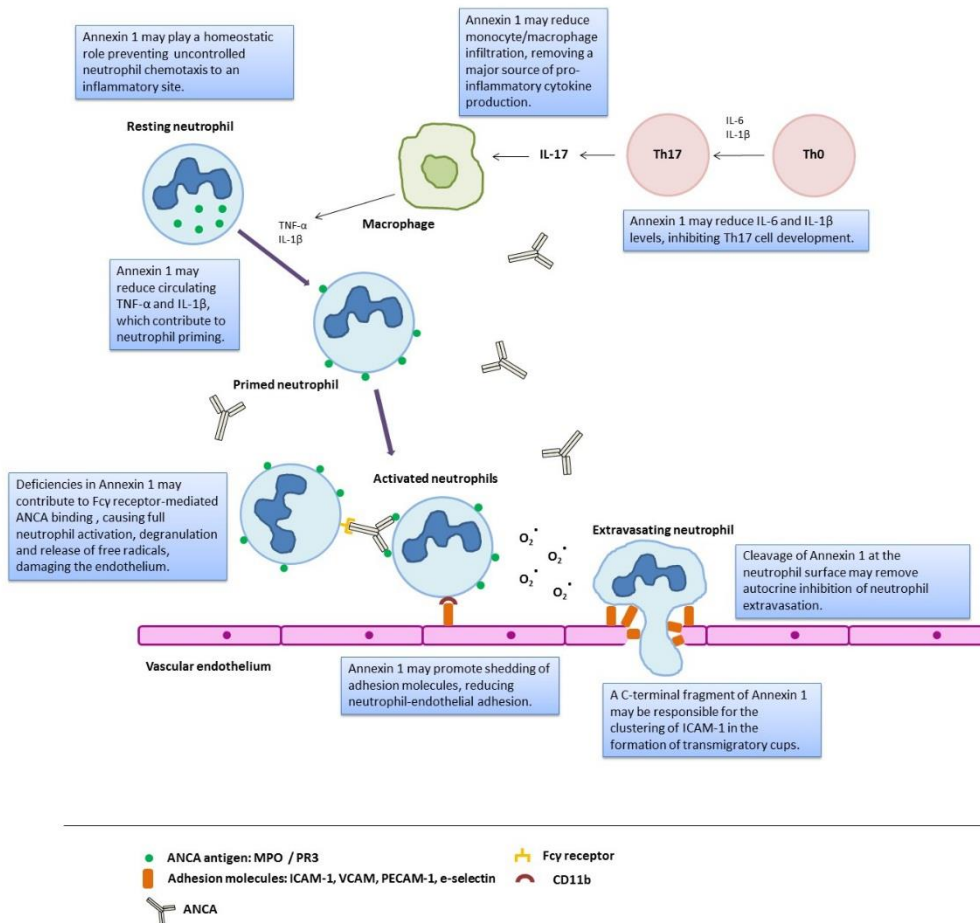
Figure 6-1 Potential influence of Annexin 1 on the innate and adaptive immune mechanisms involved in the pathogenesis of AAV

Annexin 1 may reduce IL-6 and IL-1β levels, inhibiting Th17 development and the contribution of this T cell subset to disease. Annexin 1 could reduce monocyte/macrophage infiltration, removing a major source of proinflammatory cytokine production. Annexin 1 may play a homeostatic role preventing uncontrolled neutrophil chemotaxis to the inflammatory site. Annexin 1 may reduce circulating TNF-α and IL-1β, which contribute to neutrophil priming and the externalisation of ANCA antigens. Deficiencies in Annexin 1 could contribute to Fcγ receptor mediated ANCA binding, causing full neutrophil activation, degranulation and release of free radicals, damaging the endothelium. Annexin 1 may promote shedding of adhesion molecules, reducing neutrophil-endothelial adhesion and subsequent damage to the endothelium. A C-terminal fragment of Annexin 1 may be responsible for the clustering of ICAM-1 in the formation of transmigratory cups. Cleavage of Annexin 1 at the neutrophil surface may remove the autocrine inhibition of neutrophil extravasation.

Neutrophil degranulation is a key pathological insult in AAV and MPO-expressing neutrophils are essential for the development of glomerulonephritis in mice. Increased cleavage of Annexin 1 in AAV could be a contributing factor to massive neutrophil influxes during acute disease due to the loss of Annexin 1 as a homeostatic mediator of neutrophil diapedesis (Schreiber et al., 2006). Annexin 1 may also play a role in in inhibition of PI3Kγ activation which is involved in neutrophil activation and shown to play an essential role in the development of anti-MPO induced NCGN in mice (Schreiber et al., 2010).

Research has demonstrated that Annexin 1 is upregulated in extravasated neutrophils and could play a role in promoting apoptosis of these cells, rather than allowing them to progress to a necrotic state. Focal segmental necrotising glomerulonephritis is a key feature of AAV and if there is a dysregulation of apoptosis in AAV, caused by increased cleavage of Annexin 1 by PR3, this could be contributing to the necrotising phenotype.

As mentioned above, Annexin 1 has been shown to inhibit binding of IgG to Fcγ receptors on peripheral blood monocytes and neutrophils *in vitro* through a calcium-dependent mechanism. Taken together with *ex vivo* evidence that neutrophil FcγR-mediated respiratory burst can be inhibited by treatment with the Annexin 1 N-terminal peptide Ac2-26, it is possible that Annexin 1 prevents Fcγ-mediated signalling through the MAPK pathway to prevent neutrophil activation (Goulding et al., 1998, Goulding and Guyre, 1993, van der Veen et al., 2011). If this is in fact the case *in vivo*, then Annexin 1 deficiencies in AAV could be contributing to ANCA binding to Fcγ receptors on neutrophils, leading to their activation and the subsequent inflammatory cascade.

Annexin 1 can augment IL-10 production *in vitro* and in *in vivo* models of inflammation (Cooray et al., 2013, Guido et al., 2013). IL-10 competency is diminished in AAV patients during remission and active disease and a lack of Bregs secreting IL-10 may predispose a Th1 immune response (Todd et al., 2014, Wilde et al., 2013). IL-10 can also reduce T cell expression of IFN-γ *in vitro* via IL-12 inhibition (Ludviksson et al., 1998). Excessive Annexin 1 cleavage in AAV may therefore reduce IL-10 production and contribute to disease pathogenesis.

IL-1β has an important role in immune and inflammatory responses and PR3 is known to contribute to the serine-protease dependent processing of IL-1β from its

inactive to active form (Schreiber et al., 2012). Although a direct pathogenic role for IL-1β in AAV has not been identified and IL-1β serum levels do not differ in patients with AAV when compared to healthy controls, patients with active disease have an increased number of IL-1β-expressing macrophages infiltrating the glomerulus when compared with patients with inactive disease (Nogueira et al., 2010, Noronha et al., 1993). Also, mice lacking dipeptidyl peptidase I (DPPI) which is required for PR3 activation and subsequent IL-1β generation, are protected from anti-MPO induced necrotising crescentic glomerulonephritis (Schreiber et al., 2012). In terms of its pathophysiological effects, IL-1β can enhance leukocyte rolling, adhesion and migration in a mouse model when given in combination with ANCA, possibly through the upregulation of adhesion molecule expression (Myers et al., 1992, Nolan et al., 2008). IL-1β may also contribute to Th17 cell differentiation and has thus been mentioned as a possible therapeutic target in AAV (Chung et al., 2009). In terms of Annexin 1 and IL-1β, murine models of antigen-induced arthritis and zymosan peritonitis have revealed increased levels of IL-1β in Annexin 1 deficient mice at the inflammatory site, an effect which can be rescued by administration of peptide Ac2-26 in the zymosan peritonitis model (Damazo et al., 2006, Yang et al., 2004). However, full-length Annexin 1 does not reverse IL-1β induced neutrophil infiltration into a murine air pouch (Perretti and Flower, 1993). Increased levels of PR3 in AAV patients could contribute to the generation of, and pathological effects of IL-1β in disease, in addition to reducing levels of anti-inflammatory Annexin 1, meaning that targeting Annexin 1 could be beneficial in terms of IL-1β involvement in disease.

A pathogenic link between IL-6 and AAV has not been proven, but as ANCA stimulate the release of IL-6 from B cells, driving the differentiation of Th0 to Th17 cells, this cytokine no doubt has an important role in disease. Interestingly, an IL-6 inhibitor has shown anecdotal success in the treatment of RA complicated with AAV (Sumida et al., 2011). Linking the documented effects of Annexin 1 on the MAPK pathway, a p38 MAPK inhibitor has been shown to reduce IL-6 production by glomerular epithelial cells, raising a potential role for Annexin 1 to influence not just the infiltrating cells but the microenvironment of the kidney glomerulus (van der Veen et al., 2011). In addition, in a comparison of diabetic patients with and without nephropathy, Annexin 1 levels are raised in the patients with nephropathy and positively correlate with IL-6 levels, suggesting a counter-regulatory mechanism for Annexin 1 on IL-6 levels (Pietrani et al., 2014).

Expression of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has recently been shown to associate with a number of inflammatory states and autoimmune diseases (Reviewed in (Asare et al., 2013)) and increased levels of MIF have been found to correlate with disease activity in AAV (Becker et al., 2006, Ohwatari et al., 2001). MIF has been found to counteract some of the anti-inflammatory effects of GCs, including the expression of MAPK phosphatase 1 (MKP-1) (Roger et al., 2005) and the regulation of phospholipase A_2 activity. It has been suggested that endogenous MIF limits Annexin 1 expression and MIF can counteract the GC-dependent expression of Annexin 1 (Roger et al., 2005, Sun et al., 2013). In a mouse model of AIA, levels of IL-1β, TNFα, IL-6, and MIF were all found to be increased in Annexin 1 deficient mice when compared to their wild-type counterparts (Yang et al., 2004). Increased levels of MIF in active AAV could contribute to deficiencies in Annexin 1 caused by PR3 cleavage, making Annexin 1 an even more interesting therapeutic target.

Endogenous and exogenously-administered GCs have an established role in regulating Annexin 1 levels; glucocorticoids prevent generation of proteolytic enzymes which cleave Annexin 1 as well as increasing the production of Annexin 1 and its receptor in cells of the human and mouse innate immune system and mobilizing Annexin 1 expression at the cell surface, enabling interaction with its receptor (Damazo et al., 2006, Ehrchen et al., 2007, Goulding et al., 1990, Hashimoto et al., 2007, Sawmynaden and Perretti, 2006). As well as regulating the innate immune response through Annexin 1, Perretti and D'Acquisto suggest that glucocorticoids may regulate the adaptive arm of the immune response by suppressing Annexin 1 expression, leading to reductions in T cell proliferation and activation (Perretti and D'Acquisto, 2009). Persistent T cell activation is characteristic in AAV, in active disease as well as in remission (Abdulahad et al., 2006). GCs are used in AAV to induce and maintain disease remission and a number of these anti-inflammatory effects could be mediated through Annexin 1. As there is possible dysregulation of aspects of both the innate and adaptive immune system in AAV, direct manipulation of Annexin 1 could be therapeutically beneficial across different phases of disease and could avoid adverse side effects associated with GC treatment.

6.2.1 Potential for the use of pro-resolving therapeutics in AAV

Current treatment approaches in AAV are associated with adverse side effects due to non-specific immunosuppression while achieving sustained remission is a

challenge in a significant proportion of patients. Resolution pharmacology is an emerging concept in the treatment of chronic inflammatory diseases as a mechanism to actively augment resolution processes in conjunction with the suppression of inappropriate inflammatory responses. Evidence is accumulating for the efficacy of pro-resolving mediators in treating pathologies such as asthma, polycystic kidney disease and psoriatic arthritis (for a list of clinical trials see (Perretti et al., 2015)).

Early studies utilising the N-terminal fragment of Annexin 1 (Ac2-26) in *in vivo* models of inflammation demonstrated anti-inflammatory effects (Getting et al., 1997, Perretti et al., 1993) and subsequent work has revealed the ability of Annexin 1 cleavage-resistant peptides to act as pro-resolution mediators in several models of acute and chronic inflammation (Dalli et al., 2013, Patel et al., 2012). These newly developed cleavage-resistant peptides have longer sequences and higher affinity for FPR2/ALXR than the Annexin 1 N-terminal peptides and can therefore exert more potent and sustained effects *in vivo* (Perretti and Dalli, 2009). Recently, in a mouse model of RA, the stimulation of FPR2/ALXR has also been shown to be of therapeutic benefit and inhibits TNF-α mRNA expression in the joint (Kao et al., 2014).

Pro-resolving therapeutics might encompass potentiating endogenous pro-resolving pathways, the development of new agonists designed on pro-resolving mediators or mimetics of endogenous resolution mediators. There is exciting *in vivo* evidence for Annexin 1 peptidomimetics providing therapeutic benefit when delivered within targeted nanoparticles in experimental models of disease (Fredman et al., 2015, Headland et al., 2015, Leoni et al., 2015), endorsing therapeutic prospects for this type of peptide in chronic inflammatory diseases such as AAV.

6.3 Thesis limitations

The conclusions drawn from the *in* vitro investigations in this thesis are limited due to the nature of the experiments. These experiments allow the examination of direct links between neutrophil surface PR3 expression and Annexin 1 cleavage but are constrained by the absence of other cells which may influence either factor in the physiological environment. It is possible that neutrophil surface PR3 expression and Annexin 1 cleavage are altered during the neutrophil isolation process due to a degree of neutrophil activation and it is conceivable that the amount Annexin 1 cleavage reached a maximum before cells were stimulated, masking any effect of *in*

vitro stimulation on Annexin 1 cleavage. The method of PMN isolation use for these studies would have excluded low-density granulocytes (LDGs) which co-localize with PBMCs in the separation gradient. LDGs have not yet been proven to exist in the blood of AAV patients but could play an important pro-inflammatory role in disease pathogenesis (Grayson et al., 2015). The analysis of Annexin 1 protein on Western blot was unable to encompass a loading control for calculating total protein so transcriptional/translational changes in Annexin 1 were not addressed and compared between patients and healthy controls.

6.4   Limitations of the animal models used

The use of animal models has been essential in delineating the pathology of AAV and has led to the development of novel therapeutics such as C5aR and TNF-α inhibitors. Alternative models are useful for defining separate aspects of the immune response, although do not address them all together. NTN is a straightforward model of crescentic glomerulonephritis; however the disease develops in the presence of immune-complexes unlike the pauci-immune nature of human AAV. In this model of NTN there were few crescentic glomeruli observed on histology, therefore disease was scored on glomerular thrombosis, in contrast with the fibrinoid necrosis seen in the human disease. The dose of SuperAnnexinA1 administered in NTN was based on previously published data reporting the acceleration of the resolution of inflammation in a model of inflammatory arthritis (Patel et al., 2012). Although the peptide did not affect disease severity in NTN, there was no physiological readout to demonstrate that this dose of SuperAnnexinA1 had entered the circulation or was having any systemic effect.

The passive-transfer model of MPO-associated glomerulonephritis was used to address the effect of the absence of Annexin 1 on the innate immune contribution to disease. This model is useful in examining the acute stage of disease, but due to the lack of continuing antibody production is not suitable for studying the chronic phase of disease. This disease model is labour-intensive in terms of generating MPO to inject MPO deficient mice and subsequently isolating IgG to immunise experimental animals. There were challenges in isolating good quality MPO from the mouse promyelocytic cell line and inducing an antibody response in MPO deficient animals. There were also difficulties purifying IgG from MPO deficient serum and stimulating an immune response in recipient mice. The MEV model is notoriously variable in terms of disease penetrance and large animal groups are needed to reveal true differences between experimental groups. The passive transfer model described in

this thesis required additional injections of LPS and GCSF to generate disease, which was mild in severity compared to previously published data. Due to a lack of available anti-MPO IgG, experimental groups were small and data was combined across two experiments where the same reagents were used to generate disease. There was a suggestion from the results that Annexin 1 deficient mice might be protected from disease, however this may have resulted from a lack of disease penetrance in the second experiment, incorporating the Annexin 1 deficient mice, falling short of the first experiment. More animals would be needed in each group to define a true effect.

6.5 Further questions to address

Annexin 1 cleavage in AAV

The results in this thesis revealed a skewing towards the detection of more Annexin 1 cleavage products in healthy control neutrophils compared to patient cells. This was an unexpected result and it would be informative to add more samples to the analysis to observe if there is a true disparity between the two groups. It would be interesting to follow Annexin 1 cleavage over the disease time course and compare patients with active disease to those in remission, as well as adding in a disease control group to confirm whether Annexin 1 cleavage is a general feature of inflammation or is specifically altered in AAV patients.

Human leukocyte elastase (HLE) also cleaves Annexin 1 by proteolysis and shares a cleavage site with PR3. Interestingly, both PR3 and HLE are externalised on neutrophil activation and antibodies to both proteins are found in AAV. The relevance of this is unclear, and it would be interesting to investigate whether elastase levels in neutrophils from AAV patients are also atypical and correlate with Annexin 1 cleavage.

Total Annexin 1 expression

The experiments in this thesis addressed ratios of intact to cleaved Annexin 1, but were unable to quantify total amounts of Annexin 1 in neutrophil whole cell lysates. It would be interesting to investigate whether total amounts of Annexin 1 differ between healthy controls and patients and whether total amounts of Annexin 1 change between remission and active disease states, indicating control at a transcriptional or translational level.

Annexin 1 cleavage-resistant peptides

The results described here suggest that ANCA-induced neutrophil degranulation may be inhibited by SuperAnnexinA1, however the neutrophil degranulation response to ANCA in these experiments was minimal compared to the background response to normal IgG. Compared to degranulation, intracellular ROS production is a more sensitive way of monitoring ANCA-induced activation and could provide a more robust way of measuring an inhibitory effect of cleavage-resistant Annexin 1 peptides on ANCA-induced neutrophil activation.

Nephrotoxic nephritis model

The results in this thesis demonstrated a significant exacerbation of NTN in Annexin 1 deficient animals compared to wild-type controls, but the mechanism of this protection by Annexin 1 was not apparent. It would be interesting to expand on the early time-point data to establish any effects of Annexin 1 deficiency on neutrophil infiltration early in disease and investigate Th1 responses which may have been augmented. The treatment of mice with 1μg SuperAnnexinA1 did not affect disease in this NTN model. Ultimately it would be interesting to see whether the administration of SuperAnnexinA1 could rescue the Annexin 1 deficient phenotype, providing a rationale for Annexin 1 peptidomimetics in the resolution of vascular injury.

MEV models

There were many challenges in attempting to establish MEV models for this work. It would be useful to develop on the passive transfer model of anti-MPO associated glomerulonephritis described here, adding animals to the experimental groups to observe any true effects of Annexin 1 deficiency on disease. If there was a significant effect on disease associated with the absence of Annexin 1 it would be interesting to explore whether this is due to the modulation of neutrophil trafficking in the early stage of disease. Use of alternative MEV models could provide insight into where Annexin 1 may play a role in different parts of the immune response in AAV; comparing the DTH-like injury model in wild-type and Annexin 1 animals would be informative on the effect of Annexin 1 in the adaptive immune component of disease and the BM transplant model could be utilised to determine whether Annexin 1 expression is important in BM-derived cells or intrinsic renal cells. Both the DTH-like

injury model and the BM transplant model would also be useful in examining the importance of Annexin 1 in the chronic phase of disease as antibody levels are sustained over the experimental timecourse. Once established, these models could be used to investigate the therapeutic effects of cleavage-resistant Annexin 1 apeptides on disease. It would be interesting to measure TNF-α levels in the serum and mRNA in the kidney to explore whether these change with treatment as this cytokine is relevant to AAV and has been shown to change with the presence and absence of Annexin 1.

Establishing renal expression of Annexin 1 and FPR2/AXR is important in establishing if potential therapeutics might act locally in AAV and could be achieved with immunostaining of mouse and human kidney.

6.6 Concluding remarks

The results in this thesis indicate that neutrophil surface PR3 expression is significantly higher in PR3-positive GPA patient cells compared to healthy control cells when stimulated *in vitro* and reveal a novel correlation between an increased surface PR3 expression and the detection of Annexin 1 cleavage products in neutrophil whole cell lysates.

In vivo, the genetic absence of Annexin 1 is demonstrated to result in significantly more severe disease in a murine model of immune complex-mediated crescentic glomerulonephritis, however the effect of Annexin 1 deficiency on disease in a murine model of anti-myeloperoxidase associated glomerulonephritis was inconclusive.

The data presented here demonstrate that a PR3 cleavage-resistant Annexin 1 peptide can skew infiltrating macrophages towards a less inflammatory phenotype in an *in vivo* model of crescentic glomerulonephritis and might inhibit neutrophil activation *in vitro*.

Annexin 1 has recently emerged as a key mediator in the active process of the resolution of inflammation. The protein acts to limit neutrophil trafficking to inflammatory sites, promote their apoptosis and nonphlogistic clearance and can polarize macrophages to a pro-resolving phenotype. The data in this thesis demonstrate that Annexin 1 deficiencies could play an important role in the

pathogenesis of AAV and pro-resolving therapeutics based on Annexin 1 may have potential in the treatment of this chronic disease.

This thesis is all original work. Where others have contributed it has been acknowledged.

Appendix
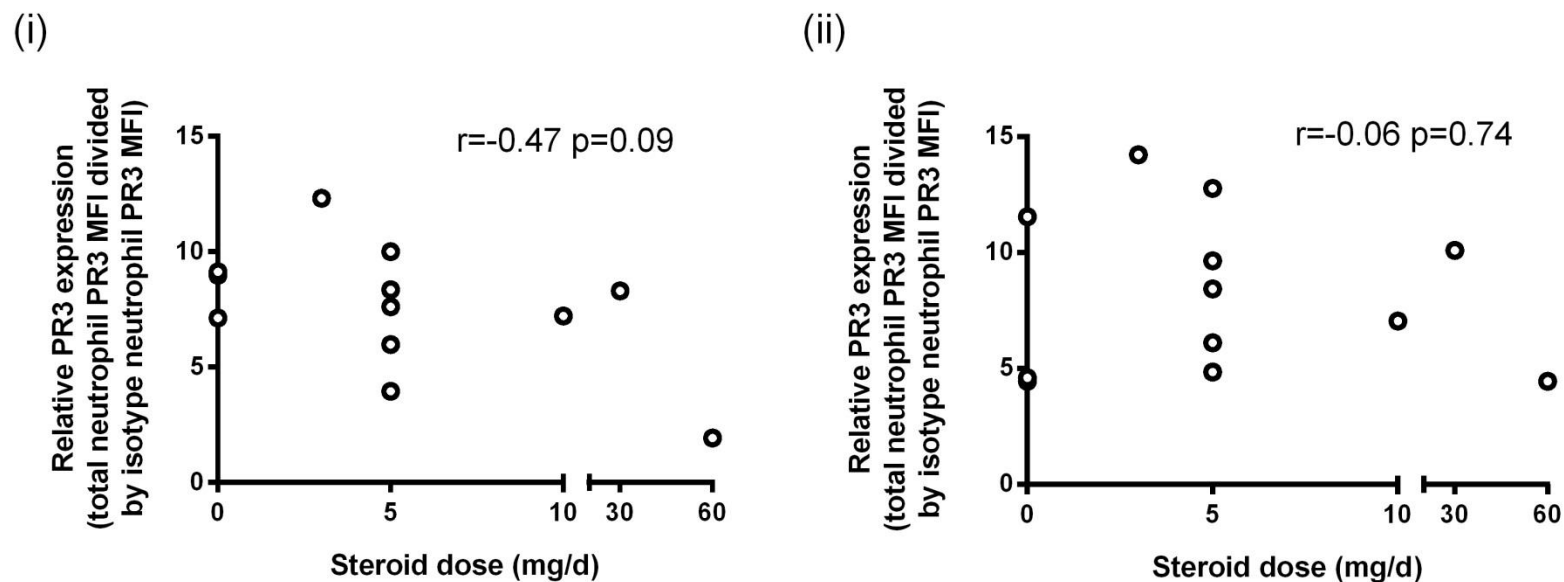
Figure 6-2 Correlation of relative neutrophil mPR3 expression and steroid dose in PR3-positive GPA patients

Neutrophils stimulated with/without TNF-α (10ng/ml) for 30 minutes at 37°C were incubated with anti-PR3 FITC antibody or isotype control. The MFI of the PR3-stained neutrophil population as a whole, was divided by the MFI of the equivalent population stained with the isotype control antibody to produce a value for relative mPR3 expression. There was no significant correlation observed between relative neutrophil PR3 expression and patient steroid dose either under unstimulated conditions (i) or with TNF-α treatment (ii).
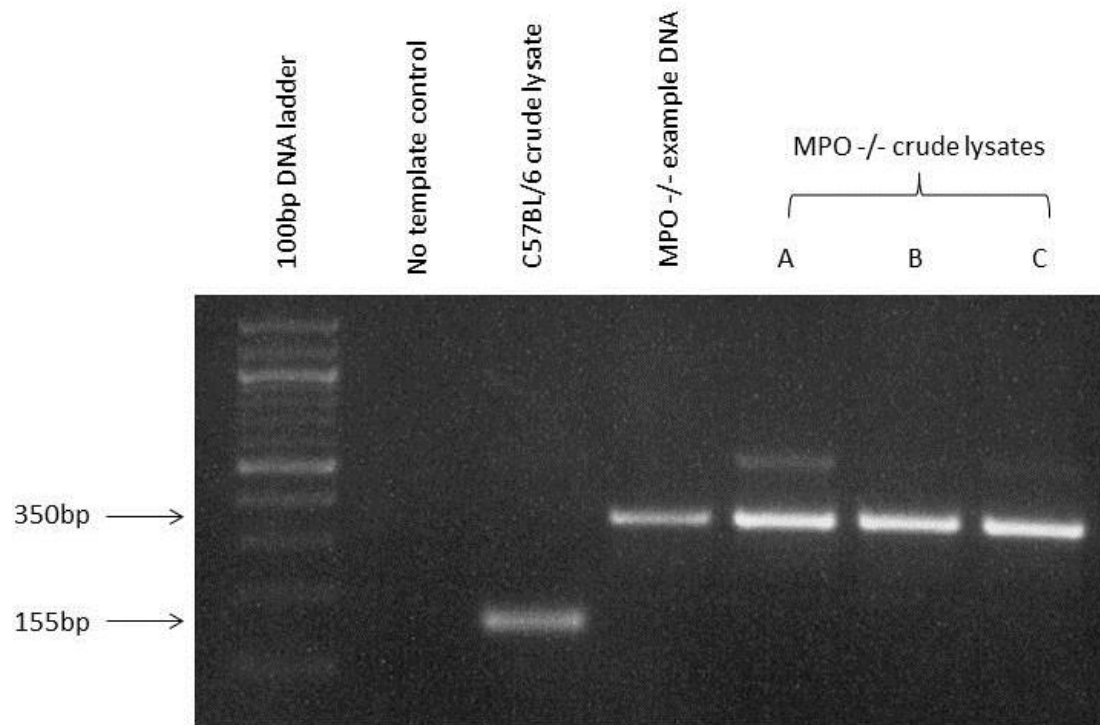
Figure 6-3 MPO-/- genotyping

Genomic DNA was obtained from the digestion of ear clips overnight in 100μl DNA lysis buffer at 55°C with 100μg/ml proteinase K. Samples were then diluted with 300μl of distilled water and heat inactivated for 15 minutes at 75°C. PCR was carried out using using REDTaq® ReadyMix™ PCR Reaction Mix with MgCl$_2$ (Sigma) and 2μl of the crude lysate template, using the following primers: MPO1 (5' TGA-CAC-CTG-CTC-AGC-TGA-AT 3'), MPO2 (5' TGC-AGG-CAG-CTG-GTC-TCG-CA- 3'), MPO3 (5' CTA-CCG-GTG-GAT-GTG-GAA-TGT- 3'). PCR products were analysed by gel electrophoresis on a 2% agarose gel with 1μl ethidium bromide (10mg/ml) per 50ml volume. Gels were run at 100V for 30 minutes and visualised using a UV light source.

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