Heterogeneity of injury in vasculitis; influence of anti neutrophil cytoplasm antibody IgG subclass and endothelial susceptibility.

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ABSTRACT

This study aimed to examine the pathogenic role of IgG subclass in ANCA associated vasculitis (AAV). Additionally, since AAV targets specific vascular beds, the concept was explored as to whether the phenotype of renal endothelial cells predisposed them to injury.

Using the flow model, interaction of neutrophils with normal immunoglobulin subclasses was compared to interaction with subclasses of ANCA IgG. Neutrophils were captured by normal IgG3, less by IgG1 and minimally by IgG2 and IgG4 when neutrophils were flowed across immunoglobulins that were bound to a solid-phase support. Blockade of CD32 was important in IgG3 interactions but CD16 more important in IgG1 and 2. When neutrophils were exposed to soluble ANCA IgG and then flowed across cytokine-activated endothelial cells, ANCA subclasses IgG1 and IgG3 activated neutrophils, causing them to adhere to endothelial cells, as did IgG4, which was surprising since IgG4 is not reported to bind to CD16 and CD32 receptors that are constitutively expressed by neutrophils. Blockade of CD32 and CD16 reduced binding of IgG3 but only CD16 was important in IgG1. Neutrophil activation by ANCA and subsequent change in behaviour from rolling to static adhesion is therefore Fc receptor dependent. IgG3 ANCA is more potent than IgG1 which is more potent than IgG4. These findings may be important in therapeutic design as targeting specific immunoglobulin subclass may enable targeting of therapies rather than blanket immunosuppression.

Glomerular microvascular endothelial cells (GEC) were compared with human umbilical vein endothelial cells (HUVEC), the umbilical vein being a large conduit vessel. VCAM-1 was reduced on GEC and in functional studies using the flow model, GEC demonstrated reduced leukocyte capture, although this was still VCAM-1 dependent. While VCAM-1 protein was detectable in both cell types (Western Blotting), it was concluded that either the isoform or post-translational modification reduces cell surface expression in GEC. RNA array analysis demonstrated similar VCAM-1 gene expression but a reduction in the ST6N gene in GEC responsible for post translational modification of the VCAM-1 protein to a sialoglycoprotein. These studies demonstrate many similarities between these endothelia but glomerular cells also display some unexpected properties, particularly low expressed levels of VCAM-1. If these findings translate *in vivo*, reduced VCAM-1 expression by glomerular endothelial cells may be a protective mechanism to reduce inflammatory responses, potentially disrupted in disease.

ANCA subclass and endothelial phenotype are important in the AAV pathogenesis: this may be useful in designing new therapies where targeted therapy reduce the need for overall immunsuppressive load. Additionally modification of specific adhesion molecule profiles on endothelial cells may enable alteration of conditions of one vascular bed whilst reducing impact on unaffected sites.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AAV	ANCA associated vasculitis
AECA	Anti endothelial cell antibodies
ANCA	anti-neutrophil cytoplasm antibody
cANCA	Cytoplasmic anti neutrophil cytoplasm antibody
CD64/32/16	Cluster of differentiation 64/32/16
CXCL	Chemokine ligand
EC	Endothelial cell
Fab	Fragment antibody binding
Fc	Fragment crystalline
FcγR	Fc gamma receptor
GEC1	Glomerular endothelial cell line 1 (immortalised)
GEC2	Glomerular endothelial cell line 2 (immortalised)
HKEC	Human kidney endothelial cell
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon gamma
IgG	immunoglobulin
IL-1β	Interleukin 1 beta
JAM	Junctional adhesion molecule
LPS	Lipopolysacchride
MPA	Microscopic Polyangiitis
MPO	Myeloperoxidase

MPO	myeloperoxidase
mRNA	Messenger ribonucleic acid
pANCA	Perinuclear anti neutrophil cytoplasm antibody
PGEC	Primary glomerular endothelial cell
PR3	Proteinase-3
PR3	proteinase-3
PTU	Propylthiouricil
TGFβ	Tumour necrosis factor beta
TNFα	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
vWF	von Willebrand Factor
WG	Wegener's Granulomatosis

1. WORK ARISING FROM THIS THESIS

1.1 Papers

- Pankhurst T, Nash G, Williams J, Coleman R, Hussain A, Savage C. Immunoglobulin subclass determines the ability of IgG to capture and activate neutrophils whether presented as normal human IgG or disease associated ANCA-IgG. *Manuscript in preparation for submission to Arthritis and Rheumatism.*

- Pankhurst T, Nash G, Williams J, Morgan M, Satchell S, Mathieson P, Kissane S, Salmon M, Savage C. Glomerular endothelial cells do not express high levels of VCAM-1 and capture peripheral blood lymphocytes with low efficiency. *Manuscript in preparation for submission to Kidney International.*

- Colman R, Hussain A, Goodall M, Young S, Pankhurst T, Lu X, Jefferis R, Savage C, Williams J. Chimeric antibodies in proteinase-3 of IgG1 and IgG3 subclass induce difference magnitudes of functional response in neutrophils. *Ann Rhem Dis published online 4 Jan 2007*

- Hussain A, Pankhurst T, Goodall M, Colman R, Jefferis R, Savage C, Williams J. Chimeric IgG4 PR3-ANCA induces selective inflammatory responses from neutrophils through engagement of Fc gamma receptors. 2008. *Immunology. In press*

1.2　Abstracts

- Pankhurst T, Nash G, Williams J, Coleman R, Hussain a, Savage COS. Is IgG Subclass of ANCA important in its pathogenicity? Medical Research Society February 2009. P73

- Pankhurst T, Nash G, Williams J, Coleman R, Hussain a, Savage COS. Is IgG Subclass of ANCA important in its pathogenicity? The British Microcirculation Meeting, March 2009. PC18

- Pankhurst T, Nash G, Williams J, Morgan M, Satchell S, Mathieson P, Savage COS. Glomerular endothelium expresses low levels of VCAM-1 that supports minimal lymphocyte binding. The British Microcirculation Meeting, March 2009. PC19

- Pankhurst T, Nash G, Williams J, Coleman R, Hussain a, Savage COS. Influence of IgG subclass on neutrophil adhesive behaviour and relevance to anti-neutrophil cytoplasm antibody (ANCA) Renal Association, April 2009. P184

- Pankhurst T, Nash G, Williams J, Morgan M, Satchell S, Mathieson P, Savage COS. Glomerular endothelium expresses low levels of VCAM-1 that supports minimal lymphocyte binding. Renal Association, April 2009. P191

- Pankhurst T, Nash G, Williams J, Coleman R, Hussain A, Savage COS. Influence of IgG subclass on neutrophil adhesive behaviour and relevance to anti-neutrophil cytoplasm antibody (ANCA) associated vasculitis. XIVth international Vasculitis and ANCA Workshop. June 2009. Oral presentation J6

- Pankhurst T, Nash G, Williams J, Morgan M, Satchell S, Mathieson P, Savage COS. Glomerular endothelium expresses very low levels of VCAM-1 that supports minimal lymphocyte binding. XIVth international Vasculitis and ANCA Workshop. June 2009. J5

1.3 Reviews

- Pankhurst T, Savage COS, Little MA. Leukocyte-endothelial dysregulation in systemic small vessel vasculitis. Nephrology. Jan 2009. Epub ahead of print

- Pankhurst T, Savage COS. Pathogenic Role of anti-nuclear cytoplasmic antibodies (ANCA) in vasculitis. Current Opinion in Pharmacology. 2006 April: 6 (2):190-196

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2. INTRODUCTION

2.1 ANCA associated vasculitis

Vasculitis is disease causing inflammation of blood vessel walls. There are a spectrum of vasculitides depending on the site and size of vessel affected and they differ in their pathologies.

In this thesis the two small vessel vasculitides associated with anti-neutrophil cytoplasmic antibody (ANCA), Wegener's Granulomatosis and Microscopic Polyangiitis, (termed ANCA-associated vasculitis (AAV)), have been studied. These frequently result in pulmonary haemorrhage and renal failure with subsequent morbidity and mortality. Current therapies based on high dose corticosteroids and cyclophosphamide are highly toxic and non-specific and in themselves cause considerable morbidity. Even with current treatments the risk of disease relapse remains high. In better understanding the pathogenesis of these diseases, more specific treatment could be designed.

These diseases are autoimmune in nature, although the exact pathogenesis remains unclear. Understanding pathogenesis has implications for other autoimmune and inflammatory diseases.

AAV is an autoimmune disease and antibodies (ANCA) directed against the neutrophil enzymes proteinase-3 (PR3) and myeloperoxidase (MPO) are found in the serum of most affected patients. One currently accepted paradigm of pathogenesis is that neutrophils at various sites, including the glomerulus, are activated by ANCA in

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the presence of inflammatory cytokines and degranulate, damaging the endothelium and setting up a cascade of inflammation drawing macrophages and other inflammatory cells into the glomerulus, leading to tissue destruction. In renal tissue biopsies taken from patients with established vasculitis, neutrophils can be found (unpublished), but not antibody. Macrophages are found in tissue biopsies, and granulomas, groups of epithelioid macrophages are found in Wegener's Granulomatosis. ANCA is directed against PR3 on macrophages (Ralston, Marsh et al. 1997) as well as neutrophils, and primed monocytes expressing surface PR3, release IL-8 in response to anti-PR3 IgG (Ralston, Marsh et al. 1997; Hattar, van Burck et al. 2005).

Neutrophils also express PR3 and MPO on their surface. This has been demonstrated by flow cytometry in our laboratory (Hewins, Morgan et al. 2006) where MPO on the surface of neutrophils was found to be up-regulated by TNFα and PR3 was found to be expressed on primed and unprimed neutrophils (Haas and Eustace 2004; Hewins, Morgan et al. 2006). This was not quantified and indeed is difficult to quantify as the amounts expressed are small.

It has been suggested that degranulation of neutrophils occurs in or at the wall of the blood vessel since ANCA and neutrophils interacting in the blood steam would be cleared by the reticular-endothelial system. Neutrophils interact with selectins on the blood vessel wall in the presence of inflammatory mediators and roll on the surface of the endothelial cell (EC) layer. Interaction with ANCA could cause the neutrophil to become abnormally activated, causing it to degranulate and move through the vessel

wall. Once in the tissue the neutrophils may undergo dysregulated apoptosis (Harper, Cockwell et al. 2001) and form a nidus for inflammation. There is currently no evidence indicating where ANCA binds to the neutrophil or where degranulation occurs, but one study found some granular deposition of PR3 in human renal biopsies of patients with AAV in a linear pattern along the basement membrane suggesting degranulation of the neutrophil at the EC surface (Brouwer, Huitema et al. 1994).

If neutrophils were degranulating in the general circulation it would be likely that an increase in PR3 and other neutrophil components would be detected. This has been reported in vasculitis, where disease controls had lower levels of PR3 (Ohlsson, Wieslander et al. 2003). Whether this is specific to vasculitis or a feature of inflammatory disease in general is yet to be established.

It remains unclear as to why vasculitis targets specific organs more than others, namely the renal and pulmonary beds. Planted antigens (Ruth, Kitching et al. 2006), where degranulated neutrophils leave MPO in the glomerulus which acts as an antigen for accumulation of ANCA, may explain the continuation of inflammation in one vascular bed. At renal biopsy however, ANCA deposition is not seen.

2.2   ANCA

2.2.1   Animal models

The pathogenicity of ANCA is debated, but supported by available evidence. ANCA are sufficient to induce vasculitis in murine models (Xiao, Heeringa et al. 2002). The use of MPO knock-out mice immunised with mouse MPO has facilitated these studies. When splenocytes from these mice were injected into recombinase-activating gene-2 (Rag 2 $^{-/-}$) deficient mice, which lack T and B cells, the mice developed necrotising glomerulonephritis and systemic vasculitis including pulmonary haemorrhage. When purified MPO-ANCA IgG was injected intravenously into Rag 2$^{-/-}$ mice or wild type mice, both strains developed crescentic glomerulonephritis. Crescentic glomerulonephritis is an advanced manifestation of vasculitis where the capillary ball of the glomerulus ruptures into Bowman's space causing cells to escape there. These cells then appear in a crescent shape around the glomerulus at biopsy. MPO-ANCA alone was sufficient to induce vasculitis, but in the presence of an intact immune system in wild type mice, the disease was more severe.

Human neutrophil extracts perfused through rat kidneys from animals previously immunized with human MPO developed anti-human MPO and anti-rat MPO antibodies. Crescentic glomerulonephritis was induced but rather than pauci-immune disease found in AAV, this was associated with immune deposits (Brouwer, Huitema et al. 1993).

Endogenous human PR3 does not elicit vasculitis in mice because there is a low homology between human and mouse PR3. PR3/neutrophil elastase double deficient mice generate murine PR3 specific polyclonal antibodies after injection of recombinant murine PR3. Antibodies transferred into wild type mice did not induce vasculitis, possibly because mouse neutrophils (in contrast to human neutrophils) do not express PR3 on their surface. However these antibodies significantly aggravated the local inflammatory response induced by subcutaneous TNFα administration, thus providing evidence to support PR3-mediated tissue damage in vivo (Pfister, Ollert et al. 2004).

Rats immunized directly with human MPO developed antibodies which could bind to human and rat neutrophils. These animals developed blood and protein in the urine, with histological inflammation in the kidney and alveolar haemorrhage (Smyth, Little et al. 2003). A further study in rats used intra-vital microscopy to directly observe the interactions between ANCA and leukocytes on EC in mesenteric venules (Little, Smyth et al. 2005). Wistar-Kyoto rats were immunised with human MPO and developed anti-MPO antibodies that cross-reacted with rat MPO. The presence of these anti-MPO antibodies increased leukocyte adhesion and migration in response to low dose mesenteric superfusion with the chemokine CXCL1 (a neutrophil chemoattractant), and induced mesenteric microvascular injury (manifested as haemorrhage). When anti-MPO immunoglobulin was transferred to naïve rats, leukocyte adhesion and migration were similarly affected. In further work, blockade of TNFα in rats resulted in reduced albuminuria, lung haemorrhage and leukocyte transmigration in mesenteric venules (Little, Bhangal et al. 2006).

Recent research in our laboratory examined the effect of murine passive transfer of high affinity anti-murine MPO IgG in further intravital microscopy studies (Nolan, Kalia et al. 2008). After pre-treatment of the cremaster muscle with the murine cytokines TNFα, IL-1β or keratinocyte-derived chemokine (the murine functional analogue of human IL-8) the animals were injected with anti-MPO IgG raised in an MPO$^{-/-}$ mouse. The number of systemic circulating leukocytes fell, rolling leukocyte numbers were reduced and the numbers of leukocytes adhering to and migrating across the endothelium increased. Blockade of the integrin component CD18 inhibited the action of the anti-MPO antibody, as did use of mice negative for the Fc receptor gamma chain. This research therefore suggested that both integrins and Fc receptor were necessary for the leukocyte activation properties of anti-MPO antibodies.

2.2.2   Immunoglobulin subclass

There are four subclasses of human IgG and their relative concentrations in human sera are IgG1>IgG2>IgG3=IgG4 (Figure 1). The subclasses differ in their Fc regions (fragment crystallisable) which are responsible for their functional responses (Burton and Woof 1992). After the antibody has engaged the relevant antigen, the Fc region can then engage the Fc receptor. The principle effector functions of human IgG are to enable phagocytosis, antibody-dependent cell killing and complement activation. The subclasses differ by deletions, substitutions and additions of amino acids in the constant heavy chain. They are in fact highly homologous in the γ-heavy chains, and are most different in their hinge regions between the Fab (fragment antibody binding)

and the Fc portion. They are able to flex at the hinge region and this may explain some of the differences in their properties. IgG3 has the longest hinge region making it the most flexible protein but also the most easily cleaved, with subsequently the shortest half life in plasma (7 days compared to 21 for the other 3 proteins). IgG1 and IgG3 have a greater response to proteins, IgG2 to polysaccharide and IgG4 to prolonged exposure to antigen. Viral antigens are proteins and therefore induce IgG1 and IgG3 responses, whereas bacterial infections cause a strong IgG1 response. IgG3 activates complement significantly more than IgG1, IgG2 activates complement minimally and IgG4 is said not to activate complement (Jefferis, Lund et al. 1990; Roux, Strelets et al. 1997). (Krapp, Mimura et al. 2003).

IgG1   IgG2   IgG3   IgG4
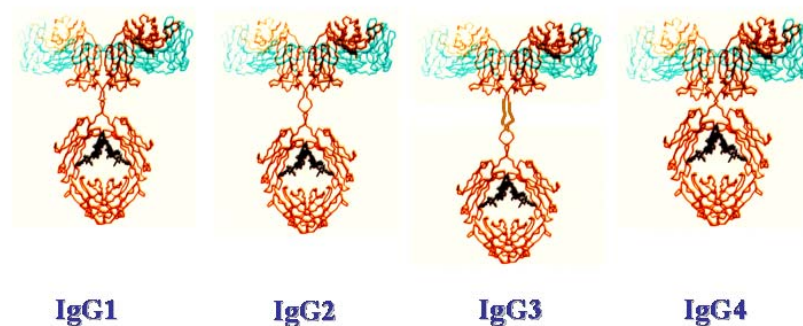
Figure 1: Four human IgG subclasses.

These are most different in their hinge regions between the heavy and light chains. IgG3 has the longest hinge region and is subsequently the most flexible protein.

IgG4 production is regulated similarly to IgE and a suggested function for IgG4 is to compete for antigen binding with IgE and therefore reduce the intensity of an allergic reaction. These antibodies are functionally monovalent and thus cannot form immune

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complexes which may be an important anti-inflammatory property. IgG4 has been reported as being a blocking/protective antibody (van der Giessen, Homan et al. 1976).

During formation of antibodies, subclass is generated by gene splicing. Somatic isotype subclass switching occurs in peripheral B cells where a cell can change production from IgG3 to 1 then to 2 then to 4 by sequentially splicing out more of the gene (Zeijlemaker 1996).This process is unidirectional and therefore continuing exposure to antigen may result in the accumulation of IgG4. It is not completely clear how this process is influenced but isotype switching to IgG4 has been shown to be dependent on IL4 (Spiegelberg 1989). This may explain why different IgG subclass populations predominate in active disease and disease in remission.

The function of IgG is largely influenced by its ability to bind Fcγ receptors and this varies across the subclasses. IgG1 is the predominant class, it binds to neutrophils constitutively expressed Fc receptors, CD16 (FcγRIIIb) and CD32 (FcγRIIa) and after activation via CD64 (FcγRI). CD64 is the only receptor that can bind soluble IgG. IgG2 is binds via CD32. IgG3 can bind CD16, CD32 and CD64. IgG4 is a smaller protein and is generally thought to bind to neutrophils via CD64 only (Stitis 1991; Roitt 1996), although there is some contrary evidence emerging (Holland, Hewins et al. 2004). It has also been proposed that subclass activity may be influenced through the ratio of binding to inhibitory and activatory Fc receptors (Nimmerjahn and Ravetch 2005).

	IgG1	IgG2	IgG3	IgG4
Binding to proteins (bacteria and viruses)	++	+/-	++	+/-
Binding to polysacchrides (bacterial cell wall)	+	++	-	-
Binding to allergens	+	-	-	++
Complement C1q binding	++	+	+++	-
Complement alternative pathway binding	++	+	+++	-
Fc receptor CD64	++	-	+++	+
Fc receptor CD32	++	+ (dependent on isotype)	+++	-
Fc receptor CD16	++	-	++	-

Table 1: IgG subclasses.

Binding abilities, complement activation and Fc Receptor binding.

2.2.3 IgG subclass and ANCA

Neutrophils are activated by ANCA IgG which complexes antigenic targets PR3 or MPO with Fcγ receptors on the neutrophil surface, triggering signalling events leading to neutrophil activation (Ben-Smith, Dove et al. 2001). These ANCA are therefore referred to as PR3-ANCA IgG or MPO-ANCA IgG and can be detected by enzyme-linked immunosorbent assay (ELISA). In addition, ANCA can also be detected by indirect immunofluorescence where a staining pattern is either seen in a perinuclear pattern (p-ANCA) or in a cytoplasmic pattern (c-ANCA). c-ANCA usually corresponds to PR3 directed ANCA and p-ANCA to MPO directed ANCA, although this is not always the case.

ANCA directed against MPO and PR3 has been detected in all four IgG subclasses. In a retrospective study of all ANCA measured in patient sera in one centre, the ANCA were predominately IgG1 and IgG4 antibodies. In MPO-ANCA, IgG2 was common and IgG3 scarce. In contrast, IgG3 was common in the PR3 group, and IgG2 rare (Segelmark and Wieslander 1993). This was supported in part by a study from our laboratory where IgG1 and IgG4 predominated in AAV, with MPO having specifically increased IgG1 predominance, and PR3, specifically IgG3 (Harper, Radford et al. 2001). In another study IgG3 was found to be reduced in the p-ANCA subset, and in c-ANCA IgG1 and IgG4 were the dominating subclasses. Despite high IgG4 activity, the sera containing c-ANCA were still able to activate complement (Mellbye, Mollnes et al. 1994).

ANCA subclass has been compared between active disease and remission (Table 2). The total IgG subclass divisions were similar to that in the normal population but the ANCA showed proportionally more IgG3 and less IgG2, which was more marked in p-ANCA. In remission, the IgG3 fell and the IgG2 increased (Jayne, Weetman et al. 1991).

This was supported by a later study where an increased capacity to induce a respiratory burst was significantly related to increases in the relative amount of IgG3 subclass of ANCA found in active disease (Mulder, Stegeman et al. 1995). Brouwer et al found IgG3 to be increased in renal limited AAV, but with overall predominance of IgG1 and IgG4, both in active and remission disease (Brouwer, Tervaert et al. 1991). This increase in the IgG4 fraction was confirmed in a later study (Cambridge, Williams et al. 1994). This may be due to the chronicity of the disease with repeated antigen stimulation accumulating IgG4, as genes coding for the IgG4 are located at the most downstream switch site of the genes coding for immunoglobulin. Variation of ANCA subclass during active disease or remission may thereby influence pathogenicity.

Study	Aims	Conclusions
Brouwer 1991	Is one ANCA subclass increased in renal limited AAV?	IgG3 increased in renal limited AAV
	Which subclasses predominate in AAV?	IgG1 IgG4 predominant in AAV
Jayne 1991	Are ANCA subclasses same as normal IgG?	ANCA IgG more IgG3, less IgG2 especially in pANCA.
	Does one subclass increase in active disease or remission?	In remission IgG3 fell and IgG2 increased
Cambridge 1994	Is IgG4 increased in AAV?	IgG4 increased proportionally in ANCA compared to normal IgG
Mellbye 1994	Which subclasses predominate in cANCA and pANCA patients?	pANCA IgG3 reduced
	If IgG4 proportion high, can compliment still be activated?	cANCA IgG1 and IgG4 dominant but still activate compliment
Segelmark 1993	Which subclasses predominate in PR3 and MPO patients?	MPO IgG2 common, IgG3 rare PR3 IgG3 common IgG2 rare
Mulder 1995	Does subclass of ANCA affect the respiratory burst?	In active disease more IgG3 and increased respiratory burst
Harper 2001	Which subclasses predominate in vasculitis, and in PR3 and MPO patients?	IgG1 and IgG4 predominant in AAV MPO increased IgG1 PR3 increased IgG3
Holland 2004	Does IgG4 activate neutrophils?	IgG4 able to activate neutrophils

Table 2: ANCA IgG subclass in vasculitis.

2.2.4 Glycosylation and galactosylation status

As well as IgG subclass, glycosylation status of the immunoglobulin has been suggested to be important in disease pathogenesis. The structure of the IgG antibody Fc includes an oligosaccharide (Figure 2). Glycosylation is essential for IgG stability and Fc mediated effector function; removal of this oligosaccharide results in decreased activation of FcγR cell mediated cytotoxicity and complement activation (Jefferis, Lund et al. 1990; Jefferis, Lund et al. 1995; Jefferis, Lund et al. 1998). This is because the carbohydrate stabilises the molecule and confers on it the correct conformation to bind to the Fc receptor. Deglycosylation of the Fc molecule reduces binding to FcγRIIa, FcγRIIIb and to a lesser extent FcγRI.

In addition to complete removal of this oligosaccharide, changes in its structure can alter the function of the IgG. In AAV disease states, it has been shown that there is a deficit in IgG galactosylation (i.e. the galactose residues on the side chains were missing from one or both side chains of the IgG) (Holland, Takada et al. 2002). A large proportion (57% in WG, 47% in MPO) of IgG in patients was agalactosylated (G0) whereas in controls the proportion of G0 IgG was 28%. The G0 glycoform of IgG has been shown to bind mannin binding lectin, activating complement and increasing uptake by macrophages and dendritic cells, thus conceivably contributing to autoimmunity (Dong, Storkus et al. 1999). Removing terminal acetylglucosamine and mannose sugars has been shown to cause conformational change which affects the interface of IgG Fc fragments and Fcγ receptors (Krapp, Mimura et al. 2003).
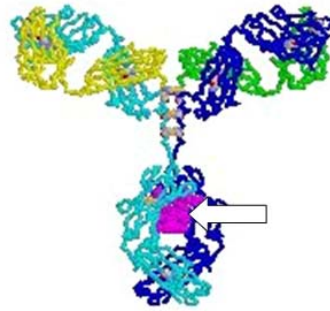
Figure 2: IgG structure showing the carbohydrate moiety.

The carbohydrate, in pink, is arrowed. The two light chains are green and yellow and the two heavy chains dark and light blue.

2.3   Neutrophils

2.3.1   Neutrophil function: superoxide and degranulation

Neutrophils act as the first line of innate immune defence against invading micro-organisms during infection and inflammation. There are two major effector functions in the neutrophil's repertoire, a respiratory burst and the use of proteolytic enzymes (degranulation) (Abbas and Lichtman 2003). Although these reactions occur simultaneously, they are distinct from each other. Priming of the neutrophil precedes these reactions and during priming granules in the neutrophil cytoplasm move towards the cell surface in readiness to fuse with the plasma membrane. Simultaneously there is adhesion molecule up-regulation on the surface of the neutrophil.

The respiratory burst can occur both inside and outside the neutrophil. In the laboratory, chemiluminesense detects this reaction inside the cell, and cytochrome C detects the reaction outside the neutrophil. The respiratory burst proceeds in three parts:

Oxygen burst: $2O_2$ (catalysed by NADPH) $\rightarrow$ $2O2^-+NAP^++H^+$

Dismutase reaction: $2O_2+2H^+$ (catalysed by superoxide dismutase) $\rightarrow$ $2O^-$. (Superoxide) + H_2O_2

Myeloperoxidase: $H_2O_2+Cl^-+H^+$ (catalysed by MPO) $\rightarrow$HOCl (hydrochloric acid) + H_2O.

In vivo the toxicity of hydrochloric acid is converted to chloramines which damage microbes but not tissues (Weiss 1989). The respiratory burst is completed within 30 to 40 minutes of activation.

The respiratory burst happens simultaneously with neutrophil degranulation. Cytoplasmic granules fuse with the cell surface and release proteolytic enzymes such as PR3, elastase, collagenase and gelatinase, which cause extracellular matrix disruption. PR3 has multiple functions and is thought to be involved in the cell cycle, differentiation and death (Yang, Preston et al. 2001). It is a substrate for transcription factors such as nuclear factor-κB (Franzoso, Biswas et al. 1994) and activates cytokines such as TNFα and IL-1β by cleaving non-soluble pro-forms of these cytokines (Coeshott, Ohnemus et al. 1999). The proteolytic activity of PR3 is principally involved, but it is thought that PR3 can also have an effect on intracellular processes independent of proteolysis.

The neutrophil has four types of granules (Faurschou and Borregaard 2003); secretory vesicles contain plasma proteins and receptors such as the $\beta 2$ integrins; primary (azurophilic) granules contain MPO and serine proteases; secondary (specific) granules contain collagenase and lactoferrin and tertiary (gelatinase) granules contain enzymes such as the gelatinase, matrix metallopeptidase. The granules are released in order of activation, so that on minimal activation the secretory vesicles move to the surface of the neutrophil and release their contents. Tertiary and secondary granules can be induced to release their contents on further activation of the neutrophil. Primary granules require the neutrophil to be very activated before they are released.

PR3 and MPO are very destructive and are limited *in vivo* by plasma anti-proteinases. However in combination with chlorinated antioxidants the anti-proteinase shield can be destroyed. Therefore the combination of the two neutrophil reactions is tissue damaging (Weiss 1989). Degranulation occurs within 15 to 20 minutes. Proteinases are essential for neutrophils to kill bacteria. Mice deficient in proteases but normal in superoxide capacity and iodinating ability were unable to kill staphylococci or candida (Reeves, Lu et al. 2002).

2.3.1.1 Proteinase 3 (PR3)

Protease -3 is a serine protease located in the azurophilic granules of neutrophils, although it is also found in the specific granules and the secretory vesicles. It is stored as a mature active protein. It is synthesized as a pre-pro enzyme and

processed in four consecutive steps into the mature form of 222 amino acids, 29-32kDa as three isoforms. Gene studies so far reveal that this protein is not very polymorphic and that polymorphisms in the gene are rare and do not seem to affect the structure at the catalytic site of the enzyme (van der Geld, Limburg et al. 2001).

As well as storage is vesicles, PR3 is expressed on the membrane of neutrophils. The interaction of PR3 with the membrane is not entirely clear, but seems to be covalent and is not dependent on sialic acid residues or via a GPI-link (Witko-Sarsat, Cramer et al. 1999). There is evidence *in vitro* that PR3 can bind to EC and EC can internalise PR3 (Yang, Preston et al. 2001).

Functionally PR3 is a degrading protease capable of killing phagocytosed microbes. This is independent of its enzymatic activity and is due to inhibition of molecular synthesis and oxygen metabolism of bacteria. Additionally some of the pro forms of the enzyme escape granular targeting and are secreted and may provide a negative feedback regulator for granulopoeisis (Skold, Rosberg et al. 1999). PR3 is involved in the growth and differentiation of granulocytes and monocytes. It is also implicated in neutrophil migration dependent on its enzymatic ability to degrade extracellular matrix proteins (Rao, Wehner et al. 1991). It is also implicated in the modulation of inflammation, degrading both TNFα (Leid, Ballieux et al. 1993) and C1 inhibitor (Leid, Ballieux et al. 1993). PR3 can also modulate the activity of platelets and endothelial cells (Renesto, Halbwachs-Mecarelli et al. 1994; Renesto, Si-Tahar et al. 1997) probably by cleavage and inactivation of protease activated receptors. PR3 can also

inhibit thrombin (Renesto, Si-Tahar et al. 1997) and neutrophil NADPH-oxidase (Tal, Sharabani et al. 1998), this latter is independent of its enzymatic activity.

The natural inhibitors of PR3 are the serpins, and the principle one of this group is α1-antitrypsin (Rao, Wehner et al. 1991). PR3 is also inhibited by elafin in the skin (Wiedow, Luademann et al. 1991), and by α2-macroglobulin (Rao, Wehner et al. 1991). Additionally, PR3 activity to large substrates such as elastin is partially inhibited by glycosaminoglycans (Fruh, Kostoulas et al. 1996). PR3 complexed to α1-antitrypsin has been found in both WG patients and healthy controls, and deficiencies in α1-antitrypsin have been reported to be increased in WG (Esnault, Testa et al. 1993).

CD177 has been reported to co-localise with membrane bound PR3 on circulating neutrophils (Witko-Sarsat, Reuter et al.). A recent paper found that membrane expression of CD177 was increased on circulating neutrophils in AAV, but interestingly that primed neutrophils from CD177-negative individuals also express membrane PR3 and are susceptible to an anti-PR3 induced oxidative burst suggesting that a CD177 independent mechanism is also involved (Hu, Westra et al. 2009). Binding partners other than CD177 for PR3 have yet to be explored.

PR3 has variable surface expression between donors, and is increased in patients with AAV (Harper, Cockwell et al. 2001). This increased expression correlates with disease activity (Muller Kobold, Kallenberg et al. 1998) and is a stable phenomenon

within individuals. In addition there is an increase in apoptotic neutrophils expressing PR3 on their surface (Harper, Cockwell et al. 2001).

2.3.1.2 Sites of binding of ANCA to PR3

Anti-PR3 ANCA recognise more than one epitope on the PR3 molecule. Five epitopes have been identified, four of which are in or near the catalytic site of the enzyme (Griffith, Coulthart et al. 2001). In the development of the monoclonal anti-PR3 antibody in our laboratory, the epitope on PR3 recognised by a mouse monoclonal antibody called 4A5 was used to create the variable region of the antibody. This epitope is recognised by more than 50% of patient ANCA and was therefore of direct pathological relevance (Colman, Hussain et al. 2007).

The binding of ANCA to PR3 potentially crosslinks both the variable and the Fc regions of the antibody. This is controversial, but is supported by work from our group in signalling pathways (see section 2.3.3.3). Other molecules on the surface of neutrophils, such as CD15 do not result in crosslinking and downstream signalling in the same way (unpublished data).

2.3.2 Fc receptor

Activation of the neutrophil occurs when the Fc portion of the immunoglobulin attaches to the neutrophil surface Fc receptors. Some Fc receptors can activate signalling pathways in the neutrophil, whilst others can inhibit these pathways.

There is variation in the ratio of activatory FcγRIIa and inhibitory FcγRIIb receptors on the surface of neutrophils between healthy people. mRNA was measured in 50 white and 10 black healthy volunteers and ratios of activatory to inhibitory receptors fell into in four groups 1:1, 2:1, 3:1 and 4:1. This was associated with functional responsiveness of neutrophils to dimeric or aggregated IgG. Elastase release and up-regulation of CD11b was greater in the group with 4:1 ratio compared to the 2:1 and 1:1 groups. The authors found a haplotype association in these groups (van Mirre, Breunis et al. 2006).

Of the activating Fc receptors found on neutrophils, there are two constitutively expressed low affinity receptors, FcγRIIa (CD32) and FcγRIIIb (CD16), and one inducible high affinity receptor, FcγRI (CD64). FcγRIIa is a transmembrane protein with a tyrosine-based activation domain (ITAM) in its cytoplasmic region. FcγRIIIb is a glycosyl-phosphatidylinositol (GPI) anchored protein which is more highly expressed on neutrophils than FcγRIIa (van Mirre, Breunis et al. 2006).

2.3.3 Neutrophils in AAV

Neutrophils appear to be essential in vasculitis. In the elegant animal model described above (Xiao, Heeringa et al. 2005), when neutrophils were depleted, mice were completely protected against anti-MPO induced glomerulonephritis.

2.3.3.1 Fc involvement in AAV

Fc involvement in ANCA induced neutrophil activation, is thought to be dependent on FcγRIIa (Mulder, Stegeman et al. 1995) and FcγRIIIb (Ben-Smith, Dove et al. 2001). However it has now been recognised that the neutrophils from patients with active vasculitis express high levels of FcγRI and this has been shown to be involved in superoxide production (Reumaux, Duthilleul et al. 2002). FcγRI is induced by interferon gamma (IFNγ) but not by TNFα.

There is no association between vasculitis and polymorphisms of FcγRIIIb (Tse, Abadeh et al. 2000) or FcγRIIa (Tse, Abadeh et al. 1999).

2.3.3.2 Apoptosis and superoxide in vasculitis

Neutrophils from vasculitis patients become apoptotic more quickly than control neutrophils (Harper, Ren et al. 2000) and during apoptosis the target antigens for ANCA become expressed on the outer cell membrane (Gilligan, Bredy et al. 1996). These antigens, PR3 and MPO, are expressed more on neutrophils from patients with vasculitis than those from controls (Harper, Cockwell et al. 2001). In active vasculitis, there is an increase in circulating apoptotic neutrophils expressing PR3 and MPO on their surface (Harper, Cockwell et al. 2001). Since ANCA can bind apoptotic neutrophils it is possible that this may affect neutrophil-endothelial cell interactions and the fate of apoptotic neutrophils within the vasculature.

The apoptosis of neutrophils in vasculitis may be dysregulated. Usually, neutrophils become apoptotic and are cleared by macrophages, inducing an anti-inflammatory response via appropriate cytokines. If neutrophils are not cleared by macrophages they undergo secondary necrosis which can induce a pro-inflammatory response. There is evidence in vasculitis that apoptosis is accelerated but externalisation of phosphatylserine is not, which may result in a reduced window of opportunity for macrophages to clear apoptotic neutrophils (Harper, Ren et al. 2000)

Neutrophils from vasculitic patients in remission produce above normal levels of superoxide (Harper, Nuttall et al. 2002).

2.3.3.3 ANCA causes distinct neutrophil signaling

Increasing evidence suggests that the signalling cascades within the neutrophil activated by ANCA, are different from those activated by normal immune complexes. Normally, immune complexes cross link two Fc receptors (Figure 3) but ANCA may cause a different activation by cross linking via the Fc and Fab portions of the immunoglobulin simultaneously (Figure 3). This theory is controversial but supported by work from our laboratory (Williams, Ben-Smith et al. 2003; Williams and Savage 2005).
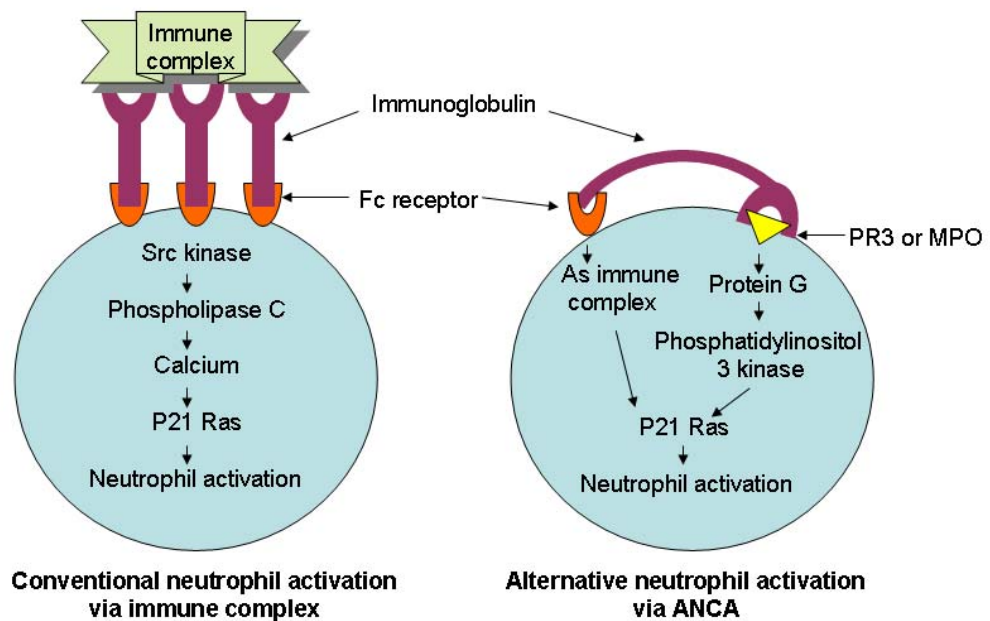
Figure 3: Conventional and alternative neutrophil signalling.

Neutrophils are conventionally activated by immunoglobulin engaged to immune complexes linking with multiple Fc receptors on the neutrophil surface. The neutrophil is activated via a phospholipase C pathway. In the alternative activation model, ANCA engages an Fc receptor and either PR3 or MPO on the neutrophils surface simultaneously activating both the conventional pathway and a protein G pathway.

In activation of the neutrophil by ANCA, as well as the normal Fc pathway resulting in tryrosine kinase, a separate pathway via Fab activates a G protein receptor coupled pathway. The Fab portion of the antibody binds the antigens MPO or PR3 on the neutrophil surface. This G protein signal is unusual and why this is activated by ANCA remains unknown. PR3 and MPO are both highly charged cationic proteins which may be why they insert into the anionic phospholipid bilayer of the neutrophil.

This charge may attract other molecules, or cause the PR3/MPO to localise in a particular sub compartment of the neutrophil membrane, thus leading to the unusual G protein signal.

The Fc portion of the antibody binds the constitutively expressed FcγRIIa and FcγRIIIb receptors of the neutrophil, activates Syk kinase and phospholipase C, and mediates calcium release.

Both the Fc and the Fab pathways must be engaged to cause a respiratory burst, that is, an intact antibody is required for activation of phosphoinositide 3-kinase and for full activation of the small GTPase, p21ras which is an important molecular switch point for a number of pathways. The FcR will not bind alone to FcγRIIa or FcγRIIIb as the affinity of the receptors is too low, it requires ligation of Fab simultaneously. Anti-CD15 antibodies are also able to bind to neutrophils but these do not cause superoxide release. They have been reported to cause neutrophil adhesion (Forsyth, Simpson et al. 1989).

The signalling pathways downstream of p21ras are not fully elucidated, but work from our laboratory suggests involvement of a number of small molecules which lead to both superoxide production and degranulation (unpublished data).

Signalling within neutrophils may be dependent on lipid rafts. Lipid rafts are cholesterol enriched microdomains within cell membranes which contain sphingolipids (lipids derived from the aliphatic amino alcohol sphingosine) and

glycolipids (carbohydrate-attached lipids). Proteins associated with signalling are thought to associate with these rafts including tyrosine kinases of the src family and glycosylphosphatidylinositol (GPI)-anchored proteins (Brown and Rose 1992). Researchers have found that by disrupting lipid rafts signalling processes can be stopped (Petrie, Schnetkamp et al. 2000). This is supported by unpublished work from our lab where the disruption of neutrophil lipid rafts destroyed the neutrophil's ability to produce superoxide. However the role of rafts remains controversial, they cannot be visualised directly and the proposed properties of them are not always supported by experimental evidence (Edidin 2003).

In addition, the $\beta2$ integrin CD18 has been shown to be essential for initiation of signalling (Reumaux, de Boer et al. 2003) and intimates the need for further molecular interactions on the neutrophil cell surface.

2.3.3.4 Degranulation in vasculitis

The site of degranulation *in vivo* in vasculitis is not clear but is obviously important. Previous work in our laboratory (unpublished data) using the flow model demonstrated PR3 smears behind neutrophils suggesting they were degranulating on the endothelium. Degranulation would be likely to result in vascular damage.

2.3.3.5 Variation in amount of PR3 and MPO on the surface of neutrophils

PR3 and MPO are expressed on the surface of normal neutrophils after they have been 'primed' and are ready to undergo superoxide production and degranulation, for

example by exposure to TNFα. Preparation of neutrophils is thought to leave the neutrophils unprimed, but previous work in our laboratory using flow cytometry has demonstrated that MPO and PR3 expression is up-regulated on the surface of unprimed neutrophils (Hewins, Morgan et al. 2006).

Patients with vasculitis have been found to have increased plasma concentrations of PR3 compared to healthy controls (Ohlsson, Wieslander et al. 2003) and also in vasculitis, greater amounts of PR3 and MPO were expressed on the surface of patients' neutrophils versus control, when analysed by flow cytometry (Harper, Cockwell et al. 2001). Vasculitis patients have also been found to have an increased number of membrane PR3 positive neutrophils (Witko-Sarsat, Lesavre et al. 1999) and these are associated with relapse (Rarok, Stegeman et al. 2002). In a recent study the role of these membrane PR3 positive neutrophils was investigated. TNFα and human anti-PR3 ANCA increased the amount of PR3 expressed on the neutrophil surface, thus providing an amplification loop where neutrophil activation by ANCA can lead to further expression of ANCA antigen (Brachemi, Mambole et al. 2007).

There have been reports of an association of AAV with a polymorphism in the PR3 gene (Gencik, Meller et al. 2000), but this has not been substantiated in a further paper (Abdgawad, Hellmark et al. 2006).

2.4 Endothelial cells

Endothelium forms the inner cell lining of blood vessels throughout the body and has many functions. It controls vasomotor tone, blood cell trafficking, coagulation and has a large role in immune function. Phenotypes of ECs change over time and according to the environment in which they exist and are therefore heterogeneous. It has been well established that chemokines and adhesion molecule expression differs between EC types and changes with the activation status of the endothelium (Lim, Garcia-Cardena et al. 2003), i.e. endothelium in its resting state expresses little in the way of adhesion molecules but these are up-regulated when activated by cytokines such as TNFα.

In vasculitis, the pulmonary and renal glomerular capillaries are particularly affected, and it is conceivable that ECs at these sites are more vulnerable to the effects of ANCA-neutrophil-EC interactions.

There is now evidence that ECs are involved in the initiation and maintenance of inflammation. They constitutively express class I MHC, but not B7. They have been classified as semi-professional antigen presenting cells as they can partially activate T cells (Segal, Bihorac et al. 2002).

Human umbilical vein ECs (HUVEC) have been widely used in *in vitro* experiments exploring EC function. They are easy to obtain, isolate and grow. Although HUVEC have been traditionally used to represent all EC function, in light of increasing evidence of diversity of function, this may no longer be acceptable practice.

2.4.1   <u>Evidence that endothelial cells have different properties</u>

Although some organelles such as Weibel-Palade bodies and fenestrae (transcellular pores, 70nm in diameter extending through the cell with a diaphragm across the opening) are typical of endothelium, these are not seen in every EC. Caveolae are 70nm vesicles which are used in transcytosis, transporting molecules across the endothelium, these are not however specific to the endothelium. Their presence varies greatly throughout the body; they are common in capillaries in non-fenestrated endothelium.

It is difficult to find a protein marker that is specific to the endothelium. CD-31 (PECAM-1) is also expressed in monocyte, trophoblasts and leukocytes; vascular endothelial cadherin is also present in trophoblasts and foetal stem cells.

2.4.1.1 Fenestrae

We know from electron microscopy studies that there are variations in fenestrae and in the continuity of the basement membrane in EC. Continuous endothelium has a continuous basement membrane in contrast to discontinuous EC, seen in liver sinusoids. EC permeated with holes is called fenestraed endothelium and this can occur in continuous or discontinuous EC, and is seen in organs where filtration is required such as the glomerulus and some renal tubules.

Fenestrae are generated and maintained by VEGF (Eremina, Sood et al. 2003) and fenestrae can be induced in some EC which do not usually have fenestrae, such as skin and cremasteric capillaries (Roberts and Palade 1995). Functional differences such as permeability must be dependent on the presence of fenestrae, tight junctions and transcellular transport mechanisms which vary greatly between vascular beds.

2.4.1.2 Fluid shear

Shear stress is known to change the phenotype of EC and the expression of ICAM-1, VCAM-1 and E-selectin (Chiu, Lee et al. 2004). Grown under conditions of high fluid shear, neutrophils are subsequently less recruited in the EC flow model (Sheikh, Rainger et al. 2003). Shear stress varies throughout the vascular tree, from more than 1 Pascal in arteries, to 0.1 to 0.5 Pa ($0.1Pa = 1dyn/cm^2$) in post capillary venules such as the kidney.

2.4.1.3 Chemokine and adhesion molecule expression

Several publications have suggested that ECs from different species and locations differ in function. Chemokines and adhesion molecules expression may differ between EC types; murine heart ECs exhibit high constitutive VCAM-1 expression while lung endothelia do not. TNFα up-regulated expression of adhesion molecules and chemokines on both cell types, but high levels of Regulation on Activation Normal T cell Expressed and Secreted (RANTES) was detected only in the heart endothelium (Lim, Garcia-Cardena et al. 2003).

Postcapillary venules are known to be 'leaky' and they have few tight junctions, high numbers of vesiculo-vacuolar organelles (VVOs) (which are probably formed from the fusion of caveolae) and increased expression of bradykinin, histamine and serotonin receptors which probably result in increased leakage of macromolecules through VVOs (Feng, Nagy et al. 1997). Cytokines such as TNFα have different effects on different ECs (Eriksson, Karlof et al. 2005).

2.4.2   Microvascular versus macrovascular cells

Macrovasular ECs such as HUVEC are conduit vessels whose function is to conduct blood from one area to another. Microvascular cells such as those found in the glomerulus are designed to allow for much more movement of cells across the endothelium into the tissue. Traditionally HUVEC have been used to represent ECs and have been presumed to act similarly to microvascular endothelium, there have been some studies which support this. McGinn et al (McGinn, Poronnik et al. 2004) found no morphological or immunohistological differences, both glomerular EC and HUVEC had a cobblestone appearance, stained positively for vWF and PECAM-1 and negatively for antifibroblast surface antigen and anticytokeratin. Studying HUS, workers found that shiga toxin 1 or 2, stimulated the rapid secretion of unusually large vWF multimeric strings from HUVEC or from GEC (Nolasco, Turner et al. 2005).Various ECs (HUVEC, dermal, aortic, pulmonary) were tested to determine their ability to form capillaries using a Matrigel-based support assay. Dermal and HUVEC were superior in their ability to develop capillary like structures but patterns of responsiveness were similar between the cell types. The authors therefore

concluded that it was justified to extrapolate from HUVEC to microvascular cells (Harvey, Welch et al. 2002). Some studies have also shown no differences between microvascular and macrovascular endothelium in terms of adhesion molecules. Brain microvascular cells were found to have similar adhesion molecules to HUVEC (Franzen, Duvefelt et al. 2003); comparing dermal microvascular cells to HUVEC, IL10 was found to induce similar amounts of E-selectin and IL-1β and TNFα were found to induce P-selectin in both types of EC (Vora, Romero et al. 1996).

In contrast, others suggest that it is not satisfactory to extrapolate from HUVEC to microvascular endothelium. HUVEC was compared to saphenous vein EC and found to demonstrate significant functional differences in adhesion (Tan, Chan et al. 2004). A comparison of human umbilical arterial cells and HUVEC established different profiles for up-regulation of vascular adhesion molecule-1 (VCAM-1) where VCAM-1 was more readily induced on the vein endothelium than the arterial (Kalogeris, Kevil et al. 1999). Auerbach (Auerbach, Alby et al. 1985) used cell cultures from multiple organ beds and reported that they expressed organ specific antigens. Glomerular EC (GEC), dermal EC and HUVEC were compared for expression of cell adhesion molecules (Murakami, Morioka et al. 2001). CD31, a molecule that can bind in a homotypic and heterotypic manner to link EC together, was constituently expressed on HUVEC, but levels were lower on GEC and dermal EC. The expression of CD31 decreased in response to a combination of TNFα and IFNγ on all cell types. TNFα induced VCAM-1 on HUVEC and GEC but not on dermal EC, while IFNγ induced VCAM-1 only on HUVEC. E-selectin was induced on all three types of EC by TNFα but not by IFNγ. In a study pursuing the pathogenesis of HUS, certain ratios of

antifibrinolytic antigens were found to be antifibrinolytic in HUVEC but profibrinolytic in GEC (Louise and Obrig 1994). DNA microarray studies have found that there are differences between microvascular and macrovascular transcription (Chi, Chang et al. 2003). These studies suggested that GEC do not express the same adhesion cell profile as HUVEC and should therefore be studied independently to determine specific roles in inflammation.

2.4.2.1 Glomerular endothelial cells

In the glomerular microcirculation the capillary wall is particularly unique as it is permeable to water and small solutes, whilst remaining impermeable to macromolecules. This is a function of the three layers of the capillary wall; endothelium, glomerular basement membrane and podocyte. The glomerular EC is a specialized cell punctuated by multiple fenestrations, trans-cellular pores 60-80nm in diameter (Deen, Lazzara et al. 2001). These fenestrations have a glycocalyx which covers the fenestrae and probably contribute to the permeability barrier (Rostgaard and Qvortrup 1997). GEC are uniformly positive for CD31 (PECAM-1) and CD34, but not vWF (Jacquemin, Neyrinck et al. 2006). The GEC sit against a collagen-rich basement membrane; the opposing epithelial cell (the podocyte) is characterised by foot process slit diaphragms, and is the subject of intense research interest by virtue of its ability to alter the GEC phenotype. For example, deletion of podocyte VEGF results in loss of GEC barrier function (Eremina, Sood et al. 2003). The unique structure of the renal endothelium is likely to be part of the explanation as to why vasculitis targets the kidney and this is the subject of ongoing research.

The diameter of the capillaries in the glomerulus is small (10-20μm) (Shiraishi, Wang et al. 2003). Normal renal blood flow 0.22m/s in intrarenal vessels (Howes, Keilty et al. 1996). As the kidney receives 20% of cardiac output, GEC are exposed to high numbers of circulating cells. It is conceivable that this endothelial bed is physiologically protected from chronic inflammation, that inflammatory cells are less easily recruited to this EC surface than to other EC surfaces. The absence of selectin expression in the renal bed under normal conditions supports such a hypothesis (Savage, Brooks et al. 1997).

2.4.3   Endothelial cell markers and markers of endothelial damage

There are many papers in the literature exploring markers for EC damage. These include adhesion molecules such as selectins, cytokines, and other proteins released from the EC during damage, most commonly used is von Willebrand factor (vWF) but other proteins such as such as tissue plasmininogen activating factor and have been explored. Circulating ECs have recently also been discussed as markers of EC damage, particularly in AAV.

2.4.3.1 CD31

CD31 (cluster differentiation molecule 31) also known as platelet EC adhesion molecule (PECAM-1) is a member of the immunoglobulin superfamily. It is expressed in high concentration on vascular ECs and in lower levels on lymphocytes, monocytes and platelets. It binds in a homotypic and heterotypic manner to join EC together affecting the growth and morphology of EC (Fawcett, Buckley et al. 1995).

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2.4.3.2 Von Willebrand Factor (vWF)

Von Willebrand Factor is a large multimeric glycoprotein produced in ECs in organelles called Weibel-Palade bodies. It has a primary function in haemostasis binding to collagen types I and III (van der Plas, Gomes et al. 2000). vWF is released from EC in vasculitis when ECs are damaged and can be therefore used as a damage marker (Savage, Pottinger et al. 1991). vWF has been used extensively as a marker of EC cell damage in many other disease processes (e.g. sepsis, cardiac failure, haemolysis, elevated liver enzymes, low platelets (HELLP) syndrome) and can be used quantifiably, where the amount of vWF released is proportional to the amount of EC damage.

2.4.3.3 Circulating endothelial cells

Large numbers of circulating EC are present in patients with active vasculitis when compared to non-ANCA associated glomerulonephritis and healthy controls, and these fall with treatment (Woywodt, Streiber et al. 2003). Circulating EC are thought to arise due to apoptosis and detachment from the basement membrane with release into the peripheral blood (Woywodt, Streiber et al. 2003). There is some debate from other studies as to the significance of these circulating EC, and it has been suggested that injured, shed cells do not account for all those found in the peripheral circulation (Holmen, Elsheikh et al. 2005). There may also be release of endothelial progenitor cells from the bone marrow in order to repopulate the denuded areas, and, indeed, these may offer therapeutic potential (Segal, Bihorac et al. 2002). In a recent

study, inflammatory EC were increased in patients with active disease compared with those in remission, and levels of these cells correlated with C-reactive protein and extent of organ involvement. They also found that patients with active disease had less endothelial progenitor cells, and that their proliferation and migration was inhibited by the circulating inflammatory EC (Holmen, Elsheikh et al. 2005).

2.4.3.4 Endothelial Microparticles

Loss of cellular phospholipid asymmetry when EC are damaged often causes shedding of lipid-symetric microvesicles from the EC surface and this may allow the cell to return to a resting state, that is, it is important in cell repair. These are less than 1 micron in diameter and may be useful in diagnosing states in which the endothelium is activated, and this has been used in Multiple Sclerosis (Larkin 2001). In AAV this has been used as a research tool and was found to correlate with disease activity (Brogan and Dillon 2004).

2.5 Interaction of leukocytes, ANCA and endothelial cells

2.5.1 Normal neutrophil adhesion cascade

Passage of leukocytes from blood to underlying tissue is a process that involves attachment, rolling, arrest and transmigration (Figure 4). Rolling adhesion is supported by selectin adhesion molecules (expressed by leukocytes and cytokine-activated ECs), which engage fast flowing cells from the circulation. E-selectin is highly restricted to ECs and is expressed in activated endothelium largely in post capillary venules. It is up-regulated by lipopolysacchride (LPS), and this varies

according to the organ (Yano, Liaw et al. 2006). Rolling adhesion is converted to firm adhesion by a second signal that results in conformational change of integrin molecules on the neutrophil surface, which bind to their ligands such as ICAM-1 on the endothelium. These ligands vary from organ to organ (Henninger, Panes et al. 1997). The neutrophil then moves through the tissue towards chemoattractants. This transmigration process is poorly understood, but is thought to involve CD31, junctional adhesion molecules (JAM) (Aurrand-Lions, Johnson-Leger et al. 2001; Johnson-Leger, Aurrand-Lions et al. 2002; Woodfin, Reichel et al. 2007) and recent work suggests that CD99 may also be involved in a homophilic interaction between neutrophil CD99 and CD99 at the EC junction (Lou, Alcaide et al. 2007). There have also been reports of leukocytes transmigrating through EC (Carman and Springer 2004).

Although this rolling-adhesion-transmigration cascade occurs in most inflamed tissues and in the high endothelial venules of lymphoid organs, the exact mechanisms of neutrophil emigration in inflamed glomeruli have not yet been established. For example, it may be that leukocyte rolling does not occur in glomeruli and that other mechanisms (e.g. direct capture using endothelial P-selectin or ICAM-1) pertain in this tissue (Kuligowski, Kitching et al. 2006). In the lungs and liver neutrophils do not roll prior to adhering and rolling is probably not selectin dependent (Wong, Johnston et al. 1997).

It is also probable that neutrophil adhesion and transmigration are dysregulated in the presence of ANCA, with consequent "bystander" injury to EC (Radford, Savage et al. 2000; Radford, Luu et al. 2001).
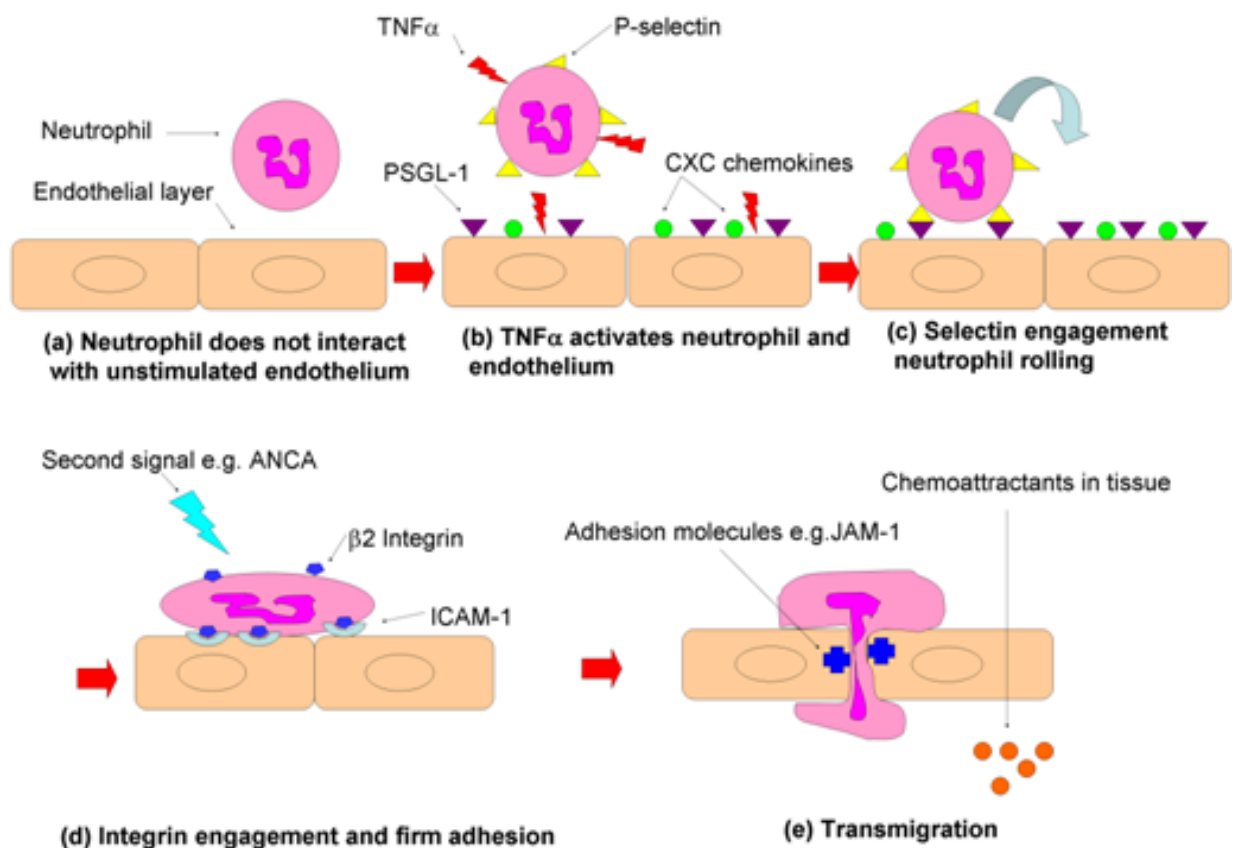
Figure 4: Neutrophil adhesion cascade.

(a) Endothelium not stimulated with cytokine does not support neutrophil rolling. (b) Cytokines such as TNFα and IL-1β induce selectins on the neutrophil surface and their ligands on the endothelial surface. Chemokines which attract neutrophils are also induced on the endothelial surface. (c) The neutrophil engages via P-selectin glycoprotein ligand–1 (PSGL-1) on the neutrophil to P-selectin on the EC and the neutrophils rolls on the endothelial surface. (d) A second signal such as ANCA induces chemokines on the endothelial surface to bind specific leukocyte receptors, initiating inside-out signalling causing conformational change in neutrophil integrin molecules. Firm adhesion takes place as integrins bind to ligands such as intercellular adhesion molecule-1 (ICAM-1) (e) Transmigration via engagement of molecules such as junctional adhesion molecule-1 (JAM-1) allows the movement of the neutrophil into the tissues towards chemoattractants.

2.5.2   Lymphocyte capture onto EC

VCAM-1 is used by peripheral blood lymphocytes for capture onto EC monolayers. Endothelial VCAM-1 can support capture from flow and rolling adhesion by interactions with lymphocyte $\alpha 4 \beta 1$ (VLA-4) integrin, and if the integrin is activated, the adhesion becomes stable and stationary. Lymphocytes can also bind CD11a/CD18 bind to endothelial ICAM-1 and this can also be used for stable adhesion and migration. The relative importance of ICAM-1 and VCAM-1 in stable adhesion and migration has not been well addressed in functional flow studies (Lalor, Clements et al. 1997).

2.5.3   Adhesion molecules and chemokines

2.5.3.1 P- selectin, E-selectin and L-selectin

E-selectin was first identified in 1987 as a molecule which, when blocked, reduced the adhesiveness of polymononuclear leukocytes and HL-60 cells (a promyelocytic cell line) to human ECs. It was initially named endothelial leukocyte adhesion molecule-1 (ELAM-1) (Bevilacqua, Pober et al. 1987) and changed to E-selectin in 1992. E-selectin was found to bind human neutrophils (Hession, Osborn et al. 1990) and eosinophils (Kyan-Aung, Haskard et al. 1991) on EC that had been activated by IL-1β, TNFα or LPS. This induction peaked at 5 hours and remained for 24 hours. Inhibition of leukocyte adhesion by blocking E-selectin was found to be additive with the addition of blockade of CD18 (Leeuwenberg, Jeunhomme et al. 1990).

Selectins are a family of cell adhesion molecules which are single chain transmembrane glycoproteins. In interacting with their ligands, the distal lectin-like domain of the selectin binds to carbohydrate groups presented on ligands on the leukocyte, which slows the cell and causes it to roll.

Three selectin genes have been identified, L-selectin, constitutively expressed on all leucocytes and whose ligands are CD34 and GLYCAM-1; E-selectin expressed on activated ECs binding to its ligand (known to bind to sialyl Lewis X on polymorphonuclear leukocytes (Munro, Lo et al. 1992) and to cutaneous lymphocyte antigen on T cells (Rossiter, van Reijsen et al. 1994)), and P-selectin expressed on platelets and ECs which binds to P-selectin glycoprotein ligand-1 (PSGL-1) (Kevil and Bullard 1999).

P-selectin is contained in Weibel-Palade bodies in platelets and in EC, along with vWF. Weibel-Palade bodies are not transcription controlled and can be recruited very quickly by histamine and thrombin and rapidly brought to the cell surface (Weller, Isenmann et al. 1992). The function of P-selectin is to capture leukocytes from flow onto the surface of ECs.

E-selectin is not stored but undergoes *de novo* synthesis in response to TNFα and IL-1β over several hours (Savage, Brooks et al. 1997). There is some evidence that certain types of endothelium, such as dermal endothelium, may have constitutively expressed E-selectin (Chong, Murphy et al. 2004). E-selectin captures neutrophils, monocytes and some subsets of T cells.

In normal renal tissue, P-selectin expression is absent. E-selectin expression is also absent but can be induced in cultured glomeruli by exposure to IL-1β, TNFα and Interferon-γ (Savage, Brooks et al. 1997).

The significance of selectins in the diseased glomerulus is not clear. In acute glomerulonephritis, P-selectin can be detected in glomeruli but this observation is complicated by the fact that this is predominantly on platelets within the glomeruli (Li, Zhou et al. 1997). P-selectin mRNA signals have been observed on capillary or venous endothelium in the interstitium, but not in glomeruli (Segawa, Wada et al. 1997).

The absence of selectin molecule expression in the glomerulus has been proposed as a protective mechanism against the development of glomerulonephritis (Harari, Marshall et al. 2001). P-selectin up-regulation in the interstitium has been associated with interstitial fibrosis and tubular atrophy (Li, Zhou et al. 1997) and selectins are increased in diabetic nephropathy (Hirata, Shikata et al. 1998). When GBM antibody was injected into mice, P-selectin increased on GEC within 30 minutes resulting in neutrophil recruitment. This was not complement or platelet dependent (Tipping, Huang et al. 1994). In a lupus model in mice, increased E-selectin, P-selectin and VCAM-1 mRNA in glomeruli were observed during development of glomerular lesions, and related to the severity of lesions (Nakatani, Fujii et al. 2004).

In contrast to these studies, P-selectin deficient mice were shown to have more severe glomerular damage in a model of glomerulonephritis (Rosenkranz, Mendrick et al. 1999). Using chimeric mice, this group showed that P-selectin on the endothelium was predominately responsible for protection from exacerbating disease. They also found that P-selectin was not expressed in the endothelium of the glomerulus and concluded that the protective effect of P-selectin may be accounted for by shedding by non-renal ECs.

P-selectin blockade made no difference in leukocyte adhesion in the glomerulus in the rat intravital model (Little, Pusey et al. 2005).

2.5.3.2 $\beta2$ Integrins and ICAM-1

As the neutrophil is engaging via ligands on its surface to selectins on the EC, chemokines bind specific leukocyte receptors, initiating inside-out signalling that brings about changes in the configuration of neutrophil integrin adhesion molecules.

Integrins are cell surface receptors with α and β subunits. There are four principle adhesion proteins that are heterodimeric, consisting of a common β subunit non-covalently attached to α subunits (CD18/CD11a-d). The integrins expressed on neutrophils are the $\beta2$ integrins: CD11a/CD18 (leukocyte- function associated antigen-1: LFA-1) which binds to ICAM-1 on the endothelial surface and CD11b/CD18 (Mac-1) which binds to ICAM-1, fibrinogen and factor X. ICAM-1 is

expressed constituitively and further upregulated by IL-1β and TNFα within 6 hours and lasts up to 72 hours (Mayadas and Cullere 2005).

The importance of integrins in neutrophil mediated endothelial damage has been known for some time (von Asmuth, van der Linden et al. 1991). In a model of TNFα activated neutrophils on EC, blocking the CD18 beta chain consistently prevented neutrophil activation, blocking the CD11b alpha chain decreased adhesion of the neutrophils to EC but did not reduce EC detachment or hydrogen peroxide release (von Asmuth, van der Linden et al. 1991). The β2 integrins have been implicated in AAV where blocking them prevented leukocyte adherence (De Vriese, Endlich et al. 1999). Polymorphisms in the CD18 gene may lead to increased adhesiveness of neutrophils in vasculitic patients (Meller, Jagiello et al. 2001). Single nucleotide polymorphisms were identified as having an association with MPO-ANCA vasculitis and these were proposed to have a dose dependent increase in adhesion of the neutrophil via CD18 facilitating degranulation and respiratory burst in these patients.

In mice, integrins have also been suggested to be involved in the capture of neutrophils, rather than selectins. LFA-1 was reported to be involved in the adherence of neutrophils and Mac-1 in neutrophil crawling (Phillipson, Heit et al. 2006).

Engagement of leukocyte integrins also affects functional response of immunological cells via outside–in signalling and many of the processes are mediated by molecules used in the classical immunoreceptor signalling pathways.

Intercellular adhesion molecule-1 (ICAM-1; CD54), a membrane glycoprotein, is an adhesion molecule which binds to the $\beta 2$ integrins. It is expressed on a variety of cells such as B and T lymphocytes, dendritic cells, macrophages, fibroblasts, keratinocytes and ECs. It is responsible for the conversion of rolling to stationary adhesion of neutrophils and is up-regulated by cytokines such as TNFα and IL-1β (Abbas and Lichtman 2003).

2.5.3.3 VCAM-1 and VLA-4 (Very late antigen 4)

Vascular adhesion molecule-1 (VCAM-1; CD106) is a cell surface sialoglycoprotein mediating leukocyte-EC adhesion and signal transduction. It is involved in the adhesion of lymphocytes, monocytes, eosinophils and basophils. It is expressed on ECs after stimulation by cytokines and is transcription up-regulated, being detectable after 8 hours and lasting for over 24 hours (Abbas and Lichtman 2003). VCAM-1 binds via the $\beta 1$ integrin VLA-4 (CD49dCD29). VCAM-1 mRNA and protein are induced via an NFκB dependent pathway (Zhou, Connell et al. 2007). Transfer of the protein to the cell surface after its manufacture is dependent on post translational modifications (Abe, Smith et al. 1999). These take place in the endoplasmic reticulum and the Golgi apparatus to convert the protein into a sialoglycoprotein by addition of various sugars and sialic acid. The sugars are added by a multitude of enzymes. The sialic acid, which is an $\alpha 2,6$-linked sialic acid is added by the enzyme Gal$\beta 1$-4GlcNAc$\alpha 2,6$-sialytransferase (ST6N), encoded by the ST6N gene (Abe, Smith et al.

1999). The addition of sialic acid may reduce the adhesiveness of the VCAM-1 protein (Abe, Smith et al. 1999).

VCAM-1 has reported to be seen in normal renal biopsies in Bowman's capsular epithelium, proximal tubule and interstitial vascular endothelium (Pall, Howie et al. 1996) and subsequently VCAM-1 has been reported to be expressed in Bowman's capsule and proximal tubules in renal tissue, but was only seen weakly on glomerular EC after upregulation by TNFα and IL-4 in combination (Savage, Brooks et al. 1997). VCAM-1 has also been reported to be induced after a 6 hour exposure to IL-1β or TNFα on cultured human GEC (Park, Chang et al. 1998; Murakami, Morioka et al. 2001). Also in cultured human GEC, TGF-β was demonstrated to blunt both TNFα induced VCAM-1 expression and IL1-β induced expression (Park, Yang et al. 2000)

In animal models VCAM-1 mRNA was increased in the kidney after uretertic ligation and immunostaining was increased in the interstium but decreased in the glomeruli (Shappell, Mendoza et al. 2000). In a mouse model of lupus nephritis, expression of VCAM-1 correlated with the severity of disease (Nakatani, Fujii et al. 2004).

In vasculitis, mouse GEC was exposed to a rabbit anti-recombinant mouse MPO antibody and this increased the expression of VCAM-1 implying that the MPO-ANCA induced activation of GEC directly (Nagao, Matsumura et al. 2007). In renal biopsies from patients with vasculitis, VCAM-1 was found in glomerular endocapillary cells and was associated with more severe lesions (Pall, Howie et al. 1996). In a subsequent study VCAM-1 was again found in vasculitis in human renal tissue (Arrizabalaga,

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Sole et al. 2008) where abnormal VCAM-1 and ICAM-1 were seen in the tubules of more than 80% of patient samples and in the glomerular tuft in more than 60%. VCAM-1 was associated with leukocyte infiltration, suggesting that VLA-4 bearing leukocytes are contributing to glomerular injury.

Very late antigen 4 (VLA-4) is a $\beta1$ integrin which binds to VCAM-1 (Abram and Lowell 2007), fibronectin and osteopontin (Khan, Allen et al. 2003) and is comprised of the $\alpha4\beta1$ subunits. The intact 150 kDa $\alpha4$ subunit can be cleaved into two non-covalently associated fragments of 70 and 80kDa each. Cleavage of this subunit which is increased following T-cell activation has been suggested to change VLA-4 functions (Teixido, Parker et al. 1992). VLA-4 has three distinct adhesion activities, it binds to a domain called CS-1 within the HepII region of fibronectin; it binds to VCAM-1 on the surface of activated ECs and it is involved in the induction of homotypic aggregation among mononuclear leukocytes and cell lines. It may also play a role in cytolytic T cell function.

The role of VLA-4 in progressive renal injury was investigated in a rat model of nephrotoxic nephritis where VLA-4 was blocked. There was a reduction in albuminuria, a higher creatinine clearance and less renal scarring in the rats who received blocking antibody, and the authors concluded that VLA-4 mediated pro-inflammatory and pro-fibrotic effects within the kidney (Khan, Allen et al. 2003).

2.5.3.4 Chemokines

Chemokines are small proteins, released locally, which control leukocyte migration. They are largely homogeneous and have four conserved cysteines with two essential disulphide bonds. Chemokines are produced under pathological conditions by tissue cells and infiltrating leukocytes (Baggiolini 1998). The family of chemokines involved with neutrophil interaction are termed CXC and IL-8/CXCL8 is a member of this group. Chemokines bind neutrophils via seven transmembrane domain G-protein coupled receptors. Neutrophils have two receptors for IL-8/CXCL8, CXC chemokine receptor 1 and 2 (CXCR1 and CXCR2). CXCR1 is involved in superoxide production and CXCR2 with neutrophil migration (Lu, Garfield et al. 2006). Chemokines initiate inside-out signalling and cause a conformational change in neutrophil integrin molecules causing firm neutrophil binding. The roles of these chemokines in AAV have been explored in the flow model (Calderwood, Williams et al. 2005).

2.5.4   Cytokines

Cytokines (Table 3) are mediators and regulators of innate and adaptive immunity, and stimulators of haematopoiesis. Many immunologically active cells produce cytokines; macrophages secrete cytokines in response to LPS from bacteria and dsRNA from viruses and T cells produce cytokines in response to foreign antigen. Cytokines regulate lymphocyte populations by recruiting, activating and regulating leukocytes such as neutrophils. Additionally cytokines act on EC and leukocytes to stimulate inflammatory reactions to microbes. Cytokine receptors are transmembrane

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proteins. Signalling pathways are activated by ligand-induced receptor clustering (Abbas and Lichtman 2003).

Cytokine	Size	Cell source	Biological effects	Receptor
TNFα	17kD 51KD homotrimer	Macrophages T cells	EC activation Neutrophil activation Hypothalamus (fever) Liver – acute phase protein production Muscle catabolism Apoptosis	TNF-R
IL-1β	17kD mature form 33-kD precursors	Macrophages EC some epithelial cells	EC activation Hypothalamus (fever) Liver – acute phase protein production Thrombosis (removes anticoagulation factors from EC)	2 members of IgG super family

Table 3: Cytokines relevant to this thesis

2.5.4.1 TNF alpha (TNFα)

TNFα is the principle mediator of the acute inflammatory response to gram-negative bacteria. The TNF family of proteins bind to the TNF receptor (TNF-R) which has conserved cysteine-rich extra-cellular domains. Activation of the receptor by ligand binding recruits intracellular cytoplasmic signalling molecules which can either induce inflammatory or apoptotic pathways. Specifically these cytoplasmic signalling molecules include TRADD (TNF receptor associated death domain), FADD (Fast-associated death domain) or RIP (receptor interacting protein). TRADD binds directly to the TNF-R cytoplasmic domain and can then bind FADD and caspase-8 which results in apoptosis. Alternatively TRADD binds directly to TRAF-2 (TNF receptor

associated factor 2) forming a heterodimeric complex with RIP-1 leading to NFκB activation, gene transcription and inflammation (Wajant, Henkler et al. 2001).

2.5.4.2 TNFα and IL-1β on EC

On EC, TNFα and IL-1β upregulate the expression of adhesion molecules such as selectins, ICAM-1 and VCAM-1. After ligand binding, they both cause vasodilatation by production of prostacyclin (PGI$_2$). EC and macrophages secrete chemokines in response to TNFα which result in leukocyte chemotaxis and recruitment. Mononuclear phagocytes are induced to secrete IL-1β. TNFα additionally induces apoptosis of some cell types (Abbas and Lichtman 2003). IL-1β causes production of nitric oxide and endothelin.

Neutrophils do not interact with an endothelial monolayer that has not been activated, and in models, activation by TNFα has demonstrated increasing capture of neutrophils with increasing concentrations of TNFα (Bahra, Rainger et al. 1998). These captured neutrophils additionally demonstrated increasing static adhesion and migration at increasing doses of TNFα. The major change in adhesive behaviour was prevented by blockade of CD18, demonstrating the key role of β2 integrins. At low concentrations of TNFα, E-selectin was predominant in capturing cells from the circulation, while at high concentrations both E-selectin and P-selectin were important. Study of the kinetics of these steps suggested that although the rolling velocity of the cells was dependent on TNFα concentration, neither the rate at which

the cells became activated nor the time spent moving over, through and under the EC was strongly influenced by TNFα concentration (Luu, Rainger et al. 1999).

TNFα and IL-1β levels are high in patients with AAV (Grau, Roux-Lombard et al. 1989). TNFα has been investigated as a potential therapeutic target (Huugen, Xiao et al. 2005) and IL-1β has been found in glomeruli of AAV patients (Noronha, Kruger et al. 1993). These cytokines were therefore chosen for investigation in this thesis.

EC can be treated with cytokines resulting in stimulation or activation. Stimulation results in the immediate release of substances already present in the cell, e.g. release of P-selectin and vWF by histamine. Activation results from transcriptional up-regulation and production of molecules such as ICAM-1 and VCAM-1, 12-24 hours after treatment with cytokines such as TNFα.

2.5.4.3 Hydrogen peroxide

Hydrogen peroxide is not a cytokine but when EC are exposed to this chemical, a product of activated neutrophils, irreversible injury and death of EC may occur. At low concentrations of hydrogen peroxide EC is activated (Bradley, Johnson et al. 1993). Treatment with 50 to 100μmol/L of hydrogen peroxide has been shown to selectively increase expression of ICAM-1 and MHC class 1 but not E-selectin or VCAM. It is also thought that hydrogen peroxide inhibits TNFα induced gene expression (Bradley, Johnson et al. 1993). Sub-injurious concentrations of hydrogen peroxide

can therefore activate endothelium but not in the same way as inflammatory cytokines.

2.5.5   <u>EC and AAV</u>

In AAV the interaction of the neutrophil with the EC is thought to involve an activated endothelium whereby selectin receptors and chemokines are up-regulated attracting neutrophils on which E-selectin has been induced by TNFα. The neutrophil after rolling encounters a second signal causing integrins to bind molecules such as ICAM-1 and resulting in firm adhesion. The neutrophil then transmigrates into the tissue, or degranulates at the endothelial surface resulting in inflammation.

ANCA may be involved at several points in this process of leukocyte-endothelial interaction (Figure 4). Traditionally, it has been thought that ANCA activate neutrophils with resultant release of superoxide or proteases at the EC surface thus resulting in EC damage, although some studies have suggested that ANCA may interact directly with EC (Muller Kobold, van Wijk et al. 1999). We shall now examine the evidence for the effect of ANCA on various steps of leukocyte-endothelial interaction.

2.5.5.1 The effects of serine proteases on EC

Elastase and PR3 (but not MPO) have been shown to be potent inducers of EC apoptosis (Yang, Kettritz et al. 1996). These proteases are released following ANCA-induced neutrophil degranulation. Yang et al have shown that PR3 and MPO are

internalised by EC, and that PR3 causes these cells to undergo apoptosis (Yang, Preston et al. 2001). This occurs via direct cleavage of p65 NF-κB, resulting in its diminished transcriptional activity, and suggests that PR3 and elastase have specific fundamental roles in EC responses during inflammation (Preston, Zarella et al. 2002). It has been suggested that, far from their role being one of simple degradation, proteases such as PR3 play a role in crosstalk between leukocytes and EC. As these proteases enter EC they can influence signalling pathways by intervening in caspase cascades and by activating pro-apoptotic signalling events (Pendergraft, Rudolph et al. 2004).

In unpublished work from our laboratory, high concentration PR3 was, as above, found to damage EC after prolonged exposure. However, when EC were exposed to concentrations that did not affect EC integrity, PR3 activated the EC and caused it to support the adhesion of neutrophils. This emphasises the complex interplay between proteases and EC and hints at subtle mechanisms by which circulating ANCA may indirectly alter endothelium.

2.5.5.2 Inhibition of respiratory burst and serine proteases by EC

Neutrophils stimulated by ANCA in suspension undergo a respiratory burst (superoxide response) and release proteases such as PR3 (Falk, Terrell et al. 1990). However, Zhao and colleagues have suggested that EC can down-regulate the superoxide response of neutrophils by the suppression of NADPH oxidase activity (Zhao, Benard et al. 2003), suggesting that this ANCA-induced effect may actually be blunted at the endothelial surface. Subsequently, Zhao et al found that the adhesion-

mediated suppression of the oxidase response could be restored to its previous rapid onset if the neutrophils had had prior exposure to cytokines and inflammatory mediators, such as TNFα (Zhao and Bokoch 2005).

In order to evaluate potential mediators of endothelial injury in vasculitis, the factors controlling the neutrophil respiratory burst and endothelial release of vWF were explored (Lu, Garfield et al. 2006). These experiments compared neutrophil interaction with fMLP, normal IgG and ANCA, on both EC and P-selectin surfaces, measuring vWF release by ELISA and serine protease activity enzymatically. EC inhibited the superoxide release from those neutrophils stimulated with both fMLP and ANCA, a finding that was reversed by the addition of adenosine deaminase, which rapidly degrades adenosine. The authors concluded that adenosine was released from EC to inhibit the potentially deleterious effects of superoxide and that it was therefore unlikely that ANCA-induced neutrophil release of superoxide radicals resulted in EC injury *in vivo*. In line with the study described above by Zhao, TNFα activation of EC did reduce the level of inhibition but, overall, this was a very small effect.

In these experiments, serine proteases were also released by ANCA during neutrophil–endothelial co-culture and thought to be mediating EC damage (as quantified by the release of vWF). Diisopropylflurorphosphate, a serine protease inhibitor, abolished this vWF release, indicating that EC damage in the context of circulating ANCA is likely to be mediated by serine proteases such as PR3 rather than superoxide.

2.5.5.3 Direct effect of ANCA on EC

ANCA may act directly on ECs and in the presence of EC the interaction of neutrophils and ANCA may be different to the action of neutrophils in suspension. ANCA from patients with AAV have been found to directly activate EC causing expression of E-selectin on the cell surface (Muller Kobold, van Wijk et al. 1999). However, these studies have not subsequently been confirmed, and indeed we were unable to reproduce this response (unpublished data).

ANCA binding to EC via Fab portion

Early studies found that TNFα and IL-1β induce PR3 expression in the cytoplasm of EC, and that this is translocated to the EC surface, thereby becoming accessible to ANCA (Mayet, Csernok et al. 1993). Affinity purified PR3-ANCA can bind to expressed PR3 and activate EC, an effect that can be blocked by co-incubation with purified PR3 (Mayet and Meyer zum Buschenfelde 1993; Sibelius, Hattar et al. 1998; De Bandt, Meyer et al. 1999). In addition, PR3 is present in serum (Henshaw, Malone et al. 1994) and thus could potentially bind to EC, complexing with ANCA. However, subsequent studies have not been able to find PR3 expression by cultured EC (King, Adu et al. 1995; Pendergraft, Alcorta et al. 2000). It may be that it is easily shed from EC during handling of these cells or that ANCA are interacting with PR3 that is bound to the EC, rather than the protease being manufactured endogenously within them.

The hypothesis that MPO-ANCA are capable of causing damage to glomerular EC (GEC) was tested by incubating mouse GEC with a polyclonal rabbit anti-recombinant mouse MPO antibody (Nagao, Matsumura et al. 2007). These workers found that mRNA for ICAM-1, VCAM-1 and E-selectin were up-regulated by 12.5, 7.5 and 10.5 fold respectively, although they did not report any endothelial damage and did not exclude an effect of lipopolysaccharide. The potential mechanisms of this EC activation are not clear as it has not been possible to demonstrate the presence of MPO on EC.

ANCA binding to EC via Fc portion

ANCA binding directly to the EC via its Fc portion would require that there were Fc receptors on glomerular cells. There are very few reports of this in the literature (Aarli, Matre et al. 1991), but Fc receptors are know to be present on liver EC (Muro, Shirasawa et al. 1993) and FcγR expression has been reported to be up-regulated on cytokine stimulated aortic EC (Pan, Kreisle et al. 1998). Based on work in our laboratory (unpublished) it appears unlikely that Fc receptors are present on GEC and, therefore, that ANCA would be interacting with GEC in this way.

2.5.5.4 Does PR3-ANCA inhibit the enzymatic reaction of PR3?

There have been some suggestions that PR3-ANCA might bind to and thereby inhibit the action of the enzyme PR3 (van der Geld, Tool et al. 2002). This appears to be dependent on the site at which the ANCA binds, and may therefore imply that that some PR3 specific ANCA may not be pathogenic. In results from our lab

(unpublished) the chimeric PR3-ANCA which is directed against PR3 does not inhibit the action of the enzyme. The chimeric PR3-ANCA binds at the 4A5 site on the PR3 enzyme (the 4A5 site is defined as that region of the protein where a mouse anti-PR3 antibody called 4A5 binds (Colman, Hussain et al. 2007)), thus implying that binding at this site does not inhibit the enzyme.

2.5.5.5 Where does ANCA-induced neutrophil degranulation occur?

It has been suggested that degranulation of neutrophils occurs within or at the wall of the blood vessel since ANCA and neutrophils interacting in the blood steam would be cleared rapidly by the reticuloendothelial system. As described above, neutrophils interact with selectins on the blood vessel wall in the presence of inflammatory mediators and roll on the surface of the EC layer. Under these circumstances, interaction with ANCA could cause the neutrophil to become abnormally activated, causing it to degranulate and move through the vessel wall. It is possible that EC injury occurs if the transmigrating cell is actively degranulating at the same time. Once in the tissue the neutrophils may undergo dysregulated (pro-inflammatory) apoptosis (Harper, Radford et al. 2001) and form a nidus for inflammation. There is evidence demonstrating neutrophil lysosomal enzymes binding to EC and basement membranes in renal biopsies of patients with AAV (Brouwer, Huitema et al. 1994). However, there is currently no firm evidence indicating at what stage ANCA binds to the neutrophil or where ANCA-induced degranulation actually occurs.

If neutrophils were degranulating in the general circulation it would be likely that an increase in PR3 and other neutrophil components would be detected. This has been

reported in vasculitis, where disease controls had lower levels of PR3 (Ohlsson, Wieslander et al. 2003). Whether this is specific to vasculitis or a feature of inflammatory disease in general is, however, yet to be established. The effects of protease damage or activation must depend on the protease/anti-protease balance, and it is likely that only proteases present in the protected microenvironment between an adhering PMN and an EC would be able to do damage. A paper which looked at the interaction between elastase, protease-3 and their inhibition by α-1antitrypsin, found that α-1antitrypsin had a higher affinity for elastase, which was therefore neutralised more quickly than PR3. PR3 was therefore able to diffuse further and do more damage than elastase (Duranton and Bieth 2003)

Transmigration of neutrophils in the glomerulus

Neutrophils recruited to the glomerulus during inflammation may consequently have several fates. They may stay within the vasculature, degranulate here and cause local destruction to the vessel wall. Deformation and shape change could potentially result in clogging of the small capillaries. Alternatively they may migrate through the vessel wall, either into Bowman's space, where they would form a component of the debris in the crescent, or into the mesangium where they would form a nidus for inflammation and further recruitment of inflammatory cells. They could then potentially migrate back into the circulation, or into the lymph system. There is so far no direct evidence for transmigration of neutrophils in AAV. Animal models have not visualised the kidney by direct intra-vital microscopy due to technical difficulties. Neutrophils are not usually found in kidney biopsies of patients with AAV, although in very early disease they are occasionally seen.

The most compelling evidence that neutrophils migrate into Bowman's capsule and are present in acute lesions in AAV, comes from the elegant animal model by Xiao et al, who demonstrated glomerular accumulation of neutrophils and macrophages (Xiao, Heeringa et al. 2005). There is no current direct evidence for transmigration of neutrophils, but in previous work in the flow model, rather than frustrating the migration of neutrophils, ANCA appeared to promote it (Radford, Luu et al. 2001).

2.5.6   Anti-endothelial antibodies

Anti-EC antibodies (AECA) have been documented (but not well characterised) in AAV (Table 4) and other diseases. Some workers suggest that AECA are pathogenic in AAV (Praprotnik, Rozman et al. 2000; Guilpain and Mouthon 2008), and may be associated with propylthiouracil induced AAV (Yu, Zhao et al. 2005). It has been proposed that AECA bind to EC in vasculitis and that these then activate the EC, as well as engaging neutrophils from the circulation via their Fc portion. AECA in vasculitis patients have been shown to induce IL-1β on EC (Carvalho, Savage et al. 1999) and have also been shown to induce apoptosis (Bordron, Dueymes et al. 1998), although this effect on apoptosis could not be replicated in our laboratory (Williams, Colman et al. 2005).

Several studies support the concept of anti-endothelial antibody binding, including work from our laboratory (Savage, Pottinger et al. 1991; Del Papa, Guidali et al. 1996; Muller Kobold, van Wijk et al. 1999), although the antigen(s) that these

antibodies are binding to remains unclear. Reports of the proportion of patients with AAV who have AECA vary in the literature from 20% to 80% (Savage, Pottinger et al. 1991; Chan, Frampton et al. 1993; Varagunam, Nwosu et al. 1993; Holmen, Elsheikh et al. 2005; Yu, Zhao et al. 2005; Oostingh, Schlickum et al. 2007; Sebastian, Mahr et al. 2007) (Table 4). Recent work examining a large cohort of AAV patients found that disease activity did not correlate with the presence or the titre of AECA, and was only positive in 20% of patients (Sebastian, Mahr et al. 2007). However, a subsequent study reported a high incidence (71%) of antibodies against GEC and demonstrated that, when the same samples were tested against HUVEC (the substrate used in earlier studies), they were detected in only 7% of samples (Holmen, Elsheikh et al. 2005). AECA may be additive to ANCA in the injury to EC, and there is evidence that they augment neutrophil/EC adhesion (Florey, Johns et al. 2007). It may be that the presence of AECA is therefore important in AAV and, in view of this study, warrants further investigation. It is possible that AECA bind to ECs from different locations to a different extent (Praprotnik, Blank et al. 2001). AECA in large vessel diseases such as Takayasu's arteritis bound to and activated HUVEC but not microvascular EC.
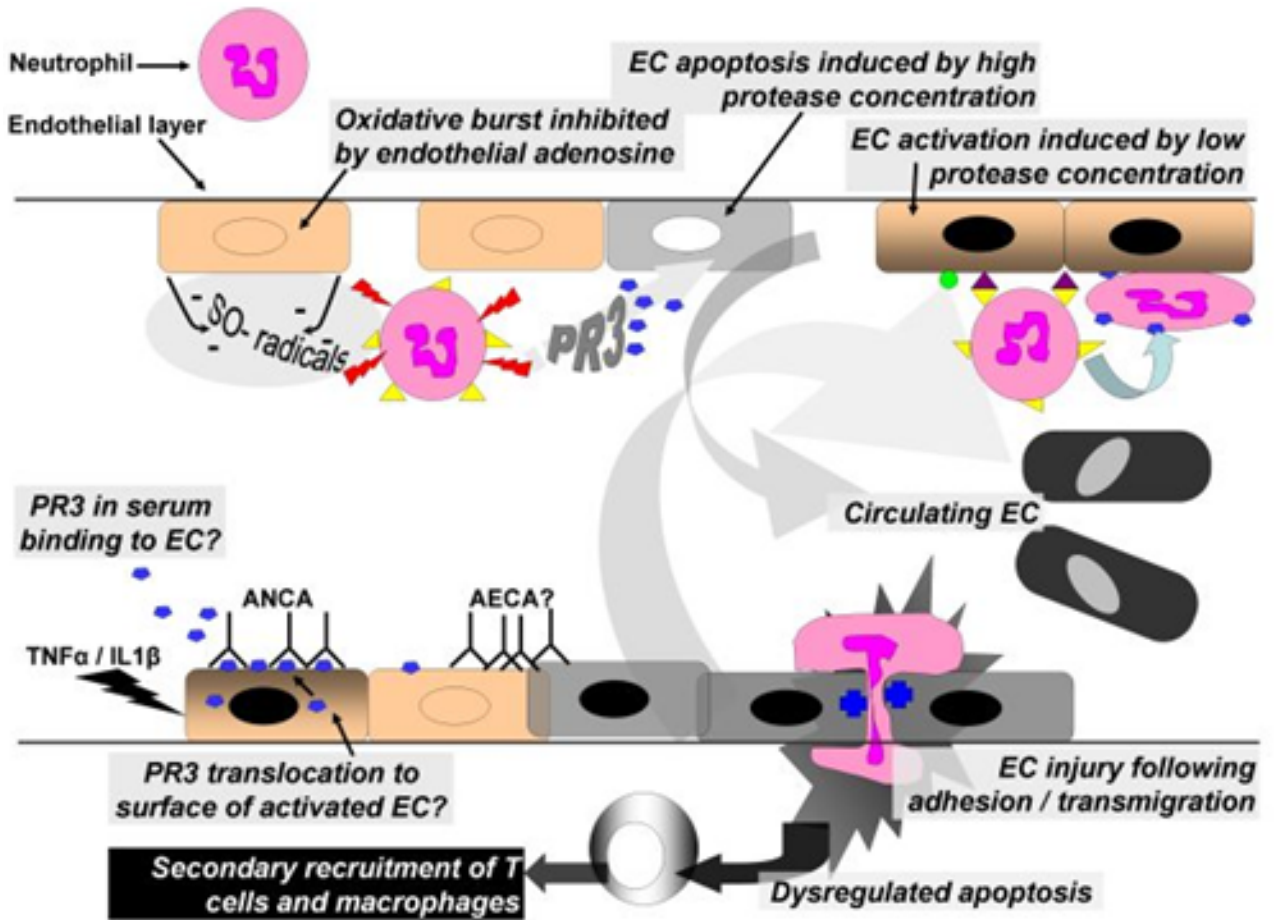
Figure 5: Potential mechanisms of endothelial injury/activation in ANCA-associated vasculitis.

Reference	Method of detection	Study group	AECA type and frequency	Correlation with disease
Savage, 1991 (Savage, Pottinger et al. 1991)	HUVEC	168 WG	59% IgG AECA 68% IgM AECA	Not investigated
Varagunam, 1993 (Varagunam, Nwosu et al. 1993)	HUVEC	43 MPA 27 WG	2% MPA 19% WG	Not investigated
Chan, 1993 (Chan, Frampton et al. 1993)	Not defined	10 AVV	80%	yes
Holmen, 2004 (Holmen, Elsheikh et al. 2005)	HUVEC, HKEC and others	10 WG 5 MPA 20 control	WG: HUVEC 7% / HKEC 71% MPA: HUVEC 0% / HKEC 60% Control: HUVEC 1% / HKEC 5%	yes
Yu, 2005 (Yu, Zhao et al. 2005)	HUVEC	11 PTU induced AAV	91%	yes
Sebastian, 2007 (Sebastian, Mahr et al. 2007)	HUVEC	173 WG	20%	no

Table 4: Summary of Literature of anti-EC antibodies (AECA).

HUVEC = human umbilical vein EC; HKEC = human kidney ECs; WG= Wegener's Granulomatosis; MPA= microscopic polyangiitis; PTU = propylthiouracil; AAV = ANCA associated vasculitis

2.5.7   Flow model of of leukocyte-endothelial cell interactions

Detailed explanation of the flow model is undertaken below in methods. Briefly it is a system whereby adhesion molecules, platelets or EC can be coated on or grown

within glass capillaries and can be viewed directly whilst neutrophils or other leukocytes are flowed through. The effects of the addition of ANCA and other substances added into the system can then be observed.

ANCA-neutrophil interactions have been studied previously in flow models using human platelets (Radford, Savage et al. 2000) and EC (Radford, Luu et al. 2001; Calderwood, Williams et al. 2005) as the adhesive substrate. In the platelet model, P-selectin was presented by the platelet monolayer and neutrophils rolled continuously on it. When ANCA were introduced to the system, rolling was converted to stationary adhesion, which was accompanied by neutrophil shape change. Antibodies blocking the Fcγ receptor IIa (CD32) or the integrin CD11b completely inhibited ANCA-mediated conversion. In the EC model, following activation of the EC by TNFα, neutrophils rolled on the surface of the monolayer and the subsequent addition of ANCA caused changes in shape and increased adhesion of neutrophils, with subsequent migration through the endothelial layer (Radford, Savage et al. 2000).

The roles of the integrins CD11a/CD18 and CD11b/CD18, and the chemokine receptors CXCR1 and CXCR2 were investigated in the same model using highly activated endothelium (treated with TNFα 100units/ml, which induces firm adhesion of the neutrophil to the EC monolayer) and minimally activated endothelium (treated with 2 units/ml of TNFα, where neutrophils roll but need a further stimulus to stop) (Calderwood, Williams et al. 2005). CD11a/CD18, CD11b/CD18 and CXCR2 were important in the adhesion and migration of neutrophils on both highly and minimally activated endothelium, whereas CXCR1 had minimal effect. However, when the

endothelium was only minimally activated with TNFα, only CD11b/CD18 stabilized the adhesion caused by the addition of ANCA. This illustrates the importance that these specific integrins and chemokines have in the interaction of ANCA-induced neutrophils with EC.

3. HYPOTHESES OF THE PHD THESIS

The hypotheses of this thesis are:

- ANCA IgG of different subclasses vary in their ability to activate neutrophils and change their interaction with ECs.

- The Fc receptor has a differential role when neutrophil interaction with ANCA IgG is compared to neutrophil interaction with IgG not containing ANCA

- Glomerular ECs are targeted in AAV because of a distinct phenotype

- It may not be relevant to use HUVEC as a substitute for GEC when investigating AAV.

4. MATERIALS AND METHODS

4.1 Cell and reagent preparation

4.1.1 Neutrophil Isolation

Blood from healthy volunteers was collected into tubes containing EDTA anticoagulant (Sarstedt, Numbrecht, Germany). Neutrophils were isolated using a two step density gradient. Whole blood was layered over equal volumes of Histopaque-1077 and Histopaque-1119 (Sigma, UK) and centrifuged at 2500rpm for 30 minutes. The middle neutrophil fraction was harvested and washed twice in phosphate buffered saline (PBS) (Invitrogen, UK) with calcium and magnesium, which had bovine serum albumin (BSA) (Sigma, UK) added to a dilution of 7.5%. The neutrophils were then re-suspended in 1 ml of PBS/BSA, counted using a haemocytometer and then re-suspended to a concentration of 1×10^6 cells per ml in PBS/BSA.

Neutrophils were not usually activated by their isolation, and if they were experiments were abandoned. It was relatively easy to assess whether neutrophils had been activated because when they were introduced into the flow model unstimulated by ANCA they appeared round and rolled on the EC surface or remained in the through flow. Activation of neutrophils resulted in shape change, they appeared 'spiky' and adhered quickly to the EC.

4.1.2   Peripheral blood lymphocyte isolation

Peripheral blood lymphocytes were isolated in the same way as neutrophils except the top mononuclear cell fraction was harvested. After washing, the cells were plated into small petri-dishes and left at 37°C for 20 minutes allowing monocytes/macrophages to stick to the plastic. The rest of the cells were collected and re-suspended to a concentration of 1×10^6 cells per ml as above.

4.1.3   Patient Immunoglobulin G isolation

ANCA positive IgG was isolated from plasma exchange fluid taken from patients during the active phase of Wegener's Granulomatosis or Microscopic Polyangiitis. Patients accorded with the Chapel Hill Consensus Conference definitions for vasculitis (Jennette, Falk et al. 1994) and all had severe disease (creatinine >500mmol/L or pulmonary haemorrhage), with evidence of renal vasculitis confirmed histologically by renal biopsy. PR3 or MPO-ANCA was confirmed in patients by indirect antigen specific ELISA and indirect immunofluorescence.

Plasma exchange fluid was aliquoted into 50ml samples. In order to remove clotting factors it was heated to 37ºC with 1ml of 1 molar calcium chloride (Sigma, UK) and the precipitant removed. The resultant serum was diluted 1:1 in PBS, 0.2μm filtered and passed manually through a 5ml volume HiTrap Protein G column (Amersham Biosciences, GE Healthcare, UK). 0.1molar glycine, pH 2.6 (Sigma, UK) was used to elute the IgG off the column and was neutralised with 1 ml of 1 molar Tris base

(Sigma, UK), pH 9.0. The sample was placed into pre boiled dialysis tubing (Medicell International Ltd.) (10-14kDa cut off) and dialysed in PBS at 4ºC overnight. The sample was then spun through a concentration column to approximately 1mL volume using vivaspin concentrators (Vivascience, Hannover, Germany) with a 10,000Da cut off, and stored at 4˚C for up to one month.

The concentration of the eluted IgG was determined by spectroscopy. IgG samples were diluted 100x and transferred to a quartz cuvette. The absorbance was measured at 280nm using PBS buffer as a blank. The protein concentration of the samples was determined using the following equation (derived from the Beer Lambert law): $A = \varepsilon \times C \times L$ (A = absorbance; ε = molar extinction coefficient; C = concentration; L = length of the light path.) The molar extinction coefficient for IgG at 280nm is 1.43 and the light path of a 1ml cuvette is 1cm. Protein concentrations using this method are expressed in mg/ml.

Normal control IgG was obtained from healthy volunteers. After consent blood was taken from a pool of at least 10 volunteers. The whole blood was then left to stand and separate, and the serum removed from the top of the sample. This was then combined into a pool and the serum processed as described above to isolate normal IgG.

4.1.4 Chimeric PR3-ANCA and control

Chimeric PR3-ANCA subclasses 1, 3 and 4 were developed in our laboratory prior to the current work commencing. Mouse/human chimeric IgG1, IgG3 and IgG4 monoclonal ANCA are directed against a single epitope on the PR3 molecule that is recognized by a mouse monoclonal antibody against human PR3, called 4A5 (Colman, Hussain et al. 2007).

Hybridoma cells (where fusion of a myeloma cell and normal cell line is produced that is capable of producing antibodies) expressing 4A5 were used to synthesize cDNA using a first strand cDNA synthesis kit. The mouse variable regions were amplified by PCR using 5' primers specific to the mouse variable region leader sequences and 3' primers specific to the mouse constant region. Fresh PCR products were cloned into sequencing vectors and transformed into E.coli. PCR screening was performed to select clones containing PCR products and plasmid DNA purified from positive clones. Automated sequencing was carried out and homology searches performed using BLAST software to confirm that mouse light and heavy chain variable regions had been amplified. The mouse light and heavy chain variable regions were subcloned into vectors containing the appropriate constant regions i.e. the IgG kappa chain or IgG1, IgG3 and IgG4 heavy chains. These were then co-transfected into CHOdhfr- cells.

Clones which produced high levels of IgG and proliferated robustly were selected and expanded into a hollow fibre bioreactor (Goodall 1998). IgG1 and IgG4 were purified

from culture supernatant using Staphylococcal protein A (SpA) coated sepharose and IgG3 purified using Streptococcal protein G (SpG) coated sepharose. IgG production was verified by ELISA, SDS-PAGE and mass spectrometry. Microtitre plates coated with PR3 were used to confirm the specificity of antigen recognition. Surface plasmon resonance was performed to ascertain antibody affinity and avidity of the chimeric antibodies to PR3 in comparison with the 4A5 anti-PR3 mouse antibody.

The chimeric PR3-ANCA were able to produce the distinct cytoplasmic staining pattern on ethanol-fixed neutrophils which is characteristic of patient-ANCA IgG using indirect-Immunofluorescence. They also recognised PR3 by ELISA and Western blot. Flow cytometry was used to confirm binding to PR3 expressed on the neutrophil cell surface.

The chimeric PR3-ANCA were compared in experiments to chimeric anti-NP (nitro-phenol) antibodies developed in the same way to the ANCA antigens (gift from Dr Margaret Goodall). Nitro-phenol is not found in the human body so is therefore an irrelevant antigen. In some experiments where difficulties with these controls were experienced, myeloma antigens of a particular subclass were used (gift from Dr Margaret Goodall).

4.1.5.1 Human umbilical vein endothelial cell (HUVEC) culture

Umbilical cords were collected with informed consent. Using a sterile technique, the cords were canulated with glass canulae and washed with sterile PBS to remove blood clots. Collagenase (Sigma) at a concentration of 1mg/ml was injected into the cords and they were incubated at 37ºC for 15 minutes. The cords were then flushed with 30ml of sterile PBS, which was collected and centrifuged at 1500rpm for 5 minutes. The pellet was then re-suspended in 4ml of medium 199 (Invitrogen, UK), which per 100ml contained 20ml of 20% heat inactivated foetal calf serum (FCS) (Sigma, UK), 10μl of hydrocortisone (Sigma, UK) 1μg/ml, 10μl of epidermal growth factor (Sigma, UK) 10μg/ml, 1ml of penicillin and streptomycin 100u/L (Sigma, UK) and 1ml of amphotericin 2.5μg/ml (Invitrogen, UK).

The isolated cells were then placed in tissue culture flasks (25cm^2; Falcoln, BD, UK/Ireland) which had been pre-coated with gelatin (Sigma, UK) and were cultured at 37ºC in a humidified CO_2 (5%) incubator. After 12 hours the medium was changed, and thereafter every 2-3 days until the cells reached a confluent monolayer.

4.1.5.2 Conditionally immortalised glomerular endothelial cells (GEC1 and 2) culture

Conditionally immortalized glomerular ECs containing a temperature sensitive transgene (simian virus 40 large tumour antigen and telomerase) have been developed by our collaborators (Satchell, Tasman et al. 2006), and were obtained

from Bristol University. Briefly, primary cell culture had been obtained from normal human kidney glomeruli that had been decapsulated by sieving. Cultures of these cells were infected with retrovirus containing supernatants from two packaging cell lines. The first, PA317 produced viral particles containing an SV40LT antigen gene containing both tsA58 and U19 mutations. This rendered the cells temperature sensitive. The second, from the packaging line TEFLY-A, produced viral particles containing hTERT, rendering the cells immortal at temperatures of 33 ºC. Cells transduced with the SV40LT construct were selected and placed at 33ºC. After reaching confluence cells also transduced with hTERT were selected with hygromycin.

They were cultured in tissue culture flasks (75cm^2, Falcoln, BD, UK/Ireland) in EBM-2 medium (Clonectics, Cambrex, UK). Exact concentrations of added reagents were unknown as the medium came in a kit, but it contained EC based medium, human fibroblast growth factor-B, hydrocortisone, endothelial growth factor vascular human recombinant, epidermal growth factor human recombinant in buffered BSA saline solution, recombinant long R insulin-like growth factor-1 in aqueous solution, ascorbic acid, gentamicin sulphate, amphotericin-B and foetal bovine serum.

When confluent (2-3 days) the cells were removed with trypsin (Sigma, UK) and EDTA (Sigma, UK), split into thirds and reseeded. Before use in experiments, the cells were grown at 37ºC for at least 30 hours to allow the SV40LT mutant to be fully arrested.

During this research two cell lines were tested, both from our collaborators in Bristol.

4.1.5.3 Primary glomerular endothelial cells (PGEC) culture

Primary cells were obtained from TSC Cellworks, UK, at passage 1. The company state that the EC are obtained by dissecting human kidney tissue and then pushing the tissue through a steel mesh to obtain glomeruli. The glomeruli are then enzyme treated and purified from primary culture using the CD31 marker.

They were grown in the EBM2 medium (as above) in tissue culture flasks ($25cm^2$ or $75cm^2$, Falcoln, BD, UK/Ireland) with pre-coating of human fibronectin (Sigma, UK) and placed in a humidified CO_2 (5%) incubator. They were grown to confluence before either splitting into 3 flasks or using in experiments. The medium was changed every 2-3 days. These became confluent in 4- 5 days.

4.1.5.4 Freezing endothelial cells

Trypsin and EDTA were used to remove cells from the flasks and they were then spun down into a pellet, to which was added 1ml of FCS with 20% DMSO (Sigma, UK). These were then placed in a cryovial and snap frozen.

4.2 Flow model methods

4.2.1 Preparation of microslides and testing with platelets

Glass microslides (rectangular cross section 0.3x3mm; length 50mm, Camlab, Cambridge, UK) were cleaned with nitric acid, washed in water and then in acetone and coated with 4% 3-aminopropyltriethoxysilane (APES) (Sigma, UK). They were then dried and autoclaved. A layer of platelets was used to check the even coating of the APES on two slides.

4.2.2 Coating microslides with P-selectin or immunoglobulin

Microslides previously coated with APES were coated with P-selectin (R&D Systems, UK) made up to the required concentration by dilution in PBS. 55μl of P-selectin solution was pipetted into the glass microslide and then incubated for 2 hours at 37ºC. Any uncoated APES on the slide was then blocked with 1% albumin (Sigma, UK) and re-incubated for a further 2 hours.

IgG coating was achieved by diluting pure human IgG (Binding Site, UK) in PBS, to the desired concentration (50-400μg/ml). This was pipetted into glass microslides as above and incubated for 1 hour at 37ºC, and APES was blocked as above.

4.2.3   Transfer of endothelial cells into microslides

The EC were grown to confluence and using sterile techniques were removed with trypsin and EDTA 0.2%. The trypsin action was stopped with medium and the cells spun at 1600rpm for 5 minutes. The pellet was re-suspended in 0.4mls of medium and drawn up into APES coated microslides in 60μl aliquots. Six slides were prepared in this way and then incubated for one hour at 37ºC allowing adherence and spreading of the ECs. The slides were then placed in a modified Petri dish full of medium (made at Birmingham University) and the slides were connected via rubber tubing to a pump which exchanged medium through the slides every hour. The endothelium was cultured in this system for 24 hours allowing formation of an endothelial monolayer.

4.2.4   Flow model set up

Flow experiments were performed in an enclosed Perspex chamber (made in house) at 37ºC (Figure 6). A microslide containing either a monolayer of ECs or coated with P-selectin was glued to a glass microscope slide, mounted on a phase-contrast microscope and viewed both in real time and recorded on video. One end of the microscope slide was attached via silicon rubber tubing to a syringe pump allowing control of the rate of flow across the microslide surface (usually 0.382mls/min resulting in 0.1Pa wall shear stress). The other end of the microslide was connected by silicon rubber tubing to an electronic valve (Lee Products, Gerards Cross, UK), which allowed switching between a reservoir of wash buffer (50ml PBS with calcium

and magnesium/1ml bovine serum albumin (BSA)) and a reservoir containing neutrophil or PBMC suspension and then subsequently any treatments used.

Once the microslide was inserted into the flow system, wash buffer was perfused to remove debris or cytokine remaining after treatment. Flow was then switched to the neutrophil/PBMC suspension and this was perfused for 4 minutes, followed by a washout of 1 minute. Video microscope recordings were then made in 4 fields along the centre of the microslide in the direction of flow. Treatment was then commenced, depending on the experiment, and further video recordings made in the same way as the first, usually at 2 minute intervals and up to 14 minutes after the initial perfusion of neutrophils.
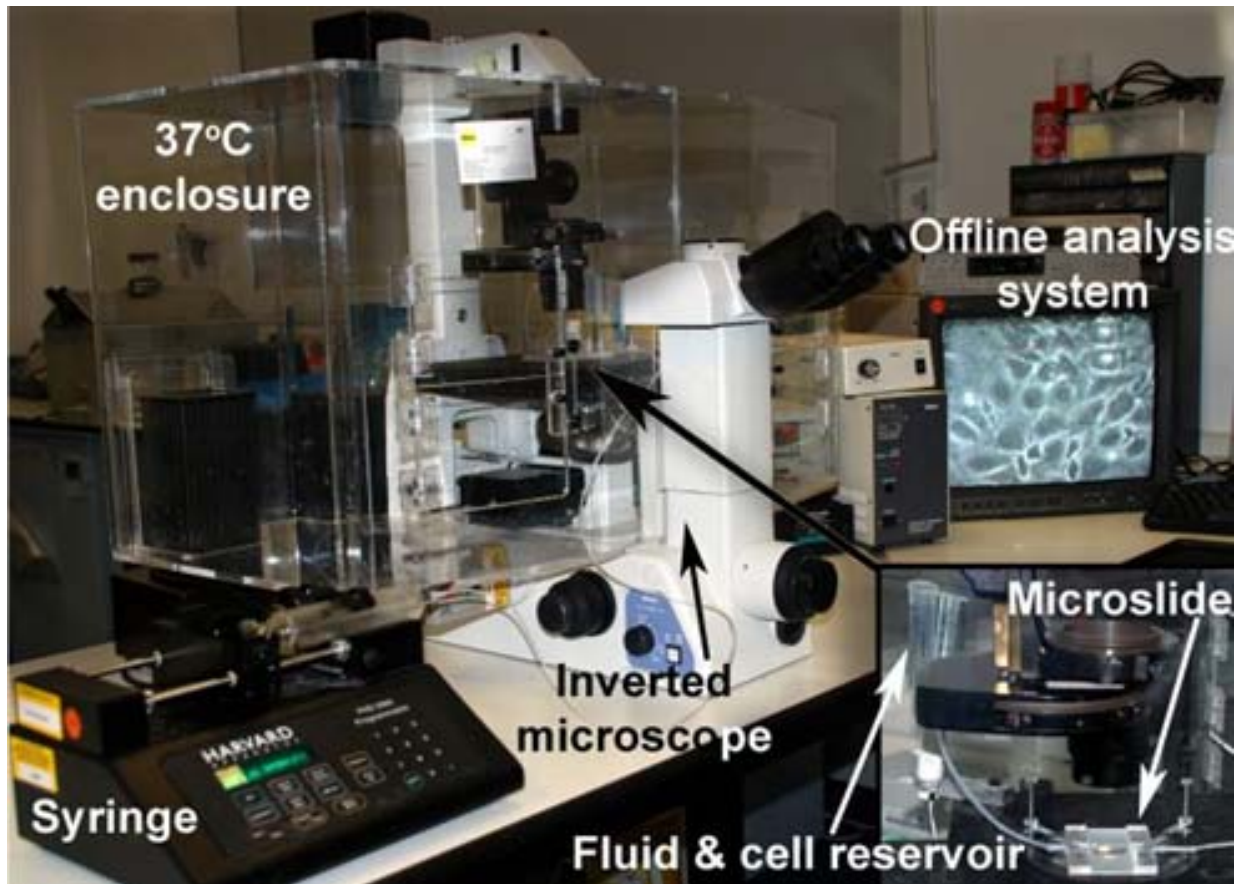
Figure 6: The flow model.

All flow experiments are performed in a Perspex box at 37ºC. The microslide, mounted on a glass microscope slide is attached to a pump and to the cell reservoir and fluid wash buffers. Events in the slide can be viewed through the microscope, recorded on video and analysed on the computer.

Video recordings were subjected to computer aided analysis off-line. Sequences of images were digitized and analysed using Image Pro-Plus software (MediaCybernetics, USA). Two populations of cells were counted on P-selectin: those rolling, which maintained their round shape and rolled on the surface and those exhibiting stable adhesion on the P-selectin surface and demonstrating shape change (Figure 7). On ECs, in addition to these populations there was another population of neutrophils which had migrated underneath the endothelial monolayer and were phase dark (Figure 7).

For each time point in the experiment, four fields had been captured and the cells in each field were counted in each population. In excel, averages were calculated for each time point and converted to neutrophils/PBMC per square mm per 10^6 cells perfused. In addition, percentage of each population was calculated, allowing expression of the relative levels of interaction (rolling, stationary-adhered and transmigrated).

On P-selectin to calculate the rolling velocity of cells, ten cells in one field were selected and start and end locations of these cells used to calculate the distance travelled in the 6 seconds of the sequence. An average was taken for the ten cells in the field.
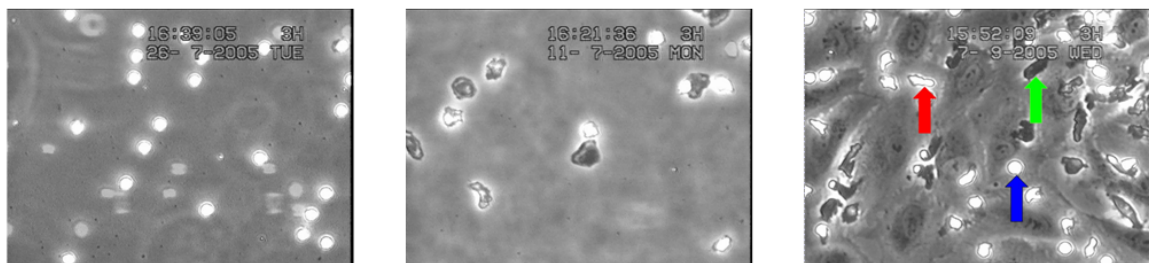
Figure 7: Neutrophil appearance.

In the flow model neutrophils rolling on a P-selectin surface appear round and white (first panel). After treatment with fMLP or ANCA the neutrophils firmly adhere and undergo shape change (second panel). On an activated EC monolayer (third panel) neutrophils roll (blue arrow), firmly adhere (red arrow) or transmigrate behind the endothelial layer (green arrow)

4.2.5   Treatment protocols

4.2.5.1 Cytokine, histamine and hydrogen peroxide

Confluent EC in microslides were treated with cytokines for four hours prior to flow assays. TNFα (concentrations between 2units/ml and 1000units/ml) (NIBSC, UK) or IL-1β (R&D systems, UK) (concentrations of 5 x 10^{-9} g/ml and 5 x 10^{-10} g/ml) were pipetted into the microslides, incubated for 1 hour, and the process repeated each hour for 4 hours. Histamine (Sigma, UK) was used to treat the EC for 20 minutes at 100μmolar and 10μmolar. In other experiments histamine was also infused with the neutrophils to allow for a continuous treatment of the EC. Hydrogen peroxide (H_2O_2) (Sigma, UK) was used at a concentration of 100μMolar for up to 1 hour to upregulate selectins on the EC. Any extension to this time resulted in damage to the ECs.

4.2.5.2 fMLP (N-formyl-methionyleucylphenylalanine)

fMLP (Sigma, UK) was added to the system, after 4 minutes of inflow of neutrophils and 1 minute of wash, at a concentration of 1x 10^{-7} molar and 4mls was infused over the rolling neutrophils.

4.2.5.3 Human ANCA

In P-selectin experiments, ANCA IgG or normal IgG at 200μg/ml (a concentration previously shown to give an optimal response (Radford, Savage et al. 2000)), diluted in PSA/BSA was added to the flow assay after 4 minutes perfusion of neutrophils and

1 minute of wash out. The IgG infusion was continued for up to 8 minutes. This allowed observation of events before and after the addition of ANCA. In HUVEC experiments, ANCA IgG or normal IgG at 200µg/ml was added to neutrophils just before perfusion over ECs for 4 minutes. The bolus of cells was then washed out with PBS/BSA. ANCA or normal IgG at 200µg/ml was then perfused over the endothelium for up to 8 minutes.

4.2.5.4 Chimeric PR3-ANCA

The same protocols as above were used for chimeric PR3-ANCA and controls (anti NP) but these were used at concentrations of 60µg/ml for IgG1 and IgG4 and 48µg/ml for IgG3 so that there was equivalent molar quantities. These concentrations had previously been shown to give an optimal response in the flow model (unpublished data).

4.2.6   Experiment design for flow experiments

4.2.6.1 P-selectin titration

Microslides were coated with P-selectin (R&D Systems, UK) at 0, 0.5, 1, 2, 5µg/ml. For each microslide, neutrophils were perfused for 4 minutes. After 1 minute of wash, 4 video microscope recordings each of 15 seconds were made along the centre line of the slide. The concentrations were tested in different orders in each experiment.

4.2.6.2 fMLP on P-selectin

Microslides were coated with P-selectin at a concentration of 2μg/ml. Neutrophils were perfused for four minutes over the slide and video recordings taken as described above. fMLP was then added to the treatment chamber and perfused over the cells for 2 minutes. Four further microscope video recordings were then taken (Figure 8).
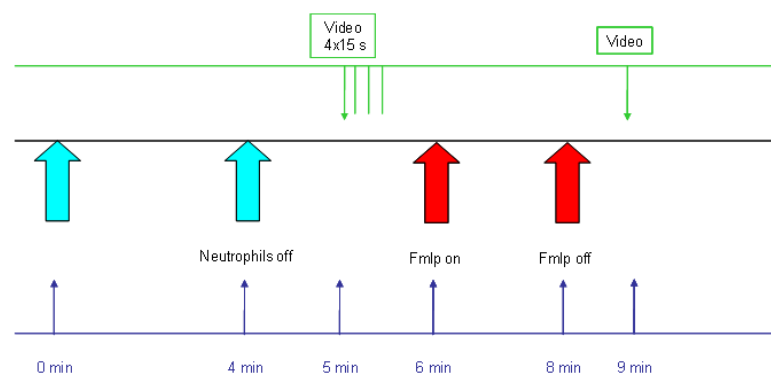
Figure 8: Experimental design for flow experiments with fMLP.

Video recordings were taken before and after fMLP was perfused over rolling neutrophils.

4.2.6.3 Immunoglobulin coating

All experiments were repeated at least 3 times. IgG1 (Binding Site, UK) was coated in increasing concentrations (50μg/ml, 100 μg/ml, 200 μg/ml and 400 μg/ml) on 4 microslides; neutrophils were flowed across for 4 minutes and video recordings taken at 5 and 10 minutes.

In the next experiments, subclasses IgG1-4 were coated at 100 μg/ml (the optimum concentration found in the gradient experiments) and neutrophils flowed across as before, video recordings were taken at 5 and 10 minutes. These experiments were then repeated using neutrophils which had been treated with anti-CD32 (clone IV.3 01470) (StemCell Technologies, France) or anti-CD16 blocking antibodies (clone 3G8) (BD biosciences, UK) both used at 10μg/ml for 30 minutes.

In the final experiments, on IgG3 coated surfaces, neutrophil were observed which had been treated with anti-CD18 at 10μg/ml for 30 minutes (clone 6.5E, gift from Dr M Robinson, Celltech, UK); both anti-CD32 and anti-CD16 together, both at 10μg/ml for 30 minutes; or with BAPTA-AM at 100mMolar for 30 minutes (Sigma, UK). BAPTA-AM was suspended in DMSO (Sigma, UK) and washed off after treatment. For the control, DMSO alone was used. For the other antibodies isotype control (mouse IgG1 isotype control) (Sigma, UK) was used as a control at 20μg/ml.

4.2.6.4 Patient and chimeric ANCA on P-selectin

Six microslides were coated with P-selectin at a concentration of 2μg/ml. Neutrophils were perfused for four minutes over the slide and video recordings taken. Previously isolated patient ANCA at 200μg/ml was added to the treatment chamber and perfused through the microslide continuously for the remainder of the experiment. Video microscope recordings were taken in four episodes every 2 minutes until 14 minutes (Figure 9). The process was repeated for 4 different patient ANCA, normal IgG and no IgG; the whole experiment was repeated at least 3 times. In experiments

using chimeric PR3-ANCA the same protocol was used, and PR3-ANCA IgG1, 3 and 4 and control chimeric NP for each subclass were perfused. Antibodies of subclass IgG1 and IgG4 were used at 60µg/ml (previously found to be a useful working concentration (Lu, Garfield et al. 2006) and IgG3 at 48µg/ml so that they were equivalent in molarity.
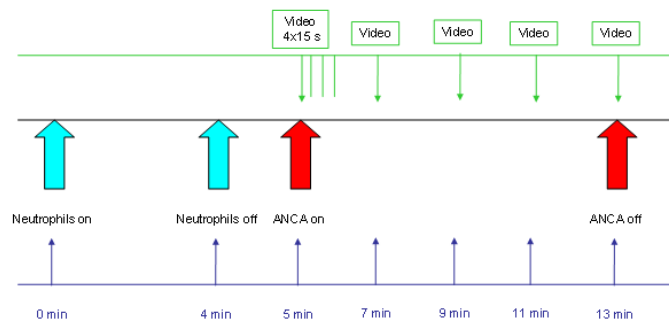
Figure 9: Experimental design for flow experiments with ANCA.

On a P-selectin surface at 2µg/ml neutrophils were perfused for four minutes. ANCA was perfused over the rolling neutrophils. Video was taken at 6 minutes, before neutrophils had been exposed to ANCA, and then at 2 minute intervals until 14 minutes

4.2.6.5 Blockade of CD32 and CD16 after chimeric ANCA on P-selectin

Experiments were undertaken to explore the role of CD32 and CD16 on neutrophils in the situation of exposure to ANCA. These experiments were limited by reagent as the chimeric ANCA were limited. The experiments on P-selectin were repeated but only two time points taken, immediately pre-ANCA and 2 minutes after exposure to ANCA. Neutrophils which had previously been treated with isotype control antibody (mouse IgG1 isotype control, Sigma, UK), or anti-CD32 (as above) or anti-CD16 (as above) were flowed across the slide and events recorded. Chimeric ANCA of

subclasses IgG1, 3 or 4 was then flowed over the slide and cell adhesion and behaviour recorded.

4.2.6.6 Endothelial cell cytokine titration

EC were grown in a monolayer in 5 microslides. These were treated with cytokines. For TNFα experiments, concentrations of 0, 1, 5, 10, 100u/ml for HUVEC, primary GEC and GEC2; 0, 100, 1000u/ml for GEC1 were applied for 4 hours. For IL-1β concentrations of 5 x 10^{-10}g/ml and 5 x 10^{-9}g/ml were applied for 4 hours. For H$_2$O$_2$, 1 millimolar was applied for 1 hour. Neutrophils were perfused for four minutes, followed by a 1 minute wash out. Video recordings were taken at 5 and 7 minutes. The process was repeated for each concentration and the order varied for each experiment repeat.

4.2.6.7 Patient and chimeric ANCA on HUVEC

Six microslides with a monolayer of ECs were treated with 100u/ml of TNFα in 3 experiments and 2u/ml in 3 separate experiments. In contrast to experiments on P-selectin, neutrophils were treated with ANCA or normal IgG (200μg/ml) just before they were perfused and in addition, ANCA or normal IgG was continuously perfused for the rest of the experiment. This was because previous published work had established these protocols were effective (Radford, Luu et al. 2001). Video microscope recordings were taken at 5 minutes and at 2 minute intervals thereafter to 14 minutes. The same protocol was used with chimeric PR3-ANCA IgG1, 3 and 4 at concentrations as in the P-selectin experiments.

4.2.6.8 Flow experiments for EC comparisons

Initial experiments compared HUVEC growing in normal medium as described above or EBM2, previously used for glomerular cells as described above. Experiments were then performed in flow stimulating the HUVEC with 100 or 2units TNFα for 4 hours and comparing neutrophil capture and behaviour as described above.

In experiments using neutrophils, three EC types, HUVEC, PGEC and GEC2 were compared. In separate experiments, these were treated with 100units or 2units/ml TNFα or 5 x10^{-9} or 5 x 10^{-10}g/L Il-1β for 4 hours, or 1 milimolar H_2O_2 for 1 hour and neutrophils flowed across. Video recordings were made at 5 minutes and 10 minutes and the capture and behaviour of neutrophils on the EC observed. The same protocol was used for experiments with PBL, where PGEC and HUVEC only were compared, EC were stimulated with TNFα at 100units/ml for 24 hours.

In blocking experiments using PBL, VCAM-1 was blocked on the EC by injecting microslides with anti-VCAM-1 antibodies (both ligands were blocked: 4B2 to domain 1 (R&D Systems, UK) and GH12 to domain 4 (a gift from Dr Roy Lobb). Both antibodies were used at 10μg/ml for 30 minutes. Isotype control (mouse IgG1 isotype control, Sigma, UK) was used as a control at 20μg/ml. In other experiments VLA-4 was blocked (MAX68P, a gift from Dr M Robinson at Celltech) on neutrophils by incubating the neutrophils with the blocking antibody at 10μg/ml for 30 minutes prior to perfusion into the flow model. Data for cell behaviour was collected as previously.

Initial experiments with GEC1 also examined the influence of human ANCA on neutrophil behaviour. In these experiments GEC1 were compared with HUVEC. GEC1 were stimulated with 1000units/ml of TNFα and HUVEC with 2units/ml. Human ANCA was added to neutrophils before perfusion, as described above, and data collected for capture and behaviour of neutrophils.

4.2.6.9 Endotoxin testing and removal

During the course of the flow experiments there were considerable problems when investigating the effects of chimeric ANCA on neutrophils on EC because control anti-NP antibodies appeared to activate neutrophils. After changing the control, and investigating the use of other myeloma controls with minimal success, all antibodies were tested for endotoxin using an E-toxate kit (Sigma, UK).

The multiple test vial and endotoxin standards were re-constituted with endotoxin free water. Two tubes of each sample to be tested were prepared. E-toxate working solution was added to one sample tube and to the standards. The other sample tube had endotoxin added to it, to check for the presence of E-toxate inhibitor. The samples were positive for endotoxin if after 1 hour, left undisturbed, a hard gel had formed in the bottom of the test tube

All samples were contaminated with endotoxin. This was removed by either perfusing the antibodies through an endotoxin removal column where packed beads coated with polymixin B columns (Detoxi-Gel affinityPak prepacked columns, Pierce, UK); or by spinning in Handee centrifuge columns (Pierce, UK) with polymixin beads (Pierce,

UK): 1ml of beads (50% suspension) to 250μl antibody at 10,000G for 5 minutes. The samples were retested to check that all endotoxin had been removed.

4.2.7   <u>Statistical analysis and graphical representation of flow experiments</u>

The number of cells per mm^2 per 10^6 perfused was used to study capture of total numbers of cells. In order to study the behaviour of the cells, the percentage of cells rolling, stationary-adhered and, in endothelial experiments, those migrated across the endothelium was calculated as a proportion of the whole captured population. This data is graphically represented throughout the thesis.

This data was entered into Excel as numbers of cells calculated for each field. Four fields were averaged for each time point, and cells per mm^2 per 10^6 perfused and percentages were calculated. Data was transferred to Prism (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California, USA) for graph construction. In concentration titration experiments, the cell number and percentage were plotted against concentration, and in comparison of antibodies, against time after exposure to antibodies. All experiments were repeated at least three times, and the standard error of the mean (SEM) calculated for each data point and plotted as error bars. Results from the flow experiments were represented in three separate ways. P-selectin experiments, where neutrophils were in contact with a P-selectin surface could either stop and adhere or roll. Graphs were created for these experiments with the percentage of cells that were rolling throughout the time course of the experiment (the remaining cells were obviously therefore static).

Results from experiments where ECs were used were represented in two ways. Firstly the total numbers of cells captured were graphed, which allows interpretation of the potency of the ECs to capture neutrophils or PBL from flow. Secondly the behaviour of the captured neutrophils/PBL was represented by the percentage of cells captured that were rolling, statically adhered or migrated across the ECs layer. By representing these as percentages of the total captured cells a comparison can then be made directly between treatments on the behaviour of the captured neutrophils, even if different total numbers of cells had been captured. The graphs are either plotted over time or for simplicity of comparison of treatments, at one time point, and the percentage of rolling cells (blue), static cells (red) and migrated cells (yellow) are represented. Error bars (SEM) for these experiments are plotted at each time point.

For statistical analysis, data were transferred to Minitab (Minitab Inc., Version 13.0, USA). Analysis of variance (ANOVA) was used to look at differences between treatments, in relation to the treatments themselves, variation over time or concentrations, and the variability between experiments. ANOVA was performed by the general linear model allowing for a 2-way ANOVA where experiment number and treatment number were used for paired data. In order to compare which treatments were significant, a post ANOVA Dunnett test was performed. In unpaired tests, a one-way ANOVA used treatment number alone.

4.3   Superoxide assay

Superoxide anion production was determined by the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c. Neutrophils were suspended to $2x10^6$ cells/ml and primed with cytochalasin B (2.5μg/ml, Sigma) for 5 minutes and then TNFα (2ng/ml NIBS, London, UK) for 15 minutes at 37ºC. PMN were stimulated with 200μg/ml of IgG.

These were then added to a reaction mixture containing cytochrome c (75μM, Sigma) and agonist, with or without SOD (150 units, Sigma). The wells without SOD were repeated in triplicate and averaged. The OD at 550nM was then taken for the wells with and without SOD and the difference calculated, to establish the change in OD. Thus delta A = (absorbance at time x – absorbance at time 0) – (absorbance in the presence of SOD at time x – SOD at time 0).

The molar extinction coefficient for ferricytochrome c is 21.1 x 10^{-3}M and the light path is 0.6cm for a final volume of 250 μl. In order to calculate production of superoxide the following is applied: change in SOD wells – change in non SOD wells/ 12.66 x 1000.

4.4   Microscopy: phase contrast and electron microscopy

ECs were grown in monolayers as described above on glass microslides. They were viewed and photographed under phase contrast microscopy with the equipment used for the flow model.

Both transmission electron microscopy (EM) and scanning electron microscopy was performed during the course of this work in order to look for fenestrations. The cells were grown in monolayers in plastic six well plates (Falcoln, BD, UK/Ireland) and were cultured in medium containing different concentrations of VEGF. Medium either contained no VEGF, the 'normal' amount which was provided in the EMB2 medium pack (10μg/ml) or high concentrations of VEGF (100μg/ml).

Preparation of samples, and help with operating the microscopes were performed by our colleagues in the Electron Microscopy laboratories.

For transmission EM, cells were fixed in 2.5% glutaraldehyde (Agar Scientific Ltd., UK) in 0.1 molar phosphate buffer (Agar Scientific Ltd., UK) and then post fixed in 1% osmium teroxide (Agar Scientific Ltd., UK), dehydrated through an ethanol series (Agar Scientific Ltd., UK) and embedded in Mollenhauer resin (Agar Scientific Ltd., UK). They were cut by the EM laboratory and transmission electron microscopy was performed on a Jeol 1200EX TEM electron microscope. The images from these techniques were not satisfactory, so we went on to perform scanning EM.

For scanning EM, cells were fixed, dehydrated and dried as above. They were then critically point dried in a Polaron Critical point drier and mounted on stubs. They were sputter coated with platinum in an Emscope SC500 sputter coater and examined in a Philips XL-30 FEG ESEM microscope in high vacuum mode.

4.5   Flow cytometry

4.5.1   Fow cytometry for surface markers and adhesion molecules on endothelial cells

ECs were grown to confluence as above and either left untreated or treated with TNFα for 4 hours if testing for E-selectin, vWF, CD31, CD16, CD32; 12 hours if testing for ICAM-1 and 24 hours when testing for VCAM-1. ECs were removed by treatment with EDTA at 4ºC for 45 minutes followed by scraping, washed and centrifuged at 1600rpm for 5 minutes and resuspended in PBS with calcium/magnesium. The cells were then counted and diluted to 10^6 per ml. 100μl of cells were added to each flow cytometry tube (12mm x 75mm 5ml round bottom polystyrene tubes, Falcoln, BD, UK/Ireland). For detection of vWF, cells were permeabilsed and fixed using Fixperm solution (Invitrogen, UK).

All primary and conjugated antibodies (negative control, mouse IgG1κ murine myeloma (Sigma, UK); monoclonal mouse anti-human CD31 (Dako UK Ltd); monoclonal mouse anti-human vWF (Dako UK Ltd); monoclonal mouse anti-human E-selectin (R&D systems, UK) monoclonal mouse anti-human ICAM-1 (abcam, USA); monoclonal mouse anti-human VCAM (R&D systems, UK); monoclonal mouse anti-human CD64 (Dako UK Ltd.)) at 1:50 and conjugated antibodies (monoclonal mouse anti-human CD16 (Dako UK Ltd.) and negative control mouse IgG2a (BD, UK/Ireland)) were added to each tube and left for 15 minutes at room temperature. 2mls of PBS was added and the cells were centrifuged, supernatants discarded and the cells resuspended by vortex. Cells treated with conjugated antibodies were fixed

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in 100ml of 2% paraformaldehyde (PFA, Sigma, UK) in PBS, and stored. Secondary antibody (anti-mouse immunoglobulins-FITC (Dako UK Ltd.) was added at 1:10 incubated for 15 minutes in the dark, washed and fixed.

Samples were acquired on a FACScalibur flow cytometer using cell quest software (Becton Dickson, Oxford, UK) with 1 x10^4 cells acquired per tube. Forward and side scatter gating excluded dead cells. List mode data was analysed using WinDMI software. Antibody staining was quantified by measuring geometric mean fluorescence.

4.6   Immunocytochemistry and immunohistochemistry

4.6.1   Immunocytochemistry for vWF; CD31; E selectin: VCAM-1 on EC

4.6.1.1 Cell smears

Confluent ECs were removed from flasks with trypsin, spun to a pellet and re-suspended in 1 ml of PBS. Using a transfer pipette, a drop of this suspension was placed onto a microscope slide and sucked back off, and this was repeated so that there were two circles of cell suspension on the slide. The slides were then left to completely dry out and were then fixed in fresh dehydrated acetone (VWR) at minus 20ºC for 20 minutes. After drying, a paraffin wax pen mark was drawn around each circle of cells. The slides were then washed in Tris buffered saline (TBS).

In a humid chamber, the primary antibodies, mouse anti-human CD31, mouse anti-human vWF, and mouse monoclonal IgG isotype control (all from Dako UK Ltd.) at 1:25 in TBS, were added to each slide for 1 hour followed by secondary antibody, rabbit anti-mouse FITC conjugated 1:25 in TBS (Dako UK Ltd.) for 1 hour in the dark. The slides were then rewashed, and citifluor (Citifluor Ltd., UK) was dropped over the circle of cells and covered with a coverslip. These were then viewed under a fluorescent microscope (Nikon Eclipse E600). Cells were viewed at X100 (MRH01902 CFI Plan Fluor 100X objective oil N.A. 1.3 W.D. 0.2mm spring loaded, stopper) in oil immersion, at room temperature, using Nikon digital camera DXM1200 and Nikon NIS elements acquisition software (Nikon, Kanagawa, Japan). All images were processed using Photoshop Elements 6.0 (Adobe, CA, USA), the sharpness of the images was adjusted and lens blur removed. Each image was adjusted to the same settings.

4.6.1.2 Whole monolayer staining

Since the images resulting from the above method were criticised as they did not demonstrate stained EC monolayers, further studies were untaken and these explored more adhesion molecules. ECs were grown to confluence on glass coverslips in 12 well plates and treated with TNFα for the appropriate time for each of the adhesion molecules (as above). The coverslips were washed in cold PBS in the wells. Primary antibodies were added (negative control, mouse IgG1κ murine myeloma at 1:20 (Sigma, UK); monoclonal mouse anti-human CD31 at 1:20 (Dako UK Ltd.); monoclonal mouse anti-human vWF at 1:25 (Dako UK Ltd.); monoclonal

mouse anti-human E-selectin at 1:100 (R&D systems, UK); monoclonal mouse anti-human VCAM at 1:100 (R&D systems, UK)) and left for 1 hour at room temperature. After washing, secondary antibody, rabbit anti-mouse immunoglobulins-FITC conjugated (Dako UK Ltd.) was added at 1:25 and left for 1 hour in the dark at room temperature. The cell layer was fixed with 2% PFA with 4% sucrose for 15 minutes. 4',6-diamidino-2-phenylindole (DAPI) (Sigma, UK) was diluted 1:50 in PBS and put onto the cell layer for 5 minutes. The coverslips were then washed, carefully removed from the wells and placed on microscope slides. A drop of Citifluor (Citifluor Ltd, UK) was placed on each coverslip, covered with a further coverslip and secured with clear nail varnish. The slides were then viewed with a fluorescent microscope as described above, and the images processed as above.

4.6.2 Immunohistochemistry for VCAM-1 in kidney biopsy

Sections of renal tissue previously obtained by workers in the laboratory, mounted on glass microscope slides and preserved in paraffin, were treated to remove the paraffin and rehydrate the sections. This was achieved by emersion for 5 minutes each in Xylene (BD chemicals, UK/Ireland) and then in an ethanol series (BD chemicals, UK/Ireland) from 100%; 90%; 80% and then running water.

Antigen retrieval was performed by microwaving the samples for 20 minutes in a 10mM citrate buffer (see appendix). After cooling and washing in running water for 10 minutes, a PAP pen was used to draw around each sample creating a well. Blocking

in 0.3% hydrogen peroxide (Sigma, UK) in methanol (BD chemicals, UK/Ireland) was performed for 30 minutes.

After washing thrice with gentle agitation in tris buffered saline (TBS) (see appendix) the samples were blocked with 20% donkey serum (Sigma, UK) and then incubated with primary antibody (goat anti-human polyclonal anti-VCAM-1 (R&D, UK)) diluted to 4μg/ml (1:300) in TBS, overnight at 4°C.

After washing the samples were incubated with secondary antibody (donkey anti-goat immunoglobulins; Santacruz Inc., USA) at 1:100, rewashed and developed cautiously with 33 diaminobenzidine (DAB) (Sigma, UK) and washed for 10 minutes. Counterstaining was achieved with haemotoxylin (Sigma, UK) following which the samples were mounted using immunomount (Thermo Scientific, USA) and viewed with a Nikon ES400 Eclipse microscope as above. Data was collected using NIS Elements version 3, F package software. Images were processed as above.

4.7   Enzyme linked immunosorbant assay (ELISA)

4.7.1   Cell ELISA for E-selectin, ICAM-1 and VCAM-1 on EC

96 well plates (BD Falcoln, UK/Ireland) were coated with gelatin or fibronectin and EC seeded 4 x 10^4 cells per well and incubated overnight in a humidified CO_2 (5%) incubator at 37°C. EC were stimulated with 100units/ml of TNFα or 5 x 10^{-9}g/ml IL-1β, for 4 hours for E-selectin; 12 hours for ICAM-1 and 24 hours for VCAM-1. In other experiments, hydrogen peroxide at 1 millimolar for 1 hour was used to induce

selectins. The cells were fixed with 2% paraformaldehyde (Sigma, UIK) with 4% sucrose in PBS for 10 minutes. Endogenous peroxidase was inhibited by 0.1% hydrogen peroxidase (Sigma, UK) in methanol (Fisher Scientific, UK) and then blocked with 10% marvel and 5% sheep serum (Dako UK Ltd.) to prevent non-specific binding of antibodies to cells for 1 hour.

Primary antibodies, monoclonal mouse anti-human human E-selectin (R&D systems, UK); monoclonal mouse anti-human ICAM (abcam, USA); monoclonal mouse anti-human VCAM (R&D systems, UK) were used at 1:1000 for one hour. Secondary antibody, anti-mouse IgG (from sheep), horse radish peroxidase (HRP) linked (Invitrogen, UK) 1:1000 was applied in all wells for one hour. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Calbiochem, Merk, UK) was then added. The colour changed quickly and stop solution was added after 5 minutes. The absorbance was then read at an OD of 450nm.

Wells were treated with secondary antibody alone as a negative control, or with primary antibodies either in normal PBS or made up in PBS or 0.1% triton to permeablised EC.

The student's T test was used to compare results (Minitab version 13.0, State College, PA).

EC were washed and 0.1% crystal violet applied for 10 minutes. After rewashing, the cells were solubilised with 1% triton (BDH chemicals, VWR, USA) and left overnight

in humid conditions. The absorbance was then read at 550nm. The results of each well were then compared by the students T test to establish that there were the same number of cells in each well.

4.8   Western blotting

EC were grown to confluence, treated with 100units/ml TNFα for 24 hours, removed with EDTA and trypsin, centrifuged and re-suspended in lysis buffer with protease inhibitors (see appendix) and left for 15 minutes on ice. Samples were then centrifuged for 10 minutes at 1500rpm, and the supernatants collected and frozen. When all samples had been collected, they were defrosted, 22μl of non-reducing loading buffer (see appendix) was added to each 100μl of sample, and for reduced samples 20μl of betamercaptoethanol (Sigma, UK) was added and the sample boiled for 5 minutes.

Glass and aluminium plates, comb, spacers and holders were cleaned with alcohol, and the plate holder was sealed with grease and assembled. Water was used to check the seal. The resolving gel (see appendix) was poured (10% for VCAM: 90-110kDa), left to set for 30 minutes and overlaid with methanol to prevent drying. This was then removed, the stacking gel (see appendix) poured on, the comb inserted, and the gel left for 15 minutes. The gel tank was assembled, running buffer put into the plates, and the gel loaded with 15μl of samples. The unit was set to 60 mVolts and 60 mAmps and run for 20 minutes, after which the voltage was increase to 120mV and run for approximately 1 hour until the dye front dropped off the gel. Nitrocellulose membrane (low fluorescence PVDF membrane, Amersham

Biosciences, UK) was cut and soaked in methanol for 5 minutes. Filter paper was cut and soaked in buffer, and stacked with 3 papers, then the membrane and then 3 further filter papers. The gel was removed from the glass plates, the resolving gel cut off, the separating gel peeled off, washed and stacked on the semi-dry unit on the filter paper and transferred at 37.8mAmps for 1 hour. Blocking was achieved with 5% marvel in TBS (see appendix) at room temperature for 2 hours. Primary antibody (murine anti-human VCAM-1, R&D Systems, UK) was diluted1:1000 in 5% marvel/TBT-T (see appendix) and incubated at 4°C overnight with agitation.

After washing, the blot was incubated with secondary antibody at 1:5000 (donkey anti-goat IgG HRP linked, Santa Cruz Biotecnology Inc, USA) in blocking buffer for 1 hour at room temperature with agitation. The substrate ECL (*E.coli* lysate, Pierce, USA) was diluted 1:1, incubated for 5 minutes, drained and wrapped in Saran wrap (VWR, UK). The membrane and photographic paper were then placed into a cassette and left for 5 minutes, developed and fixed.

4.9   RNA Arrays

RNA arrays were performed by our colleagues in the RNA extraction laboratories.

Microaaray printing was performed by 70-mer oligos consisting of the Human Genome Array Ready Oligo Set (AROS) v4.0 were supplied by Operon. These were stored in a lyophilized form at -80^{0}C.

They were then resuspended at a concentration of 40µM in 3xSSC and left to re-suspend overnight at 4^0C. The re-suspended oligos were then spotted onto UltraGAPS coated slides using a Microgrid TAS II arrayer (BioRobotics) at 70% humidity. After spotting the arrays were stabilised and immobilised using a UV cross-linker and stored in a desiccated chamber at -80^0C.

For RNA extraction, aliquots of no more than 5×10^6 cells were isolated and resuspended in 200µl of buffer (as supplied in the RNEasy Mini kit, Qiagen, UK) and RNA extraction performed with the kit. After extraction, RNA was treated to remove any contaminating DNA (DNase I kit, Ambion, USA) and the yield quantitated with spectrophotometry (Eppendorf Biophotometer) at 260nm wavelength.

Reverse transcription PCR of the RNA was achieved by adding 1-5µg of RNA to 1µl oligo(dT)$_{12-18}$ primer (Invitrogen, UK), and making the mixture up to 17µl with de-ionised water. After heating to 65^0C for 10 minutes, and ice cooled, 8µl of 1st strand buffer, 4µl 0.1M DTT, 2µl Superscript II 200U/µl (all from Superscript II RNase H Reverse Transcriptase kit, Invitrogen), 8µl 10mM dNTPs (made up from 100mM PCR Grade dNTP Set, Invitrogen) and 1µl de-ionised water were added. The reaction was then kept at 42^0C for 2 hours. The cDNA synthesized was extracted using Macherey-Nagel PCR Purification Kit, but the final elution step was performed using 35µl water rather than the supplied elution buffer. The eluted cDNA was quantitated by spectrophotometry, tested for purity by gel electrophoresis and stored at -20^0C.

Cy3-dCTP and Cy5-dCTP labelling was performed using 500ng of sample cDNA. This was added to 20µl random primer mix (Bioprime Labelling Kit, Invitrogen), made up to 43µl with deionised water, heated to 94^0C for 5mins and ice cooled. The following were then added: 1.2µl 10mM dATP; 1.2µl dTTP; 1.2µl dGTP; 0.72µl dCTP (all made up from 100mM dNTP Set PCR Grade, Invitrogen, UK); 0.68µl water; 1µl of either Cy3 dCTP (Amersham Biosciences,UK) or Cy5 dCTP (Amersham Biosciences, UK); 1µl 40U/µl Klenow enzyme (Bioprime Labelling Kit, Invitrogen, UK). The reaction was then kept at 37^0C for 8 hours.

The labelled cDNA was purified by means of Macherey-Nagel PCR Purification Kit as before and measured for pmols of labelled product by spectrophotometry (Pharmacia Biotech Ultrospec 2100 pro) at 550nm (Cy3-dCTP) or 650nm (Cy5-dCTP). pmol yield was calculated by the following: Cy3 = Abs550 x 50µl/0.15 and Cy5 = Abs 650 x 50µl/0.25.

Array hybridisation was performed. Arrays were initially prepared by a pre-soak using Corning Pronto Microarray Hybridisation Kit (Corning, USA) for 20 minutes at 42^0C, followed by three washes in de-ionised water. The slides (UltraGAPS coated slides, Corning, USA) were then pre-hybridised (2.5ml 20 x SSC; 0.25ml 20% SDS; 50µl 100mg/µl Fraction V BSA; made up to 50ml with distilled water) for 2 hours at 42^0C. Labelled cDNA for test and reference samples were taken (equal pmol), added together, concentrated with Microcon YM-30 concentration columns (Millipore, USA) and eluted in a final volume of 30µl water. 50µl of labelled cDNA mix was then made up following the instructions for the UltraGAPS coated slides, using 5µg PolyA DNA

and 8µg Cot1 DNA as nucleic acid-blockers and formamide. The 30µl labelled probe was added and the mix denatured at 95^0C for 5 minutes. The pre-hybridised slide was dipped in water, and then spun to dry. It was then placed into a corning hybridisation chamber (Corning, USA) and the preparation instructions for the chamber followed. The probe mix was placed on the slide surface and a Lifter Slip (VWR, USA) applied. The hybridisation chamber, wrapped in a damp paper towel and foil was heated in a hybridisation oven at 42^0C for 16-20 hours.

After hybridisation the coverslip was removed by immersing array in 2 x SSC and 0.1% SDS at 42^0C. It was washed in the same solution at 42^0C for 5 minutes, then at room temperature for 5 minutes (0.2x SSC), then four times in 0.05 x SSC and finally spun dry. The arrays were then scanned using a Perkin Elmer ScanArray Gx and ScanArray Express Software.

Microarray analysis was performed. Slide scans were processed first using ScanArray Express. A prepared GAL file was used to annotate the slide image and the intensity data extracted and exported as a gpr file. The results files were then uploaded into the GEPAS analysis software for normalization. First within slide normalisation was performed using print-tip normalisation with background subtraction. This was then followed by slide scale normalisation between slides. Data for the normalised data was then exported to TMEV, allowing us to perform SAM analysis, hierarchical clustering, and PCA.

5. OPTIMIZATION OF MODELS

5.1 Early experiments: setting up the systems

5.1.1 P-selectin titration: adhesion and rolling velocity

5.1.1.1 Adhesion and behaviour

A titration of concentration was performed using concentrations of P-selectin 0, 0.5, 1, 2 and 5μg/ml. The total numbers of neutrophils captured was between 300 and 600 cells per mm^2 of slide per 10^6 neutrophils perfused (Figure 10). No neutrophils were captured when no P-selectin had been applied to the slide, and increasing numbers of neutrophils were captured as the concentration of P-selectin increased between 0.5 and 2 μg/ml. Thereafter there was no increase in numbers of cells captured when the concentration of P-selectin was increased to 5μg/ml.

The behaviour of the captured cells, as illustrated (Figure 10) which presents the data of rolling cells as a percentage, demonstrates that the number of rolling cells fell between 0.5μg/ml and 1μg/ml and then levelled off with between 50 and 60% of cells rolling when the concentration of P-selectin was between 1 and 5μg/ml.
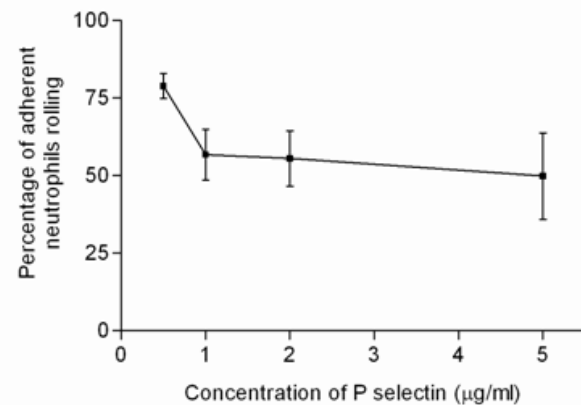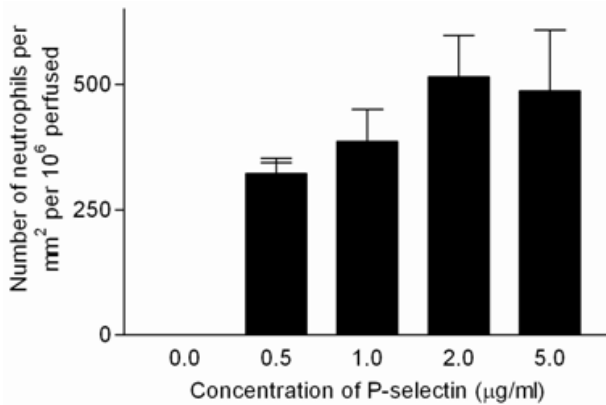
Figure 10: Neutrophil adhesion and behaviour on increasing concentrations of P-selectin.

In the flow model microslides were coated with P-selectin in increasing doses. Left hand panel: The numbers of neutrophils captured increased between 0.5 and 2.0μg/ml and thereafter did not increase. No neutrophils were captured on slides with no P-selectin coating. Right hand panel: behaviour of captured neutrophils: The percentage of captured neutrophils which roll decreases between 0.5 and 1.0μg/ml of P-selectin and thereafter does not change. There is no significant variability by ANOVA. The wall shear stress was 0.1Pa. n=10.

An analysis of variance (ANOVA) performed for this data demonstrated that there was no significant difference between different concentrations of P-selectin for capture or rolling of the neutrophils. There was no significant variability between experiments.

In summary, neutrophils are captured on P-selectin. There is an insignificant increase in the number of neutrophils captured with increasing concentrations of P-selectin and at concentrations of P-selectin between 1 and 5μg/ml about half of these cells roll.

5.1.1.2 Rolling velocity

In the P-selectin titration, the rolling velocity of moving cells was calculated, and fell as the concentration of P-selectin increased (Figure 11).
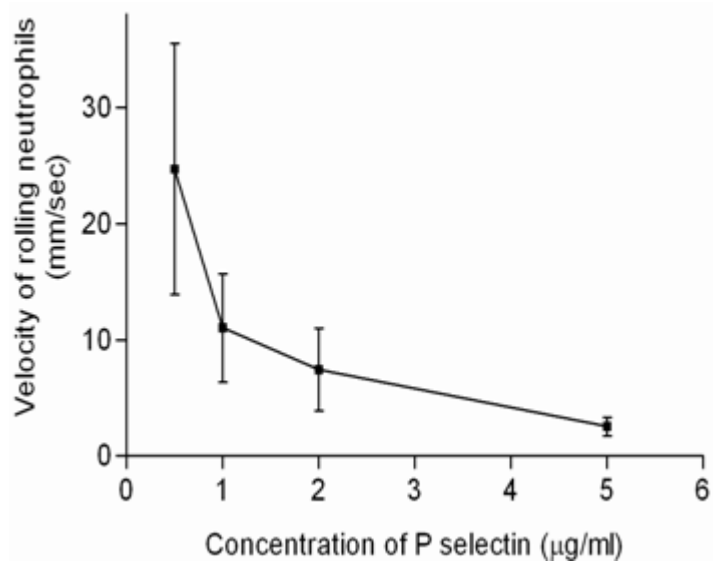
Figure 11: Rolling velocity of neutrophils on increasing concentrations of P-selectin.

Rolling velocity of captured neutrophils decreased as the concentration of P-selectin increased (p=<0.001 by ANOVA). The wall shear stress was 0.1Pa. (n=3).

In the analysis of variance for these experiments (n=3) the concentration was highly significant in determining the velocity of these cells (p=<0.001). Variation between experiments was insignificant.

In contrast to the capture and rolling behaviour of neutrophils overall, the concentration of P-selectin is thus important in the speed at which neutrophils roll. In subsequent experiments with P-selectin concentrations of 2μg/ml of P-selectin were therefore used.

5.1.2   fMLP on P-selectin

fMLP when perfused over rolling neutrophils caused any rolling cells to stop rolling, firmly adhere and undergo dramatic shape change. In three experiments the mean number of cells rolling before fMLP, were, 38%, 70% and 87% respectively and these converted to 0%, 3.85% and 0% after fMLP was added.

5.1.3   HUVEC TNFα titration: Adhesion and behaviour

In the next set of experiments HUVEC were used, grown in monolayers on glass microslides. The HUVEC were stimulated with increasing doses of the inflammatory cytokine TNFα and the number of neutrophils captured and the behaviour these neutrophils exhibited was recorded. TNFα was used in concentrations of 0, 1, 5, 10 and 100μg/ml in 12 experiments.

The total number of cells and their behaviour was recorded 7 minutes after neutrophil inflow. The total number of adherent cells (rolling, stable-adhered and transmigrated) increased with the concentration of TNFα (p=0.004). In these experiments there was some neutrophil adhesion at 0units of TNFα. This unexpected result may be due to the fact that some neutrophils were activated. These were early preparations of neutrophils and poor handling may have activated the cells. In later experiments there was no significant adhesion of neutrophils if the endothelium had not been activated with cytokines, and this is in line with previous published work. Activated

neutrophils will bind promiscuously to any albumin exposed by breaks in the endothelial monolayer.

The behaviour of these cells was not the same at all concentrations of TNFα (Figure 12). At very high concentrations of TNFα (100u/ml) there was less rolling, and more migration. These differences were significant by ANOVA (rolling cells p=0.034 and migrating cells p=0.011).
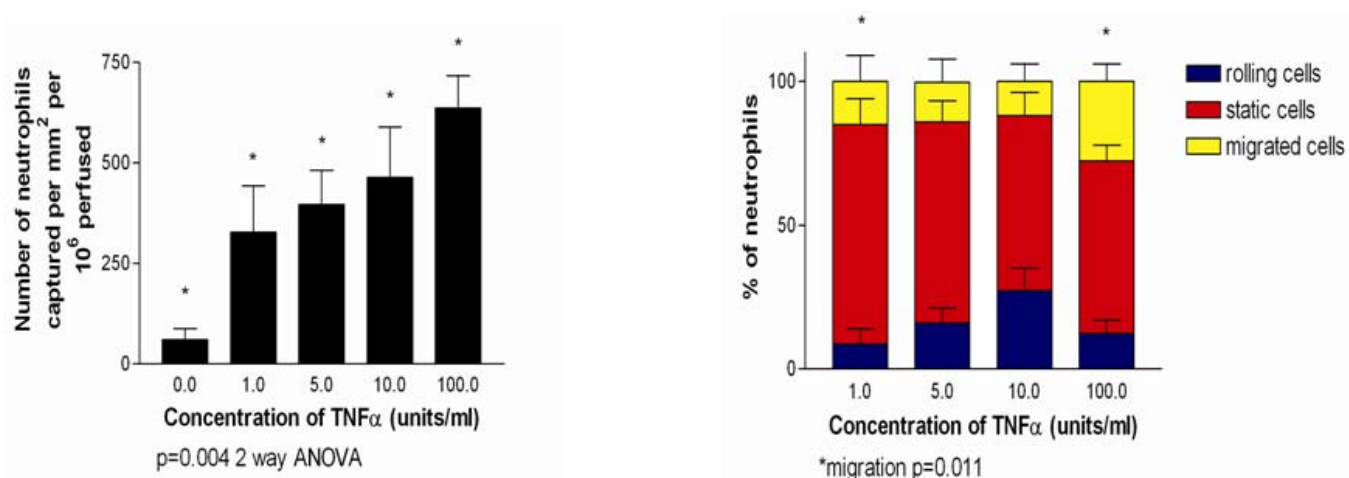
Figure 12: Neutrophil capture on HUVEC with increasing TNFα concentration.

Neutrophils were flowed over a monolayer of HUVEC using the flow model. Left hand panel: With increasing concentrations of TNFα, increasing numbers of neutrophils were captured, this was significant by ANOVA (p=0.004). Right hand panel: Behaviour of neutrophils with increasing concentrations of TNFα. There is a significant increase in migration of neutrophils at high concentrations of TNFα (p=0.011). Wall shear stress was 0.1Pa (n=5).

5.2   Patient ANCA

5.2.1   Specifics of patient ANCA

The specifics of the patient ANCA used in the experiments are as follows (Table 5).

Patient sample	Disease Type	Immunofluorescence	MPO titre (U/ml)	PR3 titre (U/ml)	Immunoglobulin subclass total IgG (g/L/ (%))			
					IgG1	IgG2	IgG3	IgG4
220115	MPA	pANCA	103	1.9	18.08 (69%)	6.91 (26%)	0.93 (3.6%)	0.06 (0.7%)
220084	WG	cANCA	4.17	83.7	7.39 (65%)	2.62 (23%)	0.57 (5%)	0.85 (7%)
220028	MPA	pANCA	140	4.7	10.12 (68%)	2.88 (19%)	0.71 (6.2%)	1.12 (10%)
220054	MPA	pANCA	>100	2.07	5.23 (37%)	6.96 (49%)	1.34 (10%)	0.54 (4%)
Normal IgG					60.3 (71.5%)	19.4 (31.0%)	5.0 (8.4%)	0.7 (4.2%)

Table 5: Properties of patient ANCA used in experiments.

IgG= immunoglobulin; MPA= microscopic polyangiitis; WG= Wegener's Granulomatosis; cANCA = cytoplasmic anti-neutrophil cytoplasm-antibody; pANCA perinuclear anti-neutrophil cytoplasm-antibody; U/ml = units per millimetre; g/L = grams per litre

5.2.2   Superoxide and patient ANCA

Neutrophils produce superoxide as part of their repertoire of defence against pathogens. Normal IgG caused neutrophils to undergo a small amount of superoxide production. In the presence of some patient ANCA, superoxide production was greater than control but there is variability between patient ANCA. Patients 220028,

220054 and 220084 had large superoxide responses which were significant by ANOVA (all p=<0.001) (Figure 13). Patient 220115 ANCA did not produce a superoxide response that was significantly larger than control. fMLP consistently causes neutrophils to undergo a large superoxide response.

*p= <0.001 by ANOVA compared to normal IgG
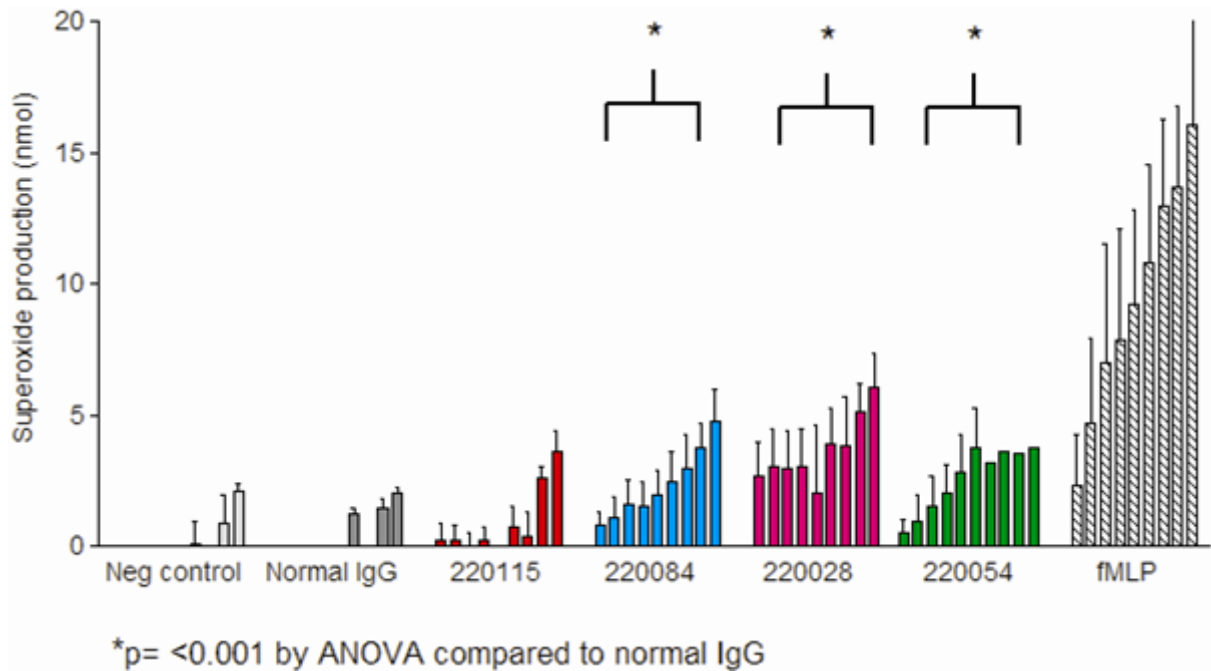
Figure 13: Superoxide production by neutrophils in the presence of normal IgG or patient ANCA.

Superoxide anion production was determined by the superoxide dismutase inhibitable reduction of ferricytochrome c. Neutrophils were suspended to $2x10^6$ cells/ml and primed with cytochalasin B (2.5μg/ml) for 5 minutes and then TNFα (2ng/ml) for 15 minutes at 37°C. PMN were stimulated with 200μg/ml of IgG. Significant levels of superoxide over control were produced by three of the four patient ANCA (p=<0.001 by ANOVA). All superoxide production represented over time up to 120 minutes. (n=5)

5.2.3   P-selectin with patient ANCA

Having tested the ability of the selected patient ANCA to cause superoxide production by neutrophils, we then proceeded to functional studies using the flow model. In the first experiments, a simple model was used, where glass microslides were coated with P-selectin and neutrophils flowed across. ANCA or control normal IgG was perfused across the neutrophils after 4 minutes of neutrophil inflow. One set of neutrophils was left untreated. The total number of neutrophils captured onto the P-selectin surface did not differ between treatments (Figure 14). When the behaviour of the neutrophils was studied, the neutrophils which had not been exposed to any treatment continued to roll (grey line Figure 14 right hand panel). Normal IgG (control in black) caused approximately half of the neutrophils to stop rolling by 8 minutes and this was compared to the four patient ANCA (220028, 220054, 220115, 220084) by two way ANOVA. There was a significant difference between normal IgG and patient 220084 (p=0.004), patient 220115 and patient 220028 (p=<0.001 in both) where the ANCA stopped cells rolling more than normal IgG. There was no significant difference in comparing patient 220054 with normal IgG.

In analysing of all the data together (n=3) there is a significant effect of time (p=<0.001) and between antibodies (p=<0.001), but there is no significant variability between experiments.
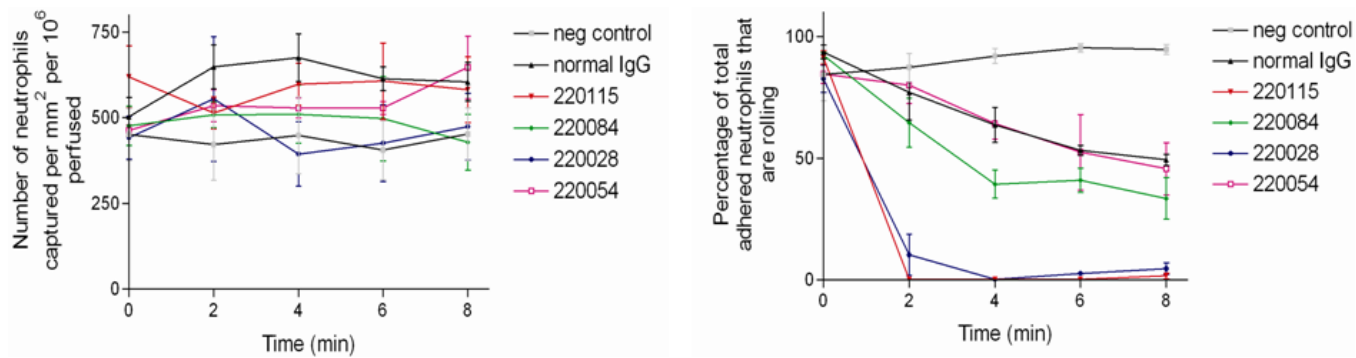
Figure 14: Influence of patient ANCA on neutrophils on P-selectin at 2µg/ml.

In the flow model, four different patient ANCA at 200µg/ml, were compared against control IgG at 200µg/ml and no IgG. In the left hand panel there is no significant difference in the total number of neutrophils captured between the treatments. In the right hand panel, behaviour of the captured neutrophils is shown as a percentage of adhered cells that are rolling. With no treatment, neutrophils continue to roll. When treated with normal IgG half the neutrophils stop rolling. When treated with patient ANCA, significantly more neutrophils stop rolling than with control normal IgG (patient 220084 p=0.004; 220028 and 220115 both p=<0.001). One patient ANCA, 220054 did not stop neutrophils more than control. Wall shear stress 0.1Pa (n=3).

5.2.4   HUVEC with patient ANCA

In the next set of experiments we used ECs which enable the study of more complex interactions. Rather than just selectins being presented to the neutrophil, other molecules now influence the interactions such as integrin/ICAM-1, chemokine and cytokine receptors. For these flow experiments data was collected at 2 minute time points over 15 minutes. All of this data was used in ANOVA analysis but for clarity the

graphs presented here are of the final time point (15 minutes after the inflow of neutrophils.

Experiments on HUVEC with patient ANCA were performed on HUVEC treated with high levels of TNFα (100units/ml) and low levels (2units/ml).

5.2.4.1 Patient ANCA on HUVEC treated with TNFα 100 units: Adhesion and behaviour

In initial experiments using highly activated endothelium, there was no significant difference in the total numbers of neutrophils captured when treated with no antibody, normal IgG or any of the four patient ANCA (Figure 15). Additionally, there was no significant difference in the number of neutrophils captured over time.

The behaviour of the neutrophils changed dependent on the antibody they were exposed to (Figure 15). The data is presented for behaviour (i.e. rolling, stationary adhered, migrated) as percentages of total neutrophils captured. No antibody or normal IgG causes approximately half of the adherent cells to roll, 25% to adhere firmly and a small proportion to migrate across the endothelial layer, which increases over time.

In these experiments, in contrast to the P-selectin experiments above, neutrophils were pre-treated with ANCA and therefore the effects can be seen immediately. The treatments were significantly different from each other when analysed together (p=<0.001).

When the patients ANCA were compared against normal antibody, ANCA isolated from patient 220054 and 220084 were similar to the controls. ANCA from patients 220028 and 220115 caused more cells to stop rolling when compared to normal IgG (220028 p=<0.001; 220115 p=0.024). Migration was not significantly different between any of the treatments.
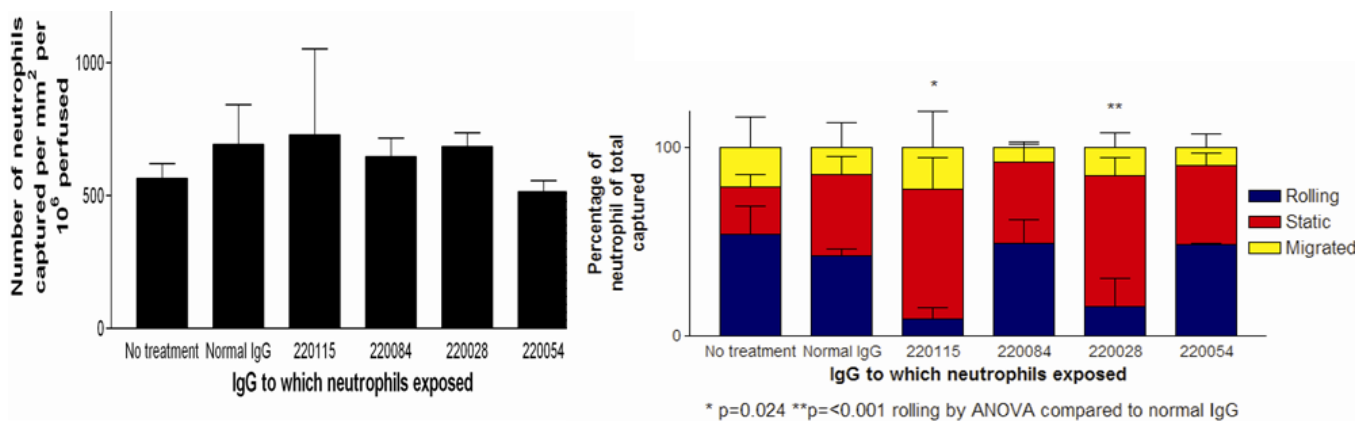
Figure 15: Neutrophil adhesion and behaviour on HUVEC treated with 100units/ml TNFα.

In the flow model there was no significant difference in the number of neutrophils captured from flow between each treatment. Behaviour of neutrophils varied between treatments. The proportion of rolling cells (blue) static cells (red) and migrated cells (yellow) are shown. With no treatment or normal IgG approximately half the cells roll. Exposure to patient ANCA 220115 (p=0.028) or 220028 (p=<0.001) resulted in a significant decrease in the proportion of neutrophils rolling. Wall shear stress 0.1Pa (n=3).

5.2.4.2 Patient ANCA on HUVEC treated with TNFα 2units: Adhesion and behaviour

When EC were treated with low levels of TNFα, in contrast to the situation on highly activated endothelium, there was a variation in the number of neutrophils captured depending on treatment (Figure 16). When neutrophils were untreated low numbers of neutrophils were captured (50-150), normal IgG captured about 250 neutrophils.

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Two of the patient ANCA captured between 300 and 500 neutrophils, significantly higher than normal IgG control (patient 220115, p= <0.001; patient 220084, p= 0.042), two patient ANCA did not capture neutrophils more than controls (220054, 220028).

The behaviour of neutrophils captured differed between the control normal IgG and patient ANCA for all four patients (Figure 16). HUVEC treated with TNFα 2u/ml supported rolling of neutrophils. Addition of normal IgG or patient ANCA converted rolling to static adhesion. Additionally ANCA IgG compared to normal IgG caused changes in behaviour, namely less rolling (patient 220115, 220028 and 220084 p=<0.001; 220054, p=0.001) and more migration in all four patient samples (220115, p=0.035; 220028, p=0.017; 220084, p=0.012; 220054, p=0.049).
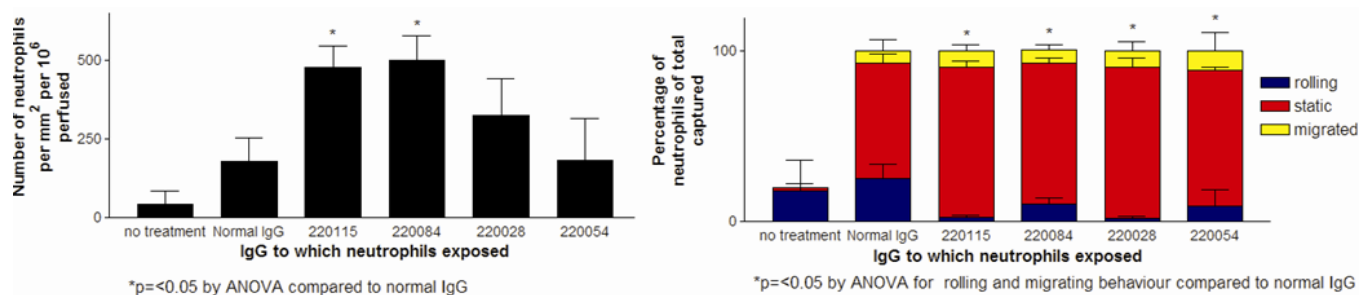
Figure 16: Neutrophil adhesion and behaviour on HUVEC treated with 2units/ml of TNFα.

In the flow model when pre-treated with no antibody, normal IgG or four different patient ANCA (all at 200μg/ml), neutrophils exposed to two patients' ANCA adhered more to the endothelium than those exposed to normal IgG (220115 p= <0.001; 220084 p= 0.042). Two patient ANCA (220054; 220028) behaved similarly to control. Behaviour of neutrophils varied between treatments. The proportion of rolling cells (blue) static cells (red) and migrated cells (yellow) are shown. Exposure to patient ANCA causes less rolling (patient 220115, 220028 and 220084 p=<0.001; 220054 p=0.001) and more migration (220115 p=0.035; 220028 p=0.017; 220084 p=0.012; 220054 p=0.049) than exposure to normal IgG. Wall shear stress 0.1Pa (n=5).

5.3   Testing controls to explore why they activate

We had considerable problems with early flow experiments as our control sera activated neutrophils and although differences between the ANCA activated neutrophils and controls were established the differences were not as great as expected. Normal IgG was not known to activate neutrophils and in previous flow experiments this had not been a problem (Radford, Savage et al. 2000). The problem was particularly marked when we began experiments using chimeric PR3-ANCA of the IgG3 subclass. We therefore undertook experiments to investigate this problem.

5.3.1   Testing controls for endotoxin

Samples of immunoglobulin were tested for the presence of endotoxin by ELISA and found to have endotoxin present. This was removed using endotoxin removal beads. The samples with and without endotoxin were then run in the flow model.

Endotoxin altered the behavior of neutrophils (Figure 17). This is seen in alteration of rolling and static behaviours, but is only seen when the system is partially stimulated. Thus the endotoxin presence makes a significant difference to the behavior of neutrophils treated with normal IgG. Normal IgG does not have a great effect on neutrophils, most remain rolling. However when the normal IgG had endotoxin in it there was a conversion to static adhesion (p=<0.001) with less rolling (p=<0.001). Migration was not significantly altered. Furthermore, if endotoxin was added to the already contaminated normal IgG, the effect was even more marked with significant increases in static neutrophils (p=<0.001) and decreases in those rolling (p=0.001).

If the system is already highly stimulated (e.g. with ANCA) the effect on the neutrophils is much less marked due to the fact that they are already mostly static without the additional stimulus of endotoxin. The only significant difference in behaviour in this experiment was when further endotoxin was added to already contaminated ANCA which significantly decreased the rolling behavior (p=0.041).
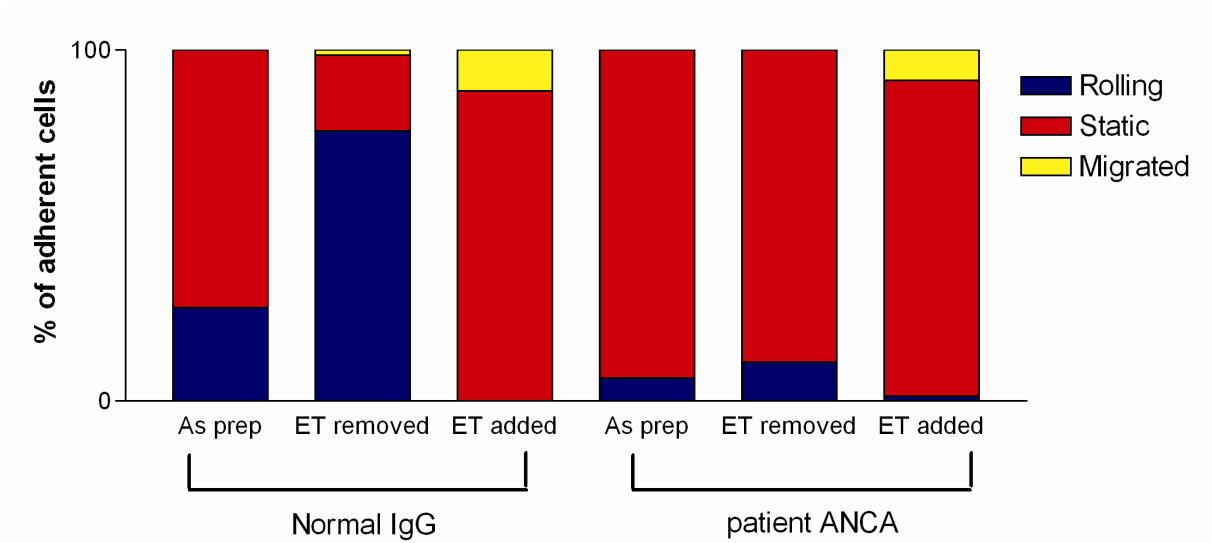
Figure 17: Effect of endotoxin in flow.

Normal IgG or ANCA was prepared and endotoxin was detected in both samples. Neutrophils were treated with either the original samples of normal IgG or ANCA, or samples where the endotoxin had been removed, or samples where endotoxin had been added. Neutrophils were flowed over HUVEC and data taken at 2 minute intervals. Data shown at 15 minutes as percentage of total adhered cells rolling (blue); static (red) and migrated (yellow). There were significant differences in behaviour after each treatment but this is more noticeable in the normal IgG samples. ET= endotoxin. n=1

5.4   Discussion

5.4.1   P-selectin titration: adhesion and rolling velocity

Neutrophils were captured on P-selectin and between a concentration of 1µg/ml and 5µg/ml there was no significant change in the numbers of cells captured or the behaviour, that is the proportion of the neutrophils rolling as opposed to stationary. The velocity of the neutrophils that were rolling decreased as the concentration of the P-selectin increased. For further experiments 2µg/ml of P-selectin was used as at

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this concentration the neutrophils adhered adequately and rolled at a speed which allowed for sensitivity in treatments

5.4.2 fMLP on P-selectin

fMLP is a powerful stimulant which stopped neutrophils rolling on P-selectin and caused firm adherence. This was accompanied by a change in shape of the cells. The addition of fMLP caused consistent reduction in rolling. This has been observed previously (Rainger, Rowley et al. 1998).

5.4.3 HUVEC TNFα titration

When HUVEC were treated with increasing concentrations of TNFα total binding increased. At high doses (100units/ml) of TNFα there was increased neutrophil migration across the endothelial monolayer. This is consistent with previous results in our laboratory. In further experiments using HUVEC, highly activated endothelium was represented by 100units/ml of TNFα, and minimally activated endothelium by 2units/ml of TNFα.

5.4.4 Patient ANCA

Four patient ANCA were studied in these experiments; three of them specific for MPO and one for PR3. Clinical presentation of the patients were similar, all had severe disease and renal failure requiring immunosuppression and plasma

exchange. ANCA was obtained for these experiments from plasma exchange fluid by isolating the whole IgG fraction. The same concentration of IgG was used in each experiment, but the proportion of the IgG which is ANCA was unknown. This may explain some of the differences in how active the ANCA were in the various experiments, although it is interesting that all the patient ANCA were more active than control in at least one of the assays (Table 6). The proportion of total IgG which is actually ANCA is small; probably less than $10\mu g/ml$, but this is likely to vary from patient to patient. It is difficult to quantify the amount of ANCA in patient serum. Immunofluorescence is subjective, and by ELISA quantification may not be accurate or comparable between patients, if there is variation in affinity of ANCA for the antigen both within, as well as between, patients.

	220115	220084	220028	220054
Superoxide	-	+	+	+
P-selectin adhesion assay: behaviour	++	+	++	-
HUVEC adhesion assay: behaviour (100units/ml TNFα)	+	-	+	-
HUVEC adhesion assay: behaviour (2units/ml TNFα)	+	+	+	+

Table 6: Summary of patient ANCA activity.

In the assays tested the different patient ANCA influenced neutrophil behaviour differently, with all ANCA activating neutrophils in at least one assay.

5.4.5   <u>Superoxide with patient ANCA</u>

Superoxide is produced by neutrophils in the respiratory burst and requires the neutrophils to be primed prior to a second stimulus. Patient ANCA provides a secondary stimulus for superoxide production and is variable between different patient samples, but is generally higher than the superoxide response elicited by normal IgG. Of the four patient ANCA tested, three caused more superoxide production than control IgG. This variation in response may be due to varying pathogenicity of patient ANCA, perhaps dependent on subclass or epitope specificity or on actual concentration of ANCA.

Superoxide experiments were performed with neutrophils in the absence of IgG Figure 13) and did not elicit a superoxide response. Control IgG from 'normal' donors does elicit a minimal superoxide response, this has been noticed in previous work in our laboratory, and reasons why this may occur is discussed further in section 5.4.6.

5.4.6   <u>P-selectin studies with patient ANCA</u>

Neutrophils rolled on P-selectin as established in the P-selectin titration, and $2\mu g/ml$ was chosen as a concentration where the cells were rolling at a suitable velocity. When normal IgG control was perfused over the neutrophils, a proportion stopped rolling when compared to experiments where no antibody was used. This non-specific response has been noted previously, but is unexplained, as with IgG isolated from normal people as opposed to those with vasculitic disease, one would not

expect neutrophil activation. Although all IgG preparations were assiduously microfuged to precipitate immune complexes, low levels of immune complex formation cannot be excluded which could activate the neutrophils. It is likely in these early experiments that the reason for this activation was contamination by lipopolysaccharide (LPS), as this was found to be a problem with later controls (see 5.3.1). This was not however explored in the early experiments.

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Three of the patient ANCA stopped the cells from rolling more than the control IgG and this reached significance and one patient ANCA did not significantly reduce rolling. This variation in response of neutrophils to different ANCA in the flow model has been previously noted, and currently remains unexplained; these differences in response are discussed below and are currently being pursued by other workers in our group.

Of the ANCA that did have an effect on the neutrophils, two ANCA were very dramatic and reduced rolling quickly with a sustained effect at 8 min. Patient 220084 seemed not to be as potent or as rapid at causing an effect despite very destructive clinical disease.

5.4.7 HUVEC studies with patient ANCA

When patient ANCA was studied on highly activated HUVEC there was no difference in the numbers of cells captured on the endothelium when comparing normal IgG with patient ANCA. In contrast to P-selectin, the neutrophils were pre-treated with

ANCA so that changes over time were not seen, and the neutrophils were affected by the treatment throughout the course of the experiment (data over time not shown). The reason for this decision was historic: although ideally experiments would be performed adding the ANCA into the system as in the P-selectin model so that changes could be seen during the experiment, this had not worked well in previous studies with HUVEC in the flow model (Radford, Luu et al. 2001). Greater changes in neutrophil behaviour were seen when ANCA was added to the neutrophils before the experiment: this method was therefore used in these experiments.

When the endothelium was treated with 100units of TNFα, two of the patient ANCA (220115 and 220028) stopped the cells rolling more than the control, and these reached significance. Migration was not affected.

HUVEC that has been pre-treated with 100units of TNFα is highly activated and is probably already capturing and activating neutrophils to a maximum amount, so against this background, it may be hard to see any additional effects of a moderate activating influence such as with ANCA. These studies were therefore repeated using minimally activated endothelium.

In studies where the HUVEC were treated with TNFα at 2u/ml greater differences were seen between patient ANCA and normal IgG. The neutrophils rolled less and migrated more in the presence of ANCA. Of note the difference between untreated neutrophils, which roll on a minimally activated endothelium, and those treated with

normal IgG, was more obvious, and normal IgG can be seen to influence the neutrophils causing more to statically adhere, but not to migrate.

It can be seen therefore that patient ANCA is able to alter the behaviour of neutrophils on P-selectin and on HUVEC and that there is a variation in the degree of affect. Although pooling all the patient data was discussed, there is an obvious variation between different patient ANCA, and thus repeats were done for each patient.

5.4.8 Patient ANCA subclasses

The relative proportions of each subclass within these ANCA varied greatly. Patient 220115 was the only ANCA where the proportions of IgG were similar to those of normal IgG. Patients 220084 and 220028 had increased proportions of IgG4. It is thought that in chronic disease because of the process of somatic isotype switching (see 2.2.2), IgG4 can accumulate. Although all these ANCA were isolated from patients who were presenting for the first time and who had active disease, it is well know that patients may have long standing chronic, low level disease prior to presentation. Patient ANCA 220054 is the most unusual as it has a reduced proportion of IgG1 and increased proportions of IgG2 and 3 compared to normal IgG.

In the superoxide experiments the patients 220028 and 220054 who have significant superoxide responses also have higher proportions of IgG3. The respiratory burst

has been reported to be increased in patients with higher proportions of IgG3, as discussed above.

In the flow experiments, patient 220084 and 220028 caused neutrophil adhesion in both highly and minimally activated systems, but all four ANCA stopped neutrophils more than control in the minimally activated system. It is interesting that patient 220054 has a higher proportion of IgG2 which is thought to have very little pathogenic activity.

Comparison of the actions of patient ANCA due to the proportions of different subclasses is obviously limited with small patient numbers and these experiments were not designed to fully explore these differences. Important to note is the fact that the concentrations of the ANCA are not the same so it is difficult to speculate as to whether the differences seen are a subclass effect or simply due to the concentration of total ANCA.

5.4.9   Summary of studies with patient ANCA

Patient ANCA is polyclonal and thus directed against multiple different epitopes on the PR3 and MPO molecules. ANCA directed against certain epitopes may be more potent than other epitopes. In addition, IgG ANCA is known to vary in subclass fraction, which may influence its pathogenesis. It is difficult to conclude very much from a sample of just four patient ANCA but it is interesting to note that the ANCA which had the least effect in the flow model had a different subclass profile to the

other three ANCA with a greater proportion of IgG2. Again this is total IgG however and may not reflect the relative proportions of each subclass of ANCA. The cause of the functional variability between ANCA preparations from different patients is the subject of ongoing studies in the laboratory and appears to be dependent on the neutrophil donor as well as on the ANCA.

These points are addressed in part by the use of standardised ANCA, which are of known subclass, are monoclonal, directed against a single epitope and can be used in exact concentrations, and to address this chimeric PR3-ANCA had been developed by workers in our laboratory.

After these experiments had been performed to set up systems and explore previous work in this area, studies were undertaken using chimeric PR3-ANCA. In the flow studies a minimally activated endothelium (TNFα 2units/ml) was used as this had been found to be the most useful method of studying differences between ANCA in these initial experiments.

5.4.10 Endotoxin effects on neutrophils in flow

In order for lipopolysacchride (LPS) to activate immunologically active cells, it is bound to CD14 on monocytes and macrophages, which then become responsive. CD14 can be shed by macrophages and taken up by other cells, such as neutrophils, which can then confer LPS responsiveness to them. Conceivably this process could take place during the neutrophil preparation so that the neutrophils become

responsive to LPS without the need for serum in the system to supply CD14 (Chaby 2004). In addition, it has been reported that L selectin (CD62L) on neutrophils can bind LPS and trigger signalling (Malhotra and Bird 1997).

Endotoxin removal or addition had a significant effect on the behaviour of the neutrophils in the flow model, especially concerning the normal IgG control, where the presence of endotoxin reduced rolling and increased static adhesion. This affect was not so marked when the system was highly stimulated, such as in the presence of ANCA and may explain why there was considerable difficulty with controls in some experiments.

The effect of endotoxin was important in systems which were not already fully stimulated. Therefore when studying the various effects of cytokines on EC, systems which are stimulated with high concentrations of TNFα or IL-1β were already fully stimulated and caused neutrophils to stop rolling whether or not endotoxin is present. Endotoxin therefore became much more important in systems with low concentrations of cytokines stimulating EC, and masked subtle differences in behaviour on these cells. Once this was established, for further experiments in flow using normal IgG or ANCA, endotoxin was routinely removed.

6. INTERACTIONS OF IMMUNOGLOBULIN AND NEUTROPHILS: NORMAL IGG VERSUS ANCA AND THE EFFECT OF IGG SUBTYPE

Having established systems and models for the study of neutrophils and their interactions with ECs we wished to proceed to answer the question 'How is the interaction of neutrophils with normal IgG in a conventional situation different from the interaction of neutrophils with ANCA?' Conventionally, neutrophils bind to immunoglobulin via their Fc receptors (FcR). Neutrophils have three activatory Fc receptors; two constitutive, CD32 and CD16 and one inducible CD64. CD64 is the only receptor that can bind to soluable IgG. ANCA are predominantly IgG and are found in all four human IgG subclasses. Several papers have discussed the importance of IgG subclass in vasculitis. IgG3 has been proposed to be more pathogenic than the other subclasses in AAV (Mulder, Stegeman et al. 1995), to fall in remission (Jayne, Weetman et al. 1991) or to predominate in renal limited AAV (Brouwer, Tervaert et al. 1991) and IgG3 has been reported to be predominant in PR3-ANCA patients (Segelmark and Wieslander 1993; Harper, Radford et al. 2001). However these findings are contradicted by other studies where IgG1 and IgG4 were dominant in patients with cANCA (largely PR3-ANCA) (Mellbye, Mollnes et al. 1994) or overall in AAV (Brouwer, Tervaert et al. 1991; Harper, Radford et al. 2001). In consequence it is not clear from the published studies which subclasses of ANCA predominate or which are the most pathogenic. Clinical studies, although informative, are not functional or mechanistic and since no clear picture emerged from these studies regarding pathogenicity based on ANCA subclass, we wished explore these questions in the laboratory.

Binding of IgG to FcR allows downstream signalling and activation of the neutrophil. Opsonisation of antigen with IgG allows recognition, binding and phagocytosis by neutrophils. FcR require cross-linking of more than one receptor to induce neutrophil activation (Ben-Smith, Dove et al. 2001). ANCA, in contrast to normal IgG can bind to neutrophils via the Fab portion of the antibody to either PR3 or MPO. We have previously postulated that simultaneous binding via the Fc receptor may cross link these receptors resulting in abnormal downstream signalling pathways and activation of the neutrophil, evidence for this has previously been published from our laboratory (Williams, Ben-Smith et al. 2003).

During formation of antibodies, subclass is generated by gene splicing. Somatic isotype subclass switching occurs in peripheral B cells where a cell can change production from IgG3 to 1, then to 2, then to 4 by sequentially splicing out more of the gene (Zeijlemaker 1996).This process is unidirectional and therefore continuing exposure to antigen may result in the accumulation of IgG4. It is not completely clear how this process is influenced but isotype switching to IgG4 has been shown to be dependent on interleukin-4 (Spiegelberg 1989). There is some evidence to support the accumulation of IgG4 in AAV (Cambridge, Williams et al. 1994). If IgG4 was less pathogenic over time the disease might reduce in severity. In anti-glomerular basement membrane (GBM) disease recurrence of IgG4 anti-GBM antibody after treatment and remission did not cause disease relapse, but IgG1 recurrence did (Bowman, Ambrus et al. 1987). Although anecdotally in AAV, patients do remain in sustained remission after years of active disease, this is not a consistent disease

pattern, and relapses are common. Presumably factors that are triggering relapse, such as infection, may cause new antibody production, perhaps against new antigen epitopes.

One proposed aspect of the pathology of AAV is that ANCA activates neutrophils and causes adherence to vessel walls, damaging the vessel. In order for neutrophils to be captured onto an EC surface, the neutrophil is engaged via selectin molecules enabling rolling on the EC. Provided an activating stimulus, such as ANCA, is passed to the neutrophils, stable, static adhesion is mediated through neutrophil integrins (CD18/CD11a and CD18/CD11b) binding to endothelial intracellular adhesion molecule-1 (ICAM-1) (Dustin, Rothlein et al. 1986). Transendothelial migration can then take place via a series of molecules such as junctional adhesion molecules (JAM) (Woodfin, Reichel et al. 2007).

Our hypothesis based on the above was that ANCA IgG subclasses have different potencies and subsequently different pathogenic effects; moreover that these may be activated through different Fc receptors. Therefore we used purified IgG subclasses in a flow model to establish the interaction of the neutrophil with normal IgG and subsequently looked at the interactions of the neutrophil with ANCA on P-selectin and EC surfaces.

Previous studies have used immobilised IgG to capture neutrophils, and explored the roles of Fc receptors and integrins, but they did not look at the effects of IgG subclass

(D'Arrigo, Candal-Couto et al. 1995; Sheikh and Nash 1996; Coxon, Cullere et al. 2001).

6.1 Studies of normal IgG interacting with neutrophils

In order to look at normal IgG and neutrophil interaction we set up studies in flow where microslides were coated with pure human IgG of subclasses 1-4 and neutrophils flowed across. These experiments explored neutrophils binding to IgG under conditions of flow, where capture is likely to involve neutrophil FcR interacting with IgG Fc presented on a solid-phase support.

6.1.1 Concentration gradient of IgG1

In the first experiments we explored the concentration of IgG needed to cover the surface to allow adequate neutrophil capture after 5 minutes of neutrophil inflow. Using concentrations of 50, 100, 200 and 400μg/ml, neutrophil binding increased (Figure 18 left hand panel). Great variability was noted between donors, but this was not further explored. In the three initial experiments, binding onto the IgG1 surface was relatively low, but it was decided that 100μg/ml would be adequate to capture enough neutrophils for study and this concentration was therefore used for all further coating experiments. Neutrophil behaviour was similar at all concentrations (Figure 18 right hand panel).
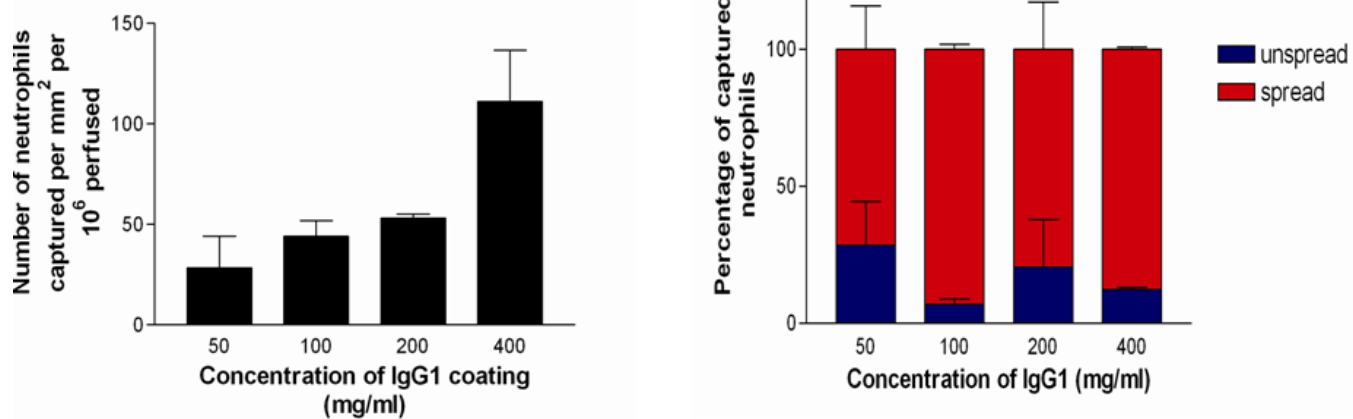
Figure 18: Concentration curve of IgG1 neutrophil capture and behaviour.

Using the flow model, neutrophils were flowed over different concentrations of coated IgG1. Left hand panel: capture increased slightly between 50-200µg/ml and was greater on 400µg/ml. Numbers of neutrophils captured was low. Right hand panel: Neutrophil behaviour was similar at each concentration, most of the neutrophils spread on the IgG1 surface. Wall shear stress 0.05kPa. n=3.

In these experiments as with others in this thesis great variability was noted between neutrophil donors in terms of neutrophil behaviour in the flow model. Some donors were more 'sticky' to surfaces than others, and the experiments were fairly consistent within donors. For this reason, experiments were repeated using at least three different donors, and this accounts for the variation in numbers between experiments, where three 'sticky' donors were used for one experiment and three 'non-sticky' for another. Within sets of experiments, e.g. testing each subclass, the same donor was used for each subclass, and at least three donors were used. The phenomenon of donor variation which was noted during this thesis was not further explored here, but subsequently ongoing work has begun in our laboratory to explore this phenomenon which may clearly have disease implications.

6.1.2   Comparison of IgG subclasses 1-4

Using concentrations of 100µg/ml for all antibody coating, the four subclasses were then compared. Neutrophils bound avidly to IgG3, less to IgG1 and minimally to IgG2 and IgG4 (Figure 19). The number of neutrophils captured onto these surfaces was increased over the initial experiments because there was variability between donors with some donor neutrophils being more 'sticky' than others.

The numbers of neutrophils captured onto these surfaces were significantly different from each other (IgG1 versus IgG2 p=0.004; IgG1 versus IgG3 p=<0.001; IgG1 versus IgG4 p=0.032; IgG2 versus IgG3 p=<0.001; IgG3 versus IgG4 p=<0.001) except IgG2 and IgG4. Once captured onto the immunoglobulin surface the neutrophils behaved similarly, changing from bright spheres to a phase-dark spread morphology with extended pseudopodia stretching across the surface (Figure 20). Movement appeared to be random on the surface which was uniformly covered with IgG. There was no difference in the behavioural pattern exhibited by the neutrophils on the different IgG subclass surfaces with most neutrophils spreading on the surface (Figure 19 right hand panel).
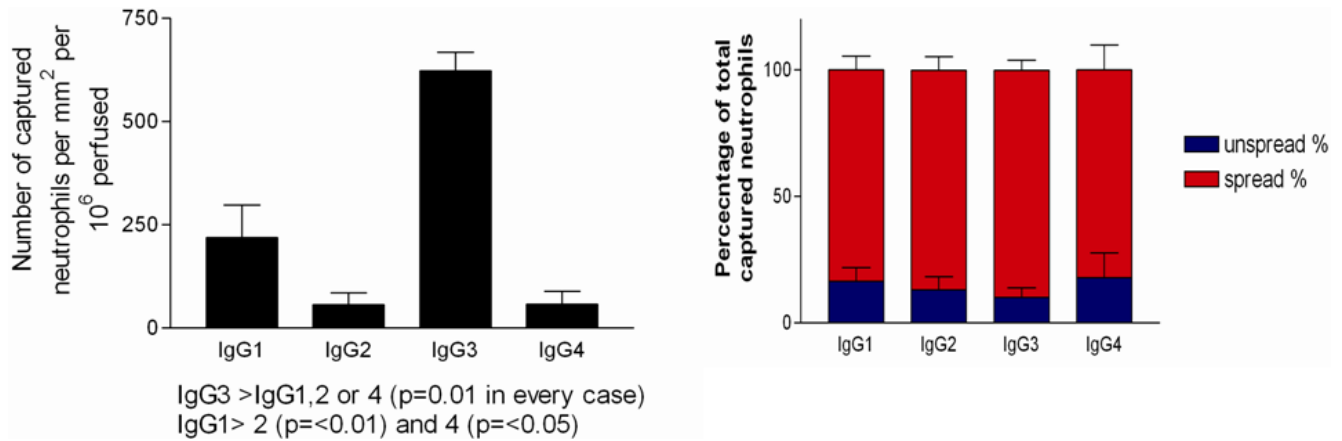
Figure 19 Binding of neutrophils to IgG surfaces of subclasses IgG1-4.

Using the flow model, neutrophils were flowed over IgG surfaces; the number of neutrophils captured and their behaviour, whether round or spread observed. Left hand panel: IgG3 avidly captures neutrophils, IgG1 captures to a lesser extent (IgG3 versus IgG1 p=<0.001) and IgG2 and 4 capture only minimally. Right hand panel: Behaviour is similar in the four subclasses. Most of the neutrophils spread. Wall shear stress = 0.05Pa; n=3.
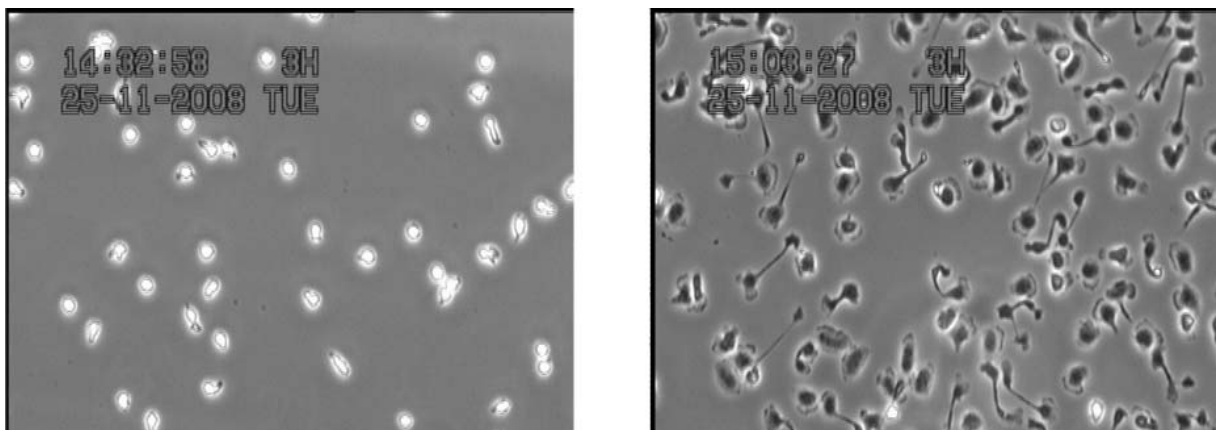
Figure 20: Behaviour of neutrophils on an IgG surface.

In the left hand panel neutrophils are adhered but round and unspread. In the right hand panel neutrophils have spread out and appear dark with extended psuedophilia.

<u>Blockade of CD32 and CD16 on neutrophils on normal IgG</u>

To establish which Fc receptors were important for the interaction of the various subclasses with neutrophils, blockade of the two constitutively expressed Fc receptors on the neutrophil surface was undertaken. Neutrophil behaviour was studied after adding antibodies to block CD32 and CD16 (Figure 21). On coated IgG3 both antibodies reduced neutrophil binding significantly compared to control antibody (CD32 $p=<0.001$; CD16 $p=<0.001$), but anti-CD16 was more effective than anti-CD32. On coated IgG1 and IgG2, the antibodies again inhibited adhesion, but now anti-CD32 was more effective than anti-CD16. Only blockade of CD32 reached significance (IgG1 $p=0.039$; IgG2 $p=0.018$). IgG4 supported such minimal capture that no effect of antibodies could be reliably observed. On IgG3, since there was residual capture after blockade of either receptor, both receptors were blocked together and this completely abrogated neutrophil capture (95%+/-3%; data not shown). Blockade of Fc receptors also changed the behaviour of the neutrophils once captured. Much less spreading was seen, especially with blockade of CD32 (Figure 22), with 70 -100% of adherent neutrophils remaining spherical.
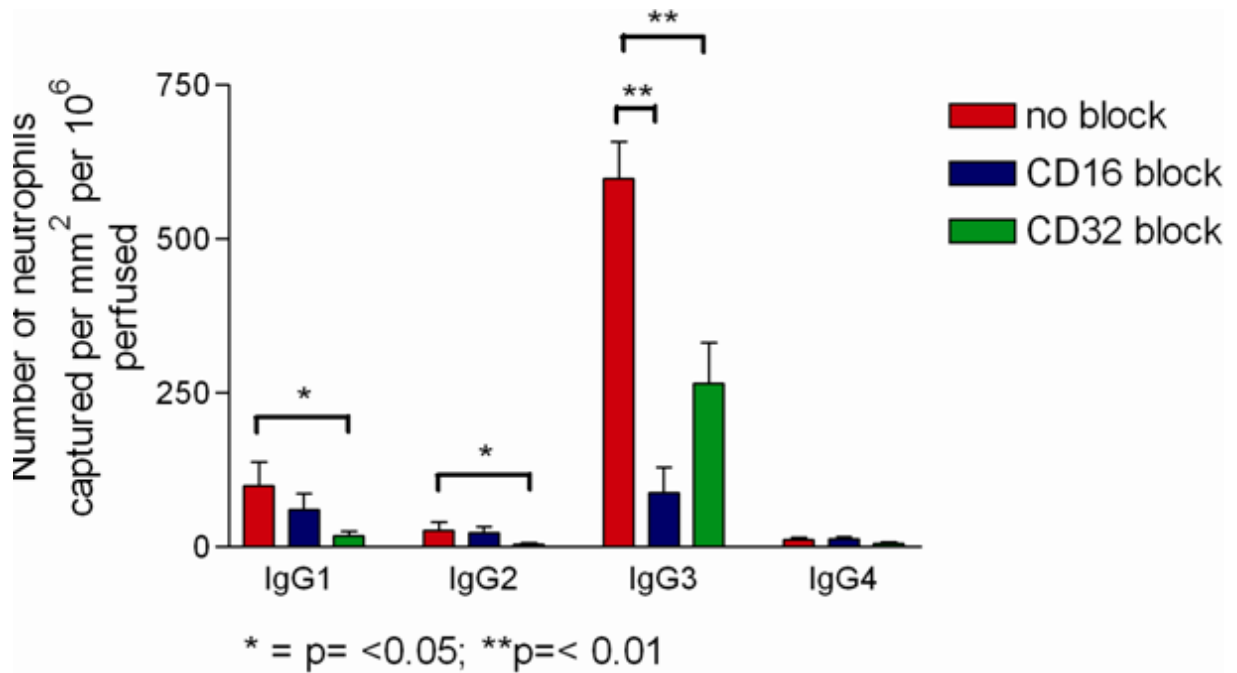
Figure 21 Neutrophil adhesion on IgG surfaces 1-4 after blockade of CD32 and CD16.

Using the flow model, neutrophils were flowed across the IgG surface after blockade of CD32 or CD16. Either blocking antibody reduced neutrophil spreading on the surface, but CD32 was more important in IgG1 (p=0.039 compared to control) and 2 (p=0.018 compared to control) and CD16 more important in IgG3 (p=<0.001 compared to control). There was no significant difference on IgG4 as binding was very minimal prior to blocking. Wall shear stress =0.05Pa; n=3.
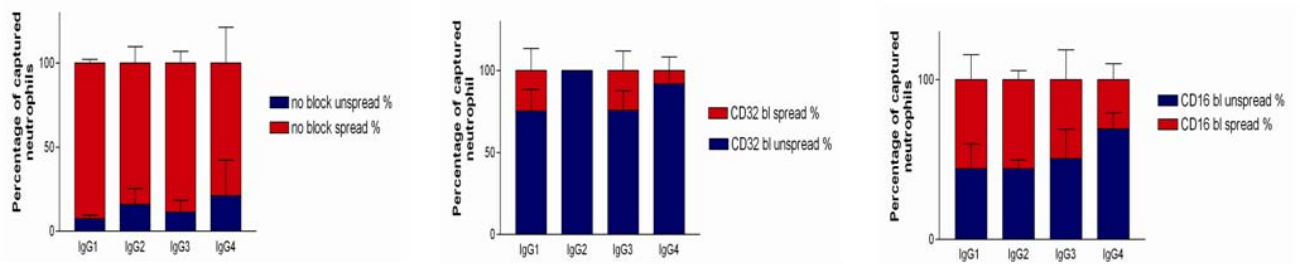
Figure 22: Neutrophil behaviour on IgG surfaces after FcR blockade.

Using the flow model neutrophil behaviour after blockade of CD32 and CD16 was observed. With no FcR blockade most of the neutrophils spread (red bars first panel). Blockade of CD32 results in most of the neutrophils remaining unspread (blue bars, second panel). Blockade of CD16 results in a reduction in spreading compared to no block, but it is not as dramatic as CD32 (third panel). Behaviour was similar in all experiments across the subclasses. Wall shear stress 0.05kPa. n=3.

6.1.4   Blockade of CD18 and treatment with BAPTA-AM

To explore the role of the integrin CD18 on the coated IgG3 surface, the neutrophils were treated with anti-CD18 (Figure 23). This did not reduce neutrophil binding to the IgG3 but it did change neutrophil spreading (p=<0.001). Thus, integrin molecules were not involved in capture but were important in subsequent behaviour of the 'activated' cells.

Finally in this set of experiments we wished to see if the binding of neutrophils to coated IgG via Fc receptors was a purely mechanical process or whether signalling of the neutrophil was important in binding. We therefore inhibited calcium signalling of

the neutrophil using BAPTA-AM which chelates intracellular calcium. This reduced the ability of the neutrophil to bind to the surface (p=<0.001) (Figure 23 left hand panel) and in addition reduced the ability of bound neutrophils to spread (p=0.001) (Figure 23 right hand panel). Thus, while FcR-IgG interaction directly captured flowing neutrophils, it also transduced a signal which stabilised adhesion through β_2-integrins.
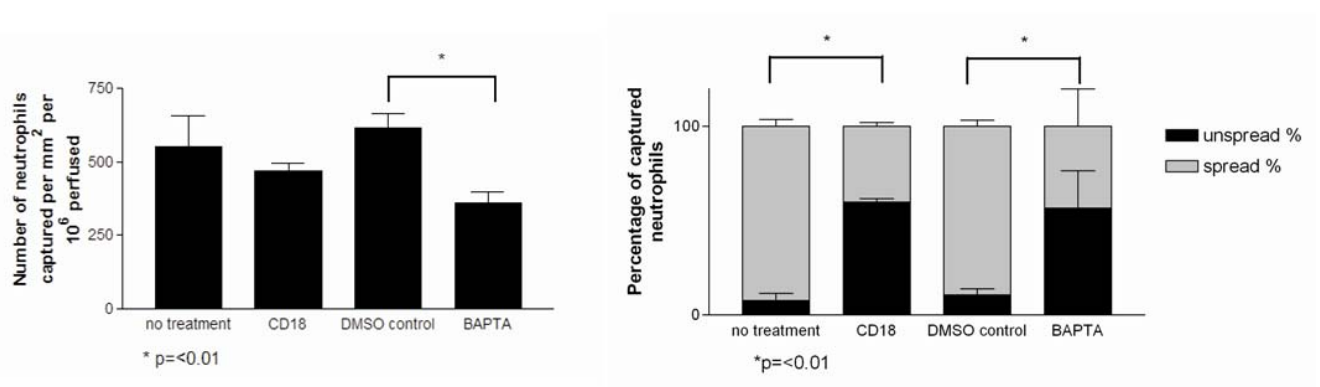
Figure 23: Blockade of CD18 and neutrophil calcium signalling inactivation.

Neutrophil adhesion and behaviour on IgG3 coated surface after blockade of CD18 and inactivation of neutrophil calcium signalling with BAPTA-AM. Left hand panel: Blockade of CD18 did not reduce binding; calcium signalling inactivation of the neutrophil by BAPTA-AM reduced neutrophil binding (p=<0.001 compared to control). Right hand panel: Blocking CD18 reduced the number of neutrophils which spread (p=0.001 compared to control). Use of BAPTA-AM reduced neutrophil spreading (p=<0.001). Wall shear stress = 0.05Pa; n=3.

6.2   Chimeric PR3-ANCA

In previous studies and in preliminary work for this thesis, it has been demonstrated that IgG isolated from patients with AAV can activate neutrophils causing a superoxide response, and can increase adhesion and migration of neutrophils on

ECs in flow. Human ANCA is difficult to isolate in a pure form and use of the IgG fraction containing ANCA means that the quantity of ANCA is unknown. Human ANCA is directed against many epitopes on the PR3 molecule, but 50% of human ANCA is directed against an epitope recognised by the mouse antibody 4A5. Workers in our laboratory previously developed a chimeric mouse/human anti-PR3 ANCA directed against this epitope in order that we could study the interactions of ANCA and neutrophils with a known concentration of ANCA and of known subclass. IgG1, 3 and 4 were developed.

6.3   Superoxide and IgG1, 3 and 4 chimeric PR3-ANCA

We firstly established that superoxide was produced by neutrophils in the presence of the chimeric ANCA. We compared superoxide responses produced by neutrophils in the presence of chimeric PR3-ANCA to those in the presence of chimeric NP control. We expected that IgG1 and IgG3 PR3 ANCA would activate the neutrophil to produce superoxide (both $p = {<}0.001$ by ANOVA compared to control), but we additionally found that IgG4 elicited a superoxide response ($p = 0.012$) (Figure 24).
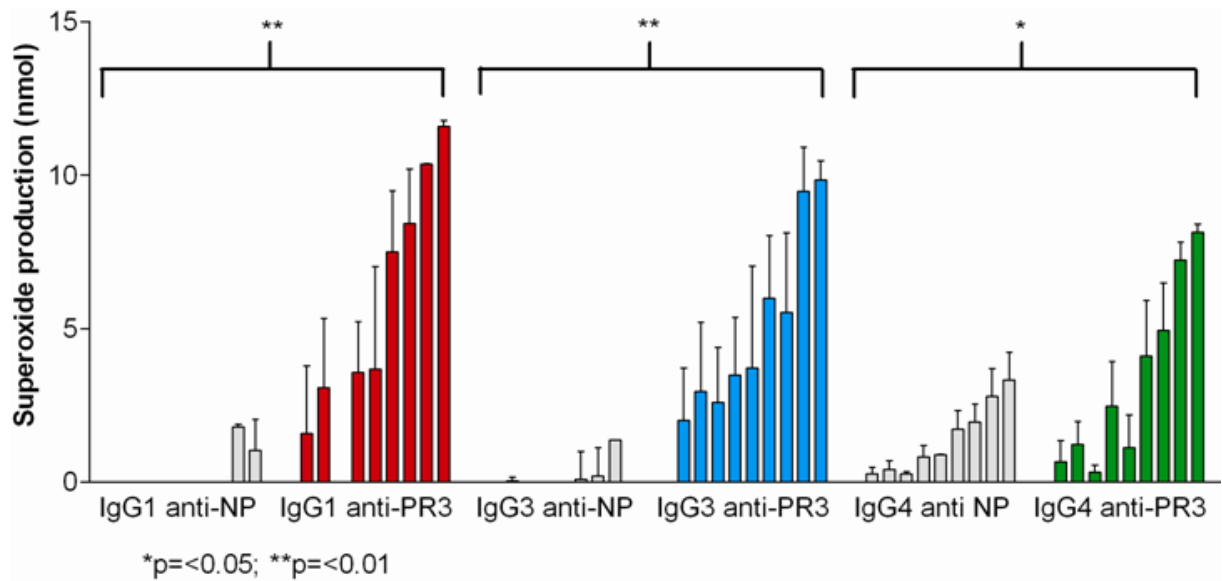
Figure 24: Superoxide production by neutrophils exposed to chimeric PR3-ANCA.

Superoxide anion production was determined by the superoxide dismutase inhibitable reduction of ferricytochrome c. Neutrophils were suspended to $2x10^6$ cells/ml and primed with cytochalasin B (2.5μg/ml) for 5 minutes and then TNFα (2ng/ml) for 15 minutes at 37°C. Neutrophils were stimulated with 60μg/ml of IgG1 and 4 and 48μg/ml of IgG3. Significant levels of superoxide over control IgG NP were produced by the three chimeric PR3-ANCA (p=<0.001 for IgG1 and 3; p=0.012 for IgG4 by ANOVA). All superoxide production represented over time up to 120 minutes. (n=4)

Superoxide experiments which had been performed with patient ANCA (section 5.4.5) had included negative controls where no IgG was used to stimulate the neutrophil and no superoxide response was seen here. In experiments where IgG control was used a small amount of superoxide is elicited, and the reasons for this are discussed previously (section 5.4.6).

6.4 Effect of fluid phase chimeric PR3-ANCA IgG on neutrophils rolling on P-selectin

Using the flow model with P-selectin coated microslides we explored the effects of ANCA IgG of different subclasses on neutrophils already rolling on P-selectin in microslides.

Typically, activation of such rolling neutrophils causes them to become stably adherent and migrate on the surface (e.g., Sheikh and Nash, 1996; Rainger et al., 1997). Here, soluble ANCA IgG is believed to bind to target PR3 antigen and then cross-link to FcR to transduce an intra-cellular signal [12].

For the total neutrophils captured, when all three PR3-ANCA (IgG1, 3 and 4) were compared together there was no statistical difference between the total number of neutrophils captured and this was also true for each PR3-ANCA individually compared to each other (Figure 25).
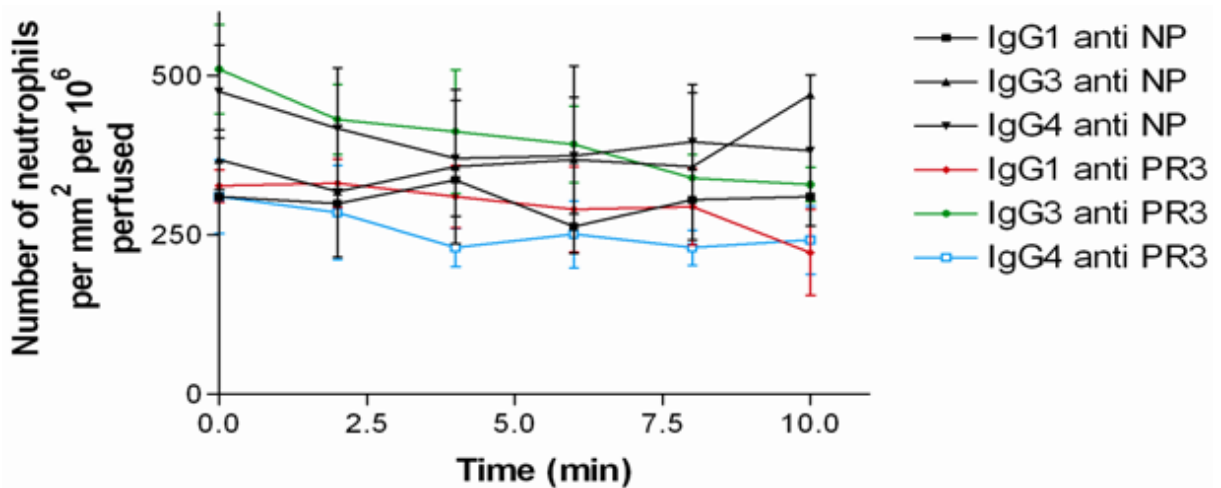
Figure 25: Neutrophils captured onto a P-selectin surface after exposure to chimeric anti-PR3 ANCA.

Using the flow model neutrophil adhesion to a P-selectin surface (2μg/ml) did not significantly vary after exposure to chimeric anti-PR3 ANCA or control anti-NP, nor did it vary over time. Wall shear stress = 0.1Pa; n=3

Behaviour of the neutrophils on the P-selectin surface did however vary with antibody exposure. IgG1 NP control was compared with chimeric IgG1 PR3-ANCA (n=3). Under the influence of IgG1 PR3-ANCA neutrophils adhered to the P-selectin surface, and by 4 minutes less than 5% of the neutrophils were rolling. When treated with control NP the neutrophils continued to roll (Figure 26). By ANOVA there was significance in variation over time (p=0.035) and the differences between the treatments were highly significant (p=<0.001). The variations between experiments did not reach significance.

The comparison of IgG3 control NP versus PR3-ANCA (n=3) also demonstrated that by 4 minutes the number of rolling cells after exposure to PR3-ANCA had fallen to less than 5%, the control antibody did not stop the cells (p=0.005) (Figure 26).

Change in cell behaviour over time was significant in these experiments (p=0.048) and when analysed separately, change over time was significant for IgG3 PR3-ANCA but not for control NP. There was no significant difference between experiments.

In the comparison between IgG4 PR3-ANCA and control NP, PR3-ANCA stopped the neutrophils rolling more than the control (p=0.01). By 8 minutes 35% of the neutrophils exposed to ANCA were rolling, whereas 70% of the control neutrophils continued to roll (Figure 26). The behaviour of the neutrophils over time varied significantly with PR3-ANCA (p=0.031), but not with the control.
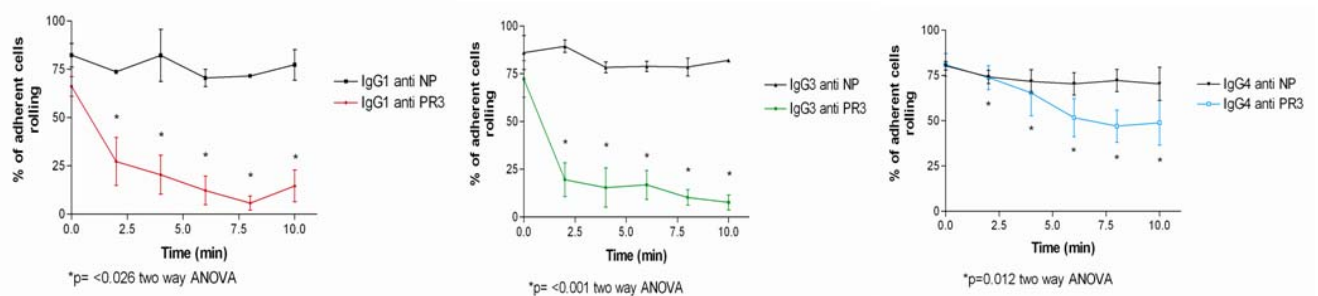
Figure 26: Neutrophils on P-selectin with chimeric PR3-ANCA.

Neutrophils are converted to static adhesion on a P-selectin surface after exposure to chimeric PR3-ANCA. Using the flow model, neutrophils were flowed across a P-selectin surface (2μg/ml). Neutrophils roll on this surface but with the addition of chimeric anti-PR3-ANCA of subclasses 1 and 3 the neutrophils stopped rolling and adhered to the surface (IgG1 p=0.026; IgG3 p=<0.001); treatment with control NP did not alter rolling. IgG4 chimeric PR3-ANCA also had this effect (p=0.01) but it was of lesser magnitude. Wall shear stress = 0.1Pa; n=3

In a comparison of all three subclasses together, there was no difference in the influence of IgG1 and IgG3 PR3-ANCA on neutrophils in this system (Figure 27). Time was significant (p=<0.001). IgG4 stopped the cells rolling less quickly than the other two subclasses and a greater proportion of cells remained rolling (both p=<0.001).
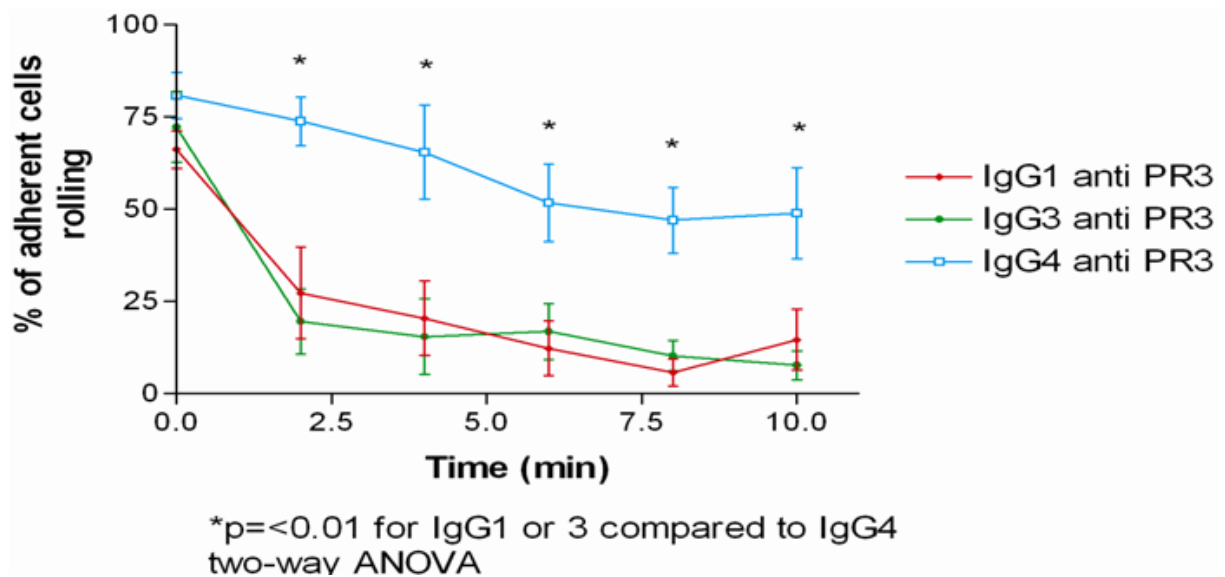
Figure 27: Comparison of PR3-ANCA IgG subclass on P-selectin.

IgG1 and 3 PR3-ANCA stop neutrophils rolling on a P-selectin surface more than IgG4 PR3-ANCA. Using the flow model, neutrophils were flowed across a P-selectin surface (2μg/ml). IgG4 chimeric PR3-ANCA stops neutrophils rolling to a lesser extent than IgG1 or IgG3. (IgG3 compared to IgG4 p=0.001, IgG1 compared to IgG4 p=0.014). Wall shear stress = 0.1Pa; n=3

6.5 Blockade of CD16 and CD32 on ANCA-stimulated neutrophils

Experiments were undertaken to explore the role of CD32 and CD16 on neutrophils in the situation of exposure to ANCA. These experiments were limited by reagent as the chimeric ANCA were limited. The experiments on P-selectin were repeated but

only two time points taken, immediately pre-ANCA and 2 minutes after exposure to ANCA. Neutrophils which had previously been treated with isotype control antibody, or anti-CD32 or anti-CD16 were flowed across the slide and events recorded. Chimeric ANCA of subclasses IgG1, 3 or 4 was then flowed over the slide and cell adhesion and behaviour recorded.

We explored the role of CD16 and CD32 in the activation of neutrophils rolling on P-selectin after exposure to the different subclasses of chimeric ANCA. Blockade of CD16 significantly reduced the effect of IgG1 ANCA on the neutrophils (p=0.03), that is, the neutrophil activation induced by ANCA which changed behaviour from rolling to static adhesion was reduced, and the neutrophils continued to roll after CD16 was blocked. For IgG3 PR3-ANCA, blockade of either CD16 (p=0.012) or CD32 (p=0.017) reduced the effect of the ANCA, allowing neutrophils to continue to roll even when ANCA was present (Figure 28). The influence of Fc receptor blockade on IgG4 was difficult to comment on since the numbers of neutrophils stopping with ANCA were low, but the blockade of either receptor did revert the rolling proportion of cells back to baseline. In summary, both IgG1 and IgG3 ANCA potently activated rolling neutrophils as judged by conversion to stable adhesion, whereas IgG4 ANCA was weakly effective. In all cases, response to ANCA was reduced by blockade of FcR CD16 or CD32, suggesting both receptors may have a role in signal transduction.
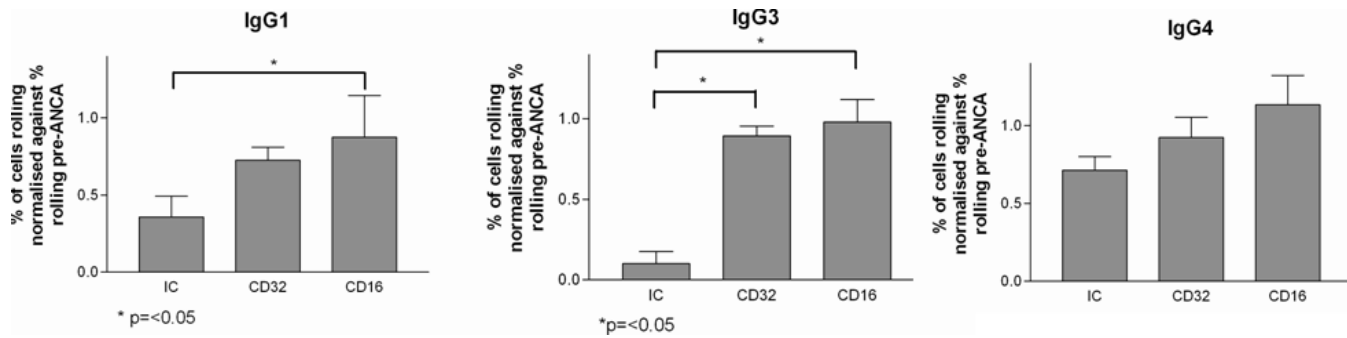
Figure 28: Blockade of CD32 and CD16 on ANCA-stimulated neutrophils.

After exposure to IgG1 chimeric PR3-ANCA, CD16 prevented ANCA stopping neutrophils more than control (p=0.028) and CD32 showed a trend. IgG3 chimeric PR3-ANCA was prevented from stopping neutrophils more than control by both CD16 (p=0.017) and CD32 (p=0.012). IgG4 chimeric PR3-ANCA was not blocked by either CD16 or CD32, although these again showed a trend. All experiments performed on P-selectin. Exposure to chimeric PR3-ANCA for 2 minutes, n=3.

6.6 IgG1, IgG3 and IgG4 PR3-ANCA with neutrophils on HUVEC

Following experiments with chimeric PR3-ANCA on P-selectin surfaces we proceeded to experiments on HUVEC in order to study the more complex interactions including integrin/ICAM-1, chemokine and cytokine influenced receptor activation.

Neutrophils were mixed with chimeric PR3-ANCA or controls just prior to perfusion into the flow model and this meant that in contrast to the P-selectin experiments there was no change over time as the ANCA were introduced, all recorded events occurred after the addition of the ANCA or its control. Although recordings were taken at multiple time points, for clarity only one time point (5 minutes after neutrophil inflow) is presented in these graphs. The EC had been activated with a low level of TNFα (2units/ml). Preliminary data was obtained comparing each chimeric PR3-ANCA to its control alone (data not shown). Multiple experiments were performed to obtain

clear comparative data and in initial experiments there were considerable problems with controls activating the neutrophils, particularly concerning the IgG3 antibody (data not shown). After using several different controls we explored the possibility of contamination by endotoxin, and all the samples were cleaned using endotoxin binding columns (see chapter 5.3). This alleviated the problem of controls activating the neutrophils and the final data presented here was a direct comparison of the three chimeric PR3-ANCA and their chimeric NP controls.

All three PR3-ANCA IgG subclasses tended to induce greater numbers of neutrophils to bind to the cytokine-activated EC surface compared to their equivalent subclass control antibodies (Figure 29).
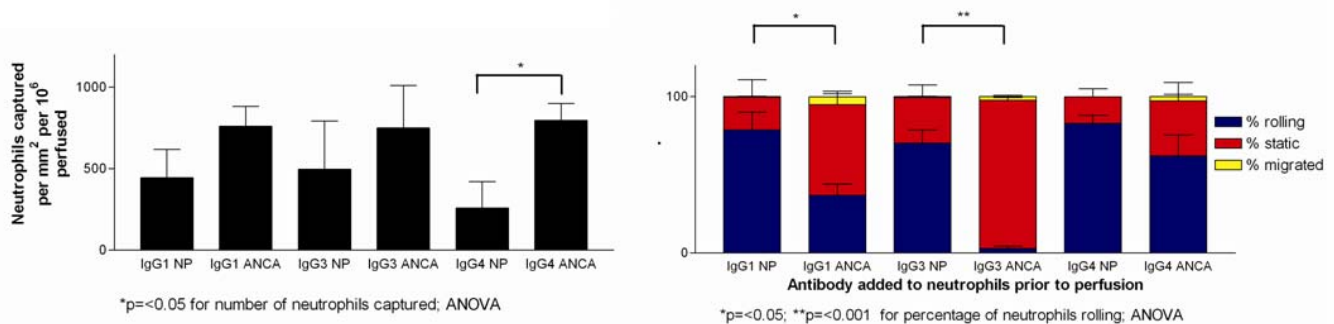
Figure 29: Neutrophils binding to and behaviour on human umbilical vein ECs.

Using the flow model neutrophils were flowed across human umbilical vein ECs which had been stimulated with TNFα at 2units/ml. Left hand panel: IgG1, 3 and 4 PR3-ANCA captured neutrophils efficiently and IgG4 captured neutrophils more than its control (p=0.048). Right hand panel: IgG3 PR3-ANCA efficiently stopped neutrophils from rolling compared to control (p=<0.001); IgG1 stopped neutrophils but to a lesser extent (p=0.036 compared to control) and IgG4 stopped neutrophils but this was not significantly different to its control. Data shown at 5 minutes after inflow. Wall shear stress = 0.1Pa; n=3

IgG3 PR3-ANCA was very potent in altering the behaviour of the captured neutrophils, causing 94% of neutrophils to stop rolling and convert to static adhesion, facilitating transmigration, and this behaviour was different to control antibody (29% neutrophils became static after exposure to the control antibody; p=<0.001) (Figure 29 right hand panel). The same was true of IgG1 PR3-ANCA but the affect was less potent (58% of neutrophils converted to static adhesion compared to 21% with control antibody; p=0.036). IgG4 PR3-ANCA also tended to convert rolling to static adhesion (35% of neutrophils converted to static adhesion with anti-PR3 ANCA IgG4 compared to 17% of neutrophils with control IgG), but this change in behaviour did not reach statistical significance (Figure 29). In general ANCA therefore increased overall binding and tended to stabilise adhesion.

In these flow experiments and in the preceding experiments on P-selectin, it should be noted that controls and chimeric PR3 ANCA were compared directly and neutrophils with no IgG were not included in these experiments. This was due to the fact that only 6 slides could be used in each experiment and these were the 3 subclasses, each with anti-PR3 and its control. Previous experiments had indicated that neutrophils with no IgG behave similarly to control IgG (section 5.2.4.1).

6.7   Discussion

This study was based on the premise that the interaction of a PR3-specific autoimmune immunoglobulin in AVV with the neutrophil is unique and is the pathogenic basis of the disease. In order to compare interactions of IgG with

neutrophils we examined neutrophils interacting with IgG that did not contain ANCA, and compared this to neutrophil interactions with ANCA IgG. Thus, normal IgG coated on a continuous presenting surface that would mimic presentation of Fc portions of fixed immune complexes was compared with control or ANCA IgG, presented in the fluid phase to flowing neutrophils, mimicking the situation in disease.

6.7.1   Experiments using normal IgG

In this study, freshly isolated neutrophils that had not been activated, bound to IgG presented on fixed surfaces and this interaction followed the conventional knowledge that neutrophils will interact avidly with IgG3, and with IgG1 and minimally or not at all with IgG2 and IgG4 (Jefferis and Kumararatne 1990) although we found increased binding to IgG3. Capture of neutrophils onto IgG surfaces has been demonstrated previously (Skilbeck, Lu et al. 2006). Greater capture of neutrophils by IgG3 may reflect the enhanced flexibility of the larger IgG3 molecule. It is unlikely that the enhanced neutrophil binding to IgG3 was due to a higher concentration of this subclass on the glass APES-coated slide as the antibodies were coated in equal molarity rather than equal concentrations which would disadvantage the larger IgG3 molecules. The differential between the antibodies is interesting particularly since in disease states, IgG3 autoantibodies have proposed to be more pathogenic (Mulder, Stegeman et al. 1995).

Having established that flowing neutrophils could be captured well by IgG3 and IgG1 Fc, the specific neutrophil receptors supporting this were investigated using blocking

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antibody. The relative roles that CD32 and CD16 play in individual subclass binding to neutrophils may be useful in the manipulation of disease states and in order to look at this we performed blocking studies. Blocking studies have been performed previously in this model using mixed immunoglobulin, and CD16 blockade was found to be of the greatest importance while CD32 also had a significant but lesser effect (Skilbeck, Lu et al. 2006). We looked at the roles of the constitutive receptors CD32 and CD16 in neutrophil adhesion in flow conditions, and found that while all subclasses are affected by blocking these, there is a differential importance in the receptors. Blocking either receptor reduced binding in all four subclasses and blocking both receptors abolished neutrophil binding. The importance of these receptors varied however, with CD16 being more important in IgG3-mediated adhesion under flow and CD32 more important for IgG1 and 2 mediated adhesion. In the previous study in flow (Skilbeck, Lu et al. 2006), the importance of CD16 over CD32 could therefore be explained by the presence of greater proportions of IgG3 subclass over IgG1. In addition, another study found that binding of subclasses on neutrophils to CD32 was highest with IgG3, then IgG1 and 2 and then IgG4. This was dependent however on allotypic form (Parren, Warmerdam et al. 1992). The present study has directly compared the relative importance of CD32 and CD16 for neutrophil adherence to IgG subclasses under flow conditions and has clearly shown that CD16 is more important for IgG3 mediated adhesion, while CD32 is more important for IgG1 and 2 mediated adhesion. Interestingly, in a previous study that examined soluble immune complex binding to neutrophils via CD16 and CD32, granule release by neutrophils was induced by all four IgG subclasses in the presence of complement, but in the absence of complement, only IgG1 and IgG3 were

functionally active. Granule release by binding of IgG3 immune complexes was mediated predominantly by CD16, whereas IgG1 immune complexes induced specific granule release by CD32, suggesting similar engagement of Fc receptors by IgG subclasses for granule release as was observed for capture and adhesion of neutrophils from flow (Voice and Lachmann 1997).

We next looked at the role of CD18 in neutrophil stabilisation and adhesion. CD18 was important in allowing the neutrophils to spread. CD18 is the common chain within the neutrophil $\beta 2$ integrin molecules comprising CD11a/CD18, which binds to ICAM-1 on the endothelial surface, and CD11b/CD18, which binds to ICAM-1, fibrinogen and factor X (Mayadas and Cullere 2005). Change in conformation of integrins on neutrophils requires inside-out signalling (Abram and Lowell 2007). In our experiments, blocking the integrin prevented the neutrophils from spreading on the IgG surface. This finding is supported by previous published work (Skilbeck, Lu et al. 2006). There are two possible explanations for this result, the first is that the Fc receptor ligation induces activation of the integrin which binds to a ligand (albumin in this case (Zhu, Subbaraman et al. 2000)) and outside-in signalling results in neutrophil spreading. The second is that the Fc receptor ligation itself causes the neutrophil to spread whilst simultaneously activating CD18 which is required to adhere to the surface.

When the neutrophil signalling was inhibited by chelating the intracellular calcium with BAPTA-AM, binding and spreading was reduced. The CD18 blocking studies indicate that spreading requires signalling within the neutrophil, but binding too is

clearly a metabolically active process and not simply mechanical; signalling is required to stabilise adhesion.

6.7.2 Studies with chimeric anti-PR3 chimeric ANCA

After establishing that solid-phase normal human immunoglobulin IgG subclasses differentially recruit neutrophils from flow by engagement with their constitutive CD16 and CD32, we wished to compare this with chimeric ANCA IgG subclasses. The ANCA IgG had to be present in the fluid phase to enable antibody Fab and Fc portions to concurrently engage with neutrophil antigen and with Fc receptors (Williams, Ben-Smith et al. 2003). The question was whether with this added complexity, neutrophil responses differed from that observed following cross-linking of neutrophil Fc receptors alone. In order to study subclass effects we used chimeric antibodies developed in our laboratory that were all directed against the same epitope of PR3 on the neutrophil surface.

All three anti-PR3 chimeric antibodies elicited a superoxide response. We wished to take the functional studies further and therefore used the flow model.

Initially we flowed neutrophils over a P-selectin surface as we have used previously ((Colman, Hussain et al. 2007) and Hussain et al in press). We verified that most of the captured neutrophils roll on this surface but with the addition of a second signal such as ANCA IgG, the rolling is converted to static adhesion. This was true of IgG1 and IgG3 anti-PR3 ANCA (Colman, Hussain et al. 2007) but also was seen with PR3-

ANCA IgG4 (Hussain et al in press) although to a lesser extent. IgG4 have been thought to be a benign blocking antibodies, and not to engage neutrophils via CD32 or CD16 (Stitis 1991; Roitt 1996; Heijnen and van de Winkel 1997), although more recent evidence suggests that this is simplistic and multiple parameters determine Fc binding and the functional outcome (Greenwood, Clark et al. 1993; Voice and Lachmann 1997). In addition IgG4 does not always behave as a conventional antibody which should be considered when comparing the IgG subclasses: polyclonal IgG4 antibodies are functional monovalent, but monoclonal IgG4 are not; IgG4 lacks the normal covalent interaction between the heavy chains and also bispecific antibodies can be found in plasma (Aalberse and Schuurman 2002). In our study, flow cytometric analysis demonstrated that CD64 was not present on the surface of freshly isolated neutrophils (data not shown), therefore PR3-ANCA IgG4 must be able to engage the constitutively expressed receptors. Indeed, previous studies confirmed that chimeric PR3-ANCA IgG4 stimulation of superoxide release and neutrophil degranulation can be blocked by antibodies to CD16 or CD32 in a manner partly dependent on the functional output, while deglycosylated PR3-ANCA IgG4, which cannot bind these receptors, is ineffective (Hussain et al in press).

We then went on to study the effects of blocking CD16 and CD32 on chimeric PR3-ANCA stimulated neutrophils. Both Fc receptors appeared to play a role when ANCA transduced an activation signal, with a hint that CD16 may have a more important role. Receptor usage for neutrophil capture from flow may be different from that in ANCA induced activation signalling. Neutrophils stopping on IgG coated surfaces may arise from activation of integrins, or simply from the ability of the Fc receptor

ligation to hold the neutrophil still. Our results with BAPTA-AM and CD18 blockade suggest that signalling is involved, so it may be that CD32 is more important for capture, and from the ANCA experiments, that CD16 is more important for neutrophil activation and subsequent conversion from rolling to static adhesion.

P-selectin is a simpler stratum for rolling neutrophils than endothelium where chemokines and adhesion molecules may alter responses. Thus, neutrophils that had been exposed to PR3- ANCA IgG subclasses, or control antibodies, were flowed over cytokine-activated HUVEC and neutrophil adhesion and behaviour observed. We suggest from these experiments that ANCA will stabilise binding of neutrophils and markedly change the behaviour of the neutrophils on the endothelial surface. The potency of ANCA IgG subclasses paralleled the findings on normal IgG, thus, the IgG3 subclass was most potent at inducing the neutrophils to become static, prior to migration, with IgG1 also being effective but to a lesser degree. These findings match the initial findings on P-selectin but, importantly, show that they also occur on a more physiological substratum that is more indicative of what may occur in the microcirculation. Again, there is the implication that IgG3 is a more pathogenic subclass in AAV (Mulder, Stegeman et al. 1995), and that IgG4 may not be as inert as previously considered, although pathogenic outcome will depend on the overall composition of the ANCA IgG subclasses between individuals.

The addition of anti-PR3 ANCA therefore activates the neutrophil and causes capture onto an endothelial surface, but it also changes the behaviour of activated neutrophils from rolling to adhesion and migration across the EC monolayers. We

have previously studied signalling pathways in the neutrophil and believe that simultaneously stimulating the neutrophil via the Fc and Fab portions of the immunoglobulin cross-links these receptors and causes aberrant downstream signalling resulting in inappropriate activation of the neutrophil (Williams, Ben-Smith et al. 2003). IgG1 and IgG3 seem to be strongly activating and even IgG4 seems to be activatory in this situation albeit weakly. We postulate this is due to different signalling strengths with recruitment of large mats causing increasingly strong signals until neutrophil activation takes place.

An argument such as this regarding cross-linking of receptors raises the question of whether cross-linking can occur with other epitopes on the neutrophil surface. We have previously tested this in our laboratory with CD15 (unpublished) and found no increased neutrophil stimulation. Blocking antibodies to CD32 and CD16 could also induce cross-linking but this was checked in previous studies in our laboratory and blocking antibodies alone or in combination did not stimulate the release of superoxide from neutrophils (Hussain in press) leading us to conclude that there was no cross-linking of the receptors in these experiments. Cross-linking therefore seems to be a unique feature of ANCA induced neutrophil stimulation.

In previous work we have demonstrated that IgG1 and IgG3 PR3-ANCA induce superoxide release, degranulation and IL8 production. IgG1 was more potent in degranulation and IgG3 more potent in IL-8 production (Colman, Hussain et al. 2007). We have also demonstrated that IgG4 anti-PR3 was able to induce superoxide in the same magnitude as IgG1, and degranulation was also similar, but

IgG4 anti-PR3 ANCA did not induce IL-8 (Hussain in press). In these studies we investigated the roles of CD32 and CD16 (Table 7) and demonstrated that superoxide production by neutrophils when stimulated with IgG1 and IgG4 PR3-ANCA was prevented by blocking CD32 but not CD16. Degranulation by IgG1 and IgG4 PR3-ANCA was also prevented by blocking CD32 but this time antibodies to CD16 also blocked the IgG1 PR3-ANCA response. IL-8 release in contrast required CD16.

	IgG1	IgG3	IgG4
Superoxide production	+	+	+
Degranulation	CD32 dependent ++	+	CD32 dependent ++
IL-8 production	CD32 dependent +	CD16 dependent ++	CD32 dependent -
Static adhesion	CD16 dependent +	CD16 dependent +	CD16 dependent
Adhesion in flow	++	+++	+
	CD16 dependent	CD16/CD32 dependent	

Table 7: Summary of chimeric ANCA action on neutrophils dependent on IgG subclass and Fc receptor engagement

In the current studies in the flow model we studied the effects of blocking CD16 and CD32 on chimeric PR3-ANCA stimulated neutrophils on adhesion. Blocking CD16 reduced activation of neutrophils by IgG1 and IgG3 PR3-ANCA, but CD32 was only affective with IgG3 PR3-ANCA. Neutrophils stimulated with IgG4 PR3-ANCA were

not influenced by CD32 or CD16 blockade, but the experiments were looking at early time points and in the previous experiments, IgG4 does not have an affect on neutrophils until later time points. It is interesting that CD32 was influential on IgG1 mediated neutrophil adhesion in initial experiments, but when IgG1 PR3-ANCA was involved, CD16 was more important. In IgG3 initially CD16 was more important, but both CD32 and CD16 had a significant effect on the ANCA-stimulated neutrophils. Previous studies in our laboratory with polyclonal patient IgG showed that the pro-adhesive effects of polyclonal human ANCA IgG, a mixture of all subclasses, in the P-selectin flow assay is particularly sensitive to inhibition of CD32 (Radford, Savage et al. 2000).

As regards the relative influence of Fc receptors in AVV, there seems therefore to be a differential effect depending on the neutrophil function. CD32 was important in superoxide production by IgG1 and 3 PR3-ANCA and in degranulation, CD16 influenced degranulation by IgG1, and IL-8 production. Neutrophil adhesion appeared to be influenced by CD16, although IgG3 was also affected by CD32. These findings are novel, and may be useful in designing therapeutic targets.

Chimeric and patient ANCA are different in that patient ANCA is polyclonal and therefore able to bind multiple antigenic targets on the PR3 molecule leading to clustering of antigen and Fc receptors to form large mats. We believe that this would be likely to amplify rather than alter the responses that we have observed.

In conclusion, these experiments showed that binding of neutrophils to IgG coated surfaces differs by IgG subclass and that Fc receptor blockade disrupts binding; CD32 is particularly important. Binding is an active process and requires signalling, and spreading on the IgG surface requires integrins. PR3-ANCA stabilises binding and activates neutrophils resulting in static adhesion, IgG3 is particularly potent and Fc receptor blockade disrupts the activation seen by ANCA. Exploration to target particular IgG subclasses, or Fc receptors may be useful in designing therapeutic intervention for AAV.

7. COMPARISON OF UMBILICAL VEIN AND GLOMERULAR ENDOTHELIAL CELL TYPES

Kidney disease is an increasing problem and the number of people commencing dialysis is rising. There is an urgent need to address preventative strategies in nephrology and the key to many renal diseases may lie with the EC. Literature regarding adhesion studies in renal endothelium is sparse and these studies aim to address this. In health, the renal circulation, due to its role in detoxification is supplied by 20% of the cardiac output. It is crucially important that leukocyte activation and recruitment does not take place within the kidney in a non-inflammatory environment. Renal capillaries are of a diameter of 10-20nm (Shiraishi, Wang et al. 2003) and require compression of leukocytes for them to pass through. If adhesion molecules were easily up-regulated this would result in clogging of glomerular capillaries with leukocytes and it may be that the glomerular EC has specific protective measures, such as reduced expression of adhesion molecules, to ensure recruitment of leukocytes is restricted to highly inflammatory conditions. In disease, disruption of such protection may explain specific targeting of the renal bed. The aim of this part of the thesis was to compare renal ECs with the standard comparator, human umbilical vein ECs (HUVEC) to establish any difference in adhesion molecule expression and functional ability in flow.

ECs are heterogeneous, changing their phenotype over time and according to the environment in which they exist. Endothelium may fluctuate between a resting and an activated state in response to inflammatory mediators, so that adhesion and other molecules can be up-regulated when EC are activated by cytokines such as TNFα

(Cotran and Pober 1990). Indeed, chemokines and adhesion molecule expression can differ between EC types as well as with changes of the activation status of the endothelium (Lim, Garcia-Cardena et al. 2003).

In the glomerular microcirculation the endothelium is particularly unique as it is permeable to water and small solutes, whilst remaining impermeable to macromolecules. Glomerular ECs are punctuated by multiple fenestrations, trans-cellular pores 60-80nm in diameter (Deen, Lazzara et al. 2001). These fenestrations have a glycocalyx that covers the fenestrae and probably contributes to the permeability barrier (Rostgaard and Qvortrup 1997).

Traditionally HUVEC have been used to model microvascular EC and have been presumed to behave similarly and although some studies have concluded similarities between ECs others have suggested that extrapolation from one to the other is not satisfactory, as discussed in the introduction.

This chapter explores studies in which we aimed to compare three types of EC: HUVEC obtained from primary culture within our institution, primary glomerular ECs (PGEC) and conditionally immortalized glomerular ECs containing a temperature sensitive transgene (called GEC2 here). We compared the cells by microscopy and presence of fenestrations. We then characterised them for the expression of CD31 and vWF; explored functional differences by determining the expression profiles of the cytokine-modulated adhesion molecules, E-selectin, ICAM-1 and VCAM-1, and by comparing their performance under conditions of flow for capture of neutrophils

and peripheral blood lymphocytes. We performed RNA arrays to compare the gene profile of the three EC types.

Cytokines were used to stimulate the endothelium to ascertain if these induced different patterns of behaviour dependent on the EC type. The cytokines chosen were TNFα and IL-1β which are both up-regulated in AAV, as discussed in the introduction. In order to induce selectins on the ECs, hydrogen peroxide was also used to treat the EC as this is known to induce selectins by up-regulation of histamine (Okayama, Coe et al. 1999). This can only be used for a short time as it is rapidly toxic to endothelium. In AAV the superoxide response produces hydrogen peroxide.

The comparisons in this chapter were complicated by the use of two different transformed (immortalised) glomerular cell lines. The first line (GEC1) was tested in flow initially and then tested in E-selectin ELISA and found to not express E-selectin. A second cell line (GEC2) was then tested, and comparisons made between this, primary GEC and HUVEC. All of these data are presented below.

7.1   Appearance of endothelial cells

7.1.1   Phase contrast microscopy

The 4 different cell types were grown in culture flasks and then transferred to glass slides. The monolayers of the cell types looked similar under phase contrast

microscopy, the cells grew in a single layer and were small, homogeneous and 'cobblestone' in appearance.
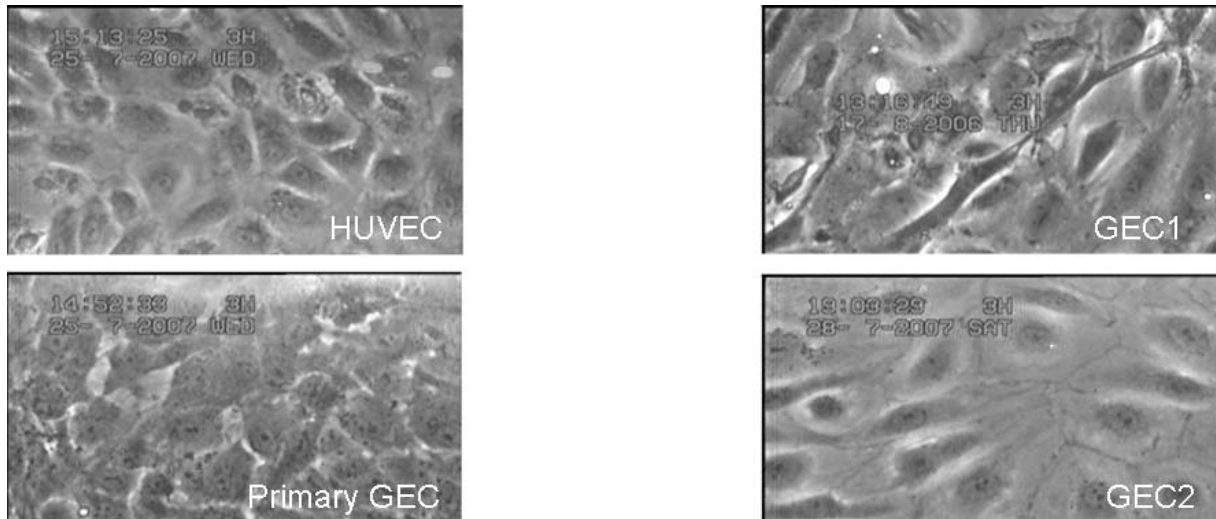
Figure 30: Phase contrast microscopy of ECs growing in monolayers.

Clockwise from left: HUVEC, immortalised glomerular cell lines GEC1, GEC2 and primary glomerular cells. All cells were grown in EBM-2 medium at 37°C. The immortalised lines were cultured at the permissive temperature (33°C) initially. Magnification x20, cells grown on glass slides.

7.1.2   <u>Electron microscopy</u>

HUVEC, PGEC and GEC2 were examined by electron microscopy (EM). We wished to see if the glomerular cells had fenestrations, a distinct feature of these cells. In order to induce fenestrations all cells were grown in medium containing high concentrations of vascular endothelial growth factor (VEGF), one hundred times the concentration of that in EBM2 medium and compared to those grown in EBM2 alone. Our initial studies used transmission EM in an attempt to see fenestrations through the whole cell, but these were unsuccessful, the cells fragmented and fenestrations

were not obvious. We therefore repeated the studies using scanning EM, where the surface of the cells could be seen. Fenestrations were observed on the primary glomerular cells, and these increased in the high VEGF medium. GEC2 demonstrated obvious fenestrations after high VEGF treatment, with a suggestion of these at normal VEGF concentrations. Fenestrations could not be induced on HUVEC (Figure 31).
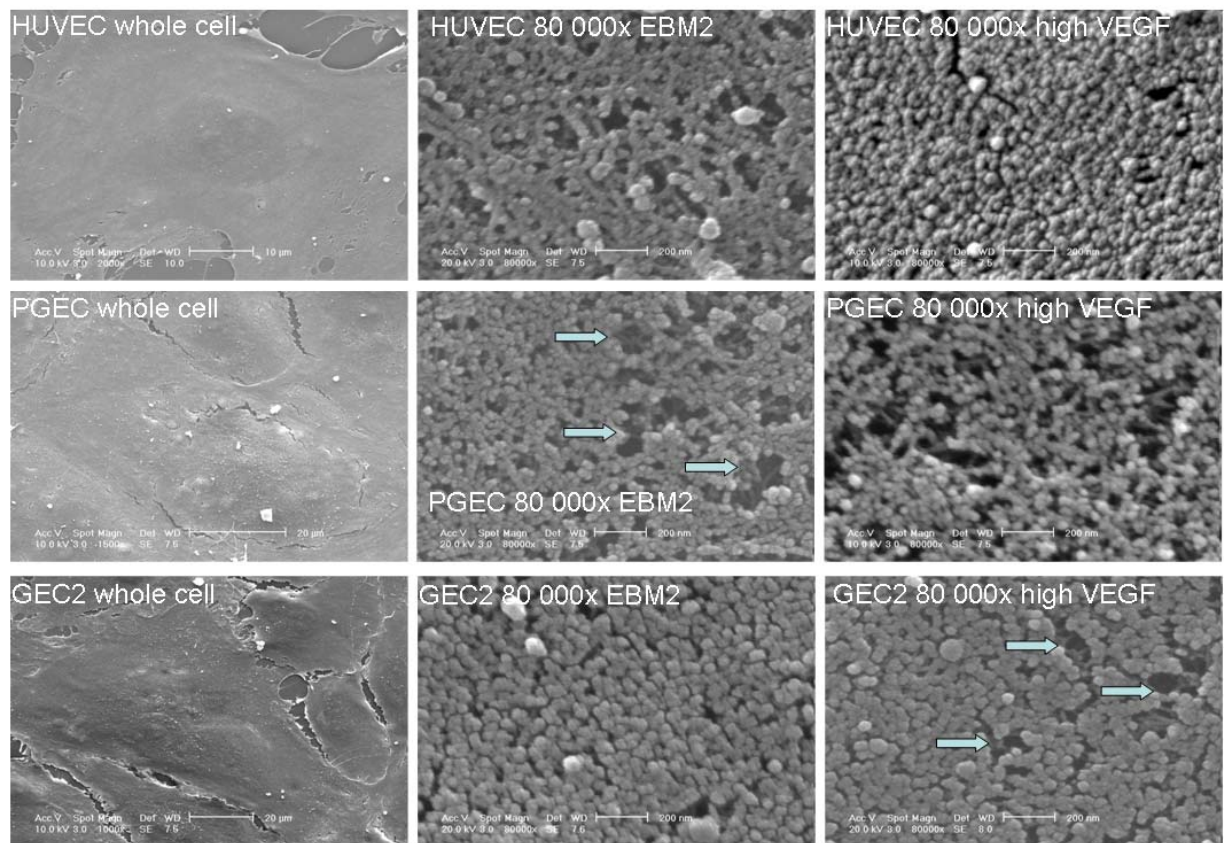
Figure 31: Scanning Electron Microscopy of ECs.

Three ECs (EC) types were grown on glass coverslips either in EBM2 medium or EBM2 with vascular endothelial growth factor (VEGF) at 100x the normal concentration. In the left hand panels whole cells are seen. In the middle panel the surface of the cells at 80 000 times magnification, and in the right hand panels at the same magnification after treatment with high VEGF. The cells have villi on their surface and on PGEC fenestrations are seen (arrowed) which increase after treatment with VEGF. In GEC2 fenestrations are seen only after high VEGF treatment. HUVEC do not demonstrate fenestrations. HUVEC = human umbilical vein EC; PGEC = PGEC; GEC2 = immortalised glomerular EC line.

7.2   Characterisation: presence of CD31 and vWF

Characteristic features of EC include expression of CD31 (important for endothelial-endothelial as well as leukocyte-endothelial interactions) and vWf (that is stored within Weibel Palade bodies and released as multimeric molecules in response to EC

stimulation). The presence of CD31 was confirmed on all three EC types by flow cytometry and immunocytochemistry (

Figure 34). As expected, levels of CD31 were not increased by TNFα stimulation since CD31 is not influenced by TNFα stimulation alone.

In addition to these studies, we checked that the commercial primary GEC were a pure CD31 exressing population by cell sorting on MoFlo (by another laboratory at our request). The population was 93% pure (data not shown).

Flow cytometry plots were gated to exclude dead cells and debris. Examples of forward/side scatter plots with gating are shown (Figure 32).
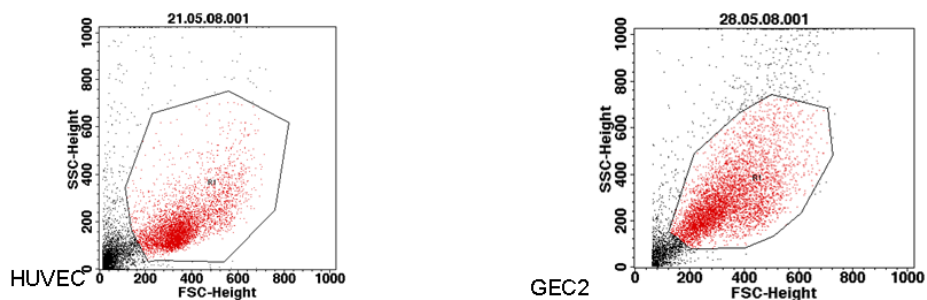
Figure 32: Forward side scatter plots for flow cytometry.

Cell populations were gated to exclude dead cells and cell debris. Examples are shown for HUVEC and GEC2 for unpermeabilised experiments.

Immunocytochemistry was undertaken using IgG1 antibody; isotype controls for experiments with and without TNFα and permeabilised cells are shown below (Figure 33) for comparison to all following immunocytochemistry data.
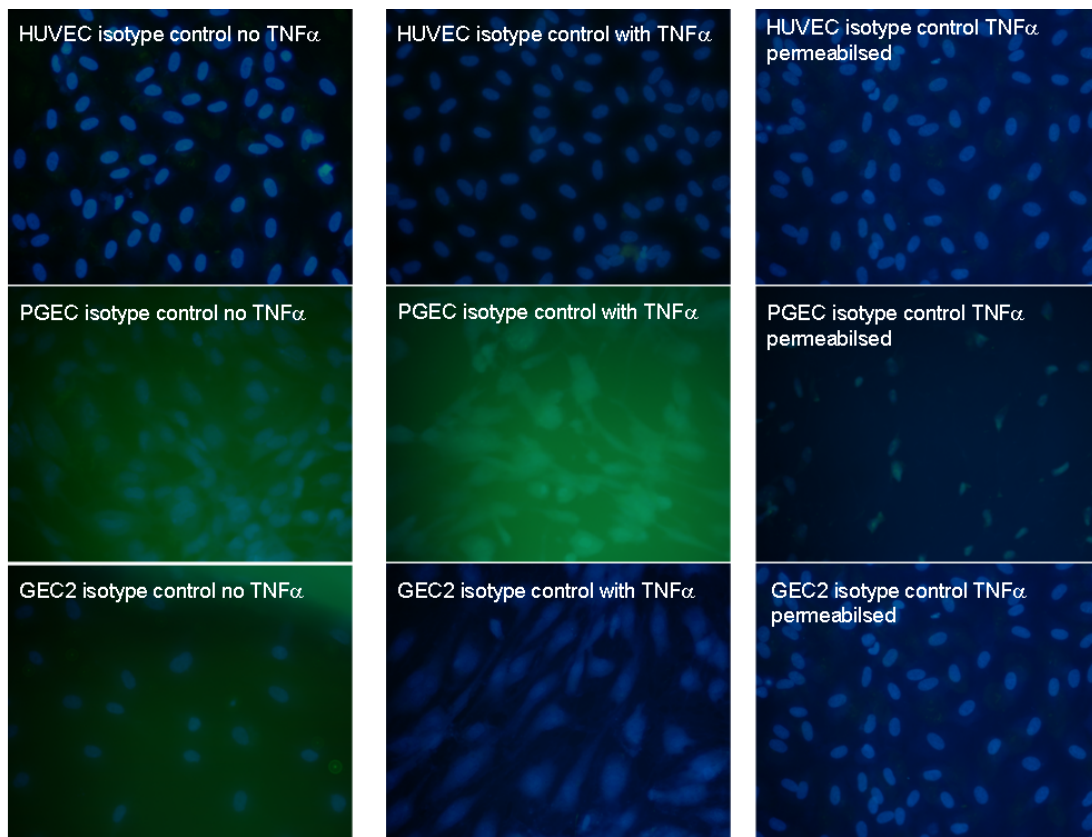
Figure 33: Immunocytochemistry controls.

All experiments for immunocytochemistry were performed with IgG1 antibodies and all control antibodies are for cells before and after treatment with TNFα and after permebilisation.
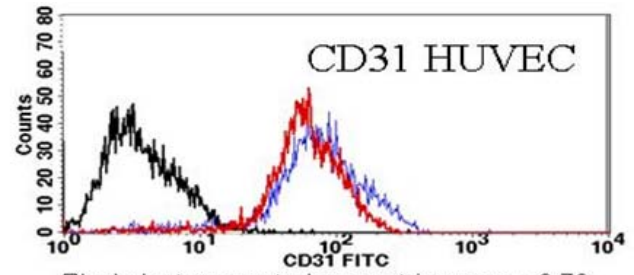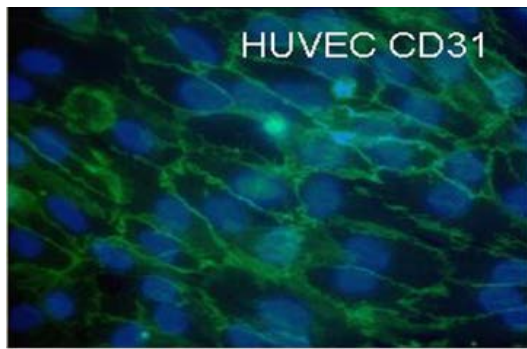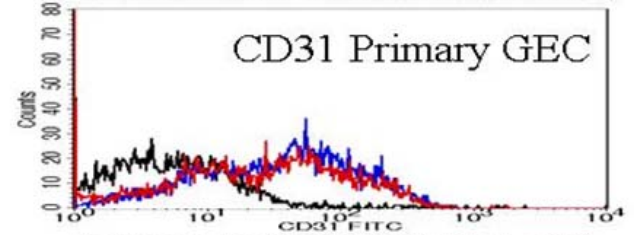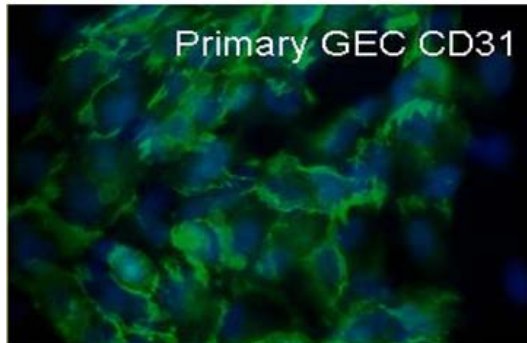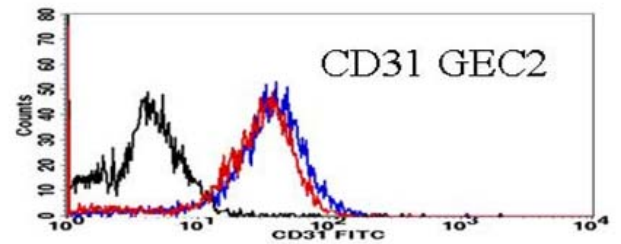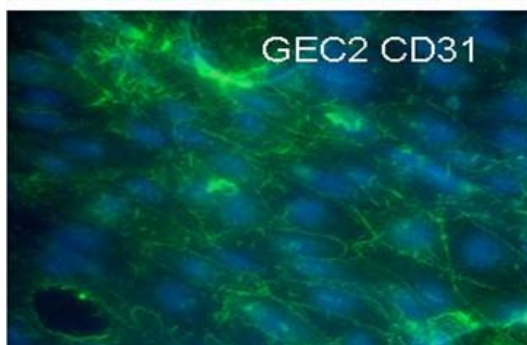
Figure 34: Expression of CD31 in all three cell types confirms their endothelial derivation.

CD31 is expressed in HUVEC, primary GEC and GEC2. It is seen around the edges of the cells by immunohistochemistry staining (primary antibody anti-CD31; secondary rabbit anti-mouse immunoglobulins-FITC conjugated) and is present by flow cytometry (primary antibody, anti-CD31; secondary anti-mouse immunoglobulins-FITC) compared to an isotype control (mouse IgG1κ murine myeloma). Expression of CD31 was unaffected by TNFα.

vWF was also found in all three cell types by flow cytometry and immunocytochemistry. With immunocytochemistry, the vWF appears punctuate

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within the Weibel-Palade bodies that are stained after permeabilising the cells (Figure

35).
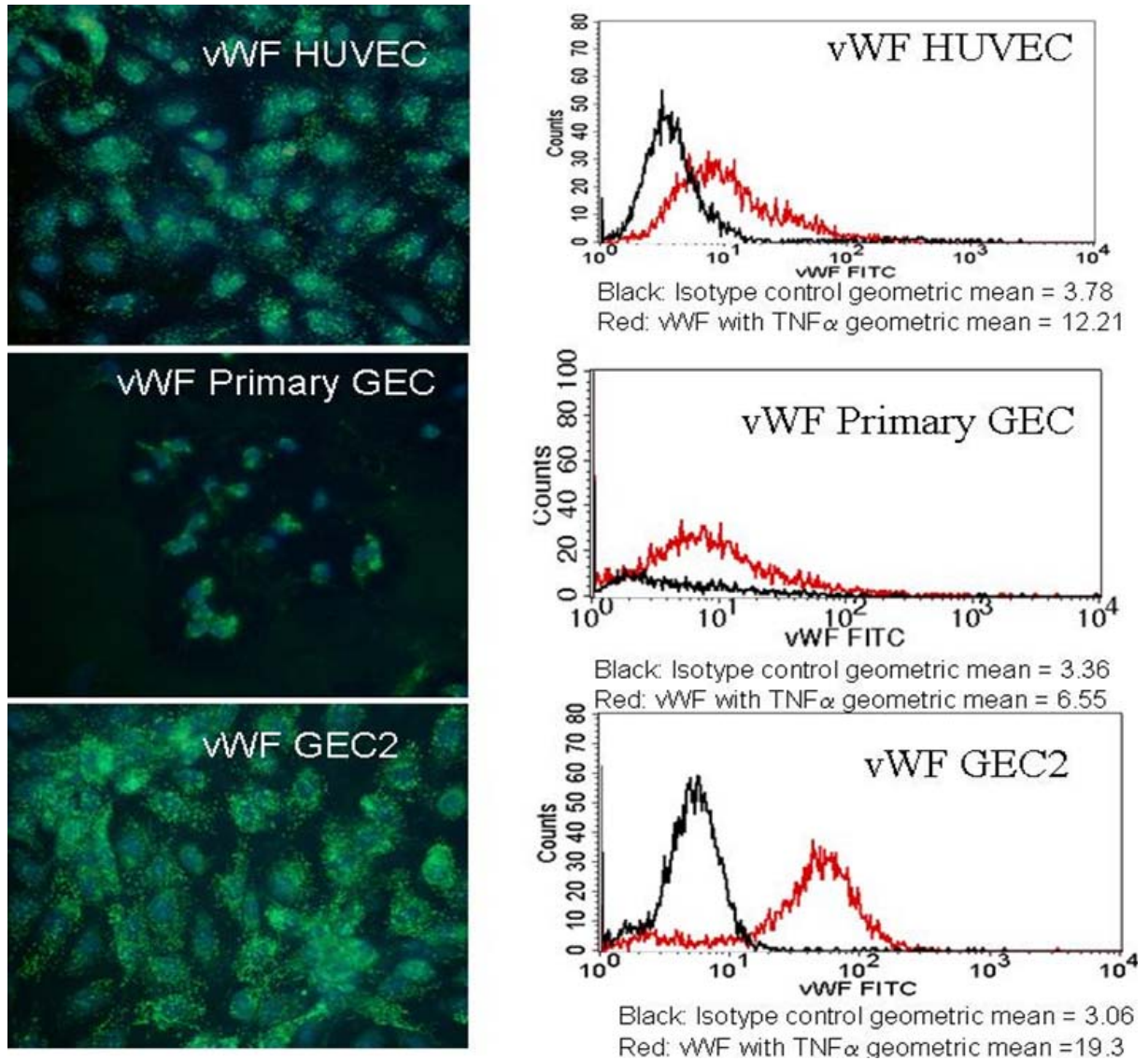
Figure 35: Expression of vWF in all three cell types confirms their endothelial derivation.

vWF is expressed in HUVEC, primary GEC and GEC2. It appears punctuate in the Weibel Pallade bodies by immunohistochemistry staining after cell permeabilisation (primary antibody anti-vWF; secondary rabbit anti-mouse immunoglobulins-FITC conjugated) and is present by flow cytometry (primary antibody, anti-vWF and secondary anti-mouse immunoglobulins-FITC) compared to an isotype control (mouse IgG1κ murine myeloma)

7.3 Expression of adhesion molecules: E-selectin, ICAM and VCAM

7.3.1 <u>E-selectin</u>

E-selectin expression was confirmed on all three cell types by flow cytometry, immunocytochemistry (Figure 36) and ELISA (Figure 37). E-selectin was up-regulated on all three cell types by TNFα and IL-1β.
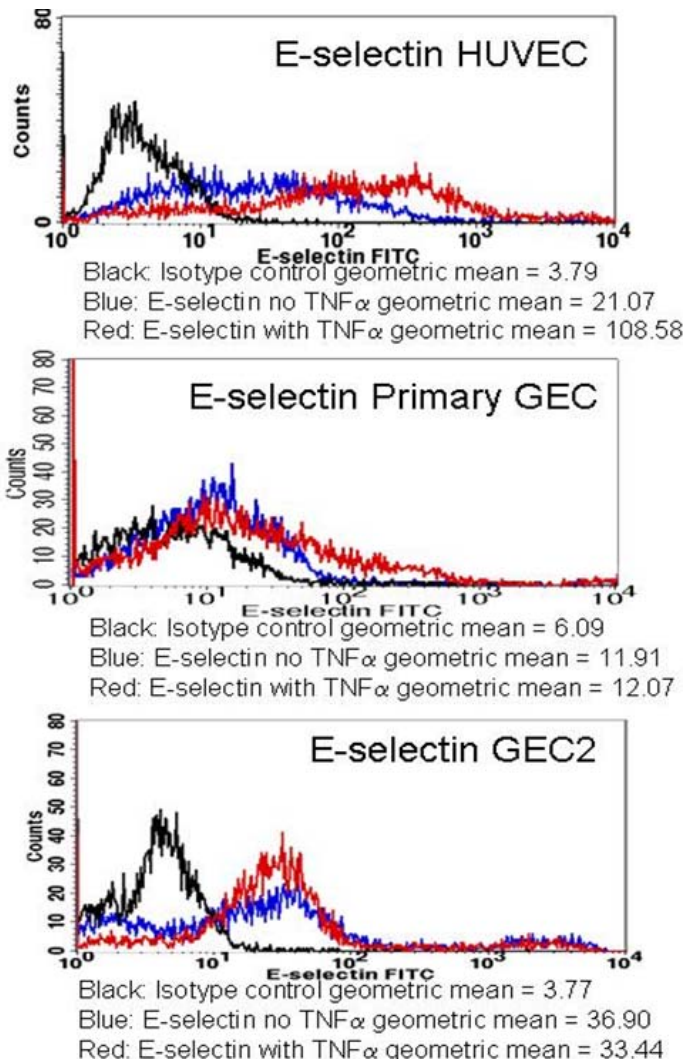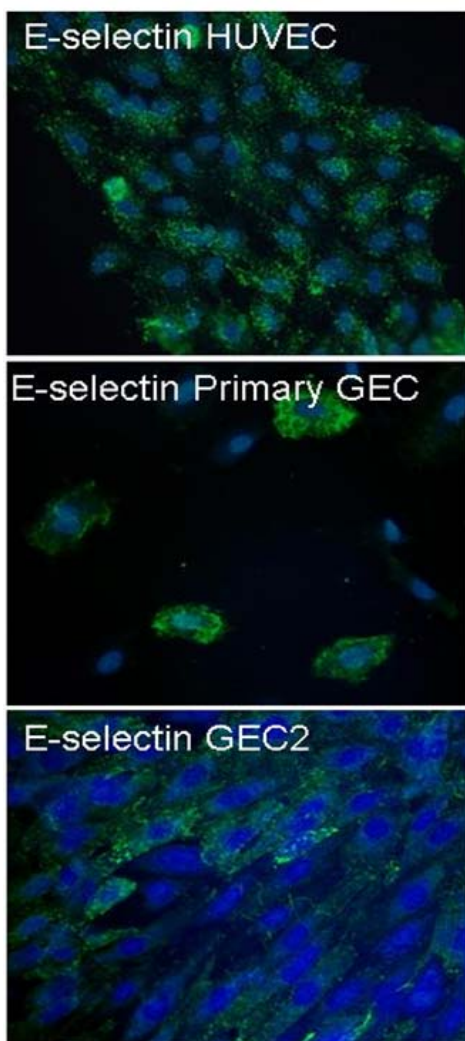
Figure 36: E-selectin expression on HUVEC, primary GEC and GEC2 by immunohistochemistry and flow cytometry.

E-selectin is seen by immunocytochemistry staining (primary antibody anti-E-selectin; secondary rabbit anti-mouse immunoglobulins-FITC conjugated) and is present by flow cytometry (primary antibody, anti-E-selectin and secondary anti-mouse immunoglobulins-FITC) compared to an isotype control (mouse IgG1κ murine myeloma). All cells were treated with TNFα prior to immunocytochemistry staining. By flow cytometry, expression of E-selectin was increased by TNFα in HUVEC andGEC2 but is less convincing on PGEC.

In ELISA, EC were either left unstimulated or stimulated with TNFα at 100u/ml, or IL-1β at 5×10^{-9}g/ml for four hours (Figure 37). Of the two sets of stimulated wells, one set looked for surface expression of E-selectin and the other was permeabilsed to

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look for total cell E-selectin. The basal levels were compared with the stimulated or permeabilsed levels to ascertain if there was significant up-regulation of E-selectin. HUVEC up-regulated E-selectin in response to TNFα, basal expression of E-selectin was significantly different than surface expressed (p=0.005) or total E-selectin (p=0.03). This was also true of primary GEC (up-regulation p=0.0003; permeabilsed p=0.006) and GEC2 (up-regulation p=0.0002; permeabilsed p=0.0003). IL-1β also caused significant up-regulation of E-selectin in all three cell types (HUVEC, surface p=0.003; permeablised 0.011; PGEC surface p=<0.001; permeablised <0.001; GEC2 surface p=0.009; permeablised 0.003). There were equal numbers of cells in each well within each plate when compared with crystal violet.
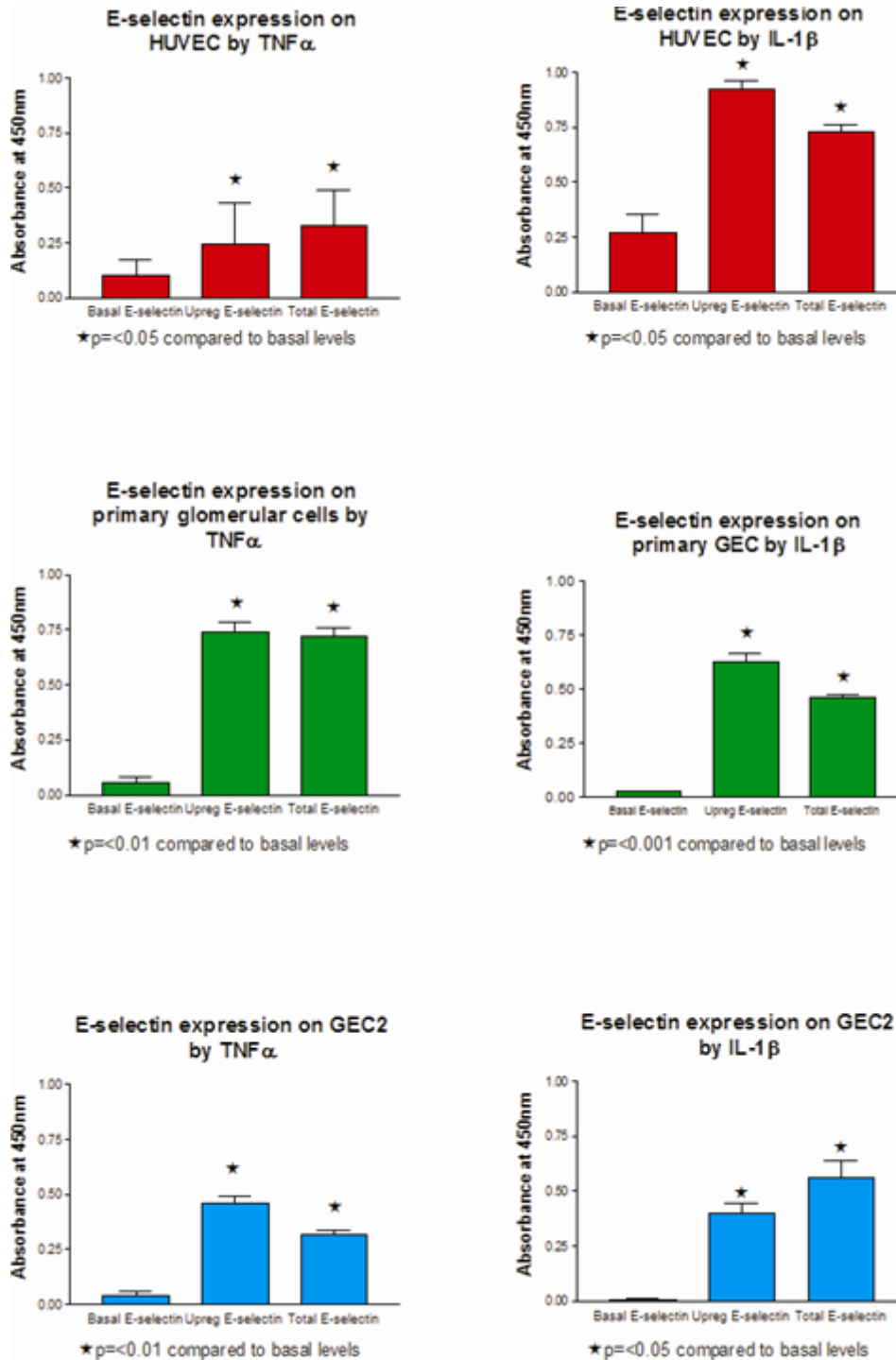
Figure 37: E-selectin is expressed in HUVEC, primary GEC and GEC2 by ELISA.

E-selectin was detected by ELISA (primary antibody, anti-E-selectin; secondary antibody, HRP-conjugated sheep anti-mouse IgG) and was up-regulated by TNFα and IL-1β. Upreg E-selectin = after treatment with cytokines; Total E-selectin = permeabilised cells enabling detection of intracellular E-selectin. (n=3). Comparison with T test.

ELISA was also performed on HUVEC and GEC2 after treating the cells with hydrogen peroxide at 1 millimolar for 1 hour in order to induce selectins (Figure 38). Selectins were induced on GEC2 but not on HUVEC by this method. It is possible that the hydrogen peroxide damaged the HUVEC and this was why selectins were not up-regulated. From other experiments the GEC2 seemed to be more robust.
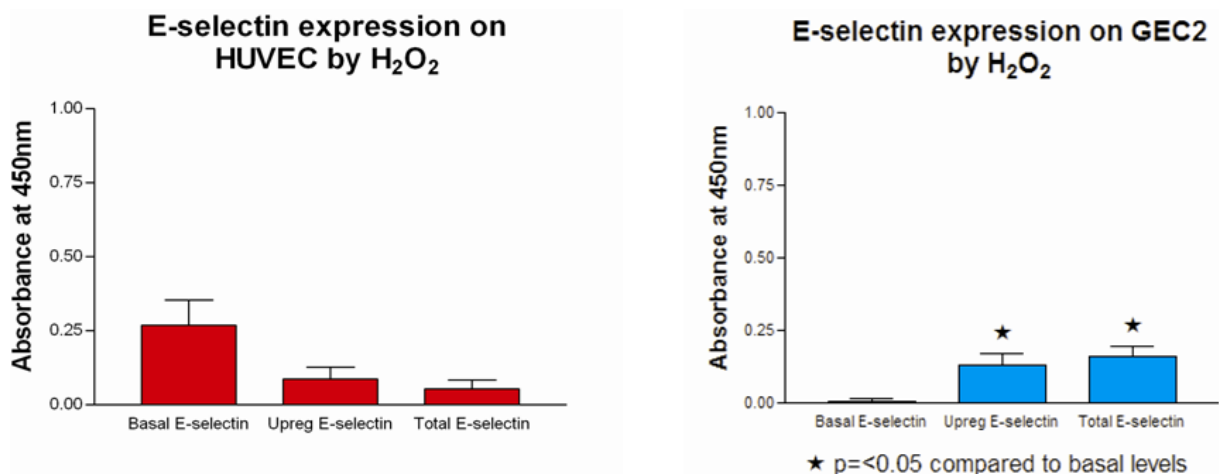
Figure 38: E-selectin is expressed in GEC2 after treatment with hydrogen peroxide.

E-selectin was up-regulated by hydrogen peroxide at 1millimolar on GEC2 but not on HUVEC by ELISA (primary antibody, anti-E-selectin; secondary antibody, HRP-conjugated sheep anti-mouse IgG). Upreg E-selectin = after treatment with cytokines; Total E-selectin = permeabilised cells enabling detection of intracellular E-selectin. n=3. Comparison with T test.

In initial ELISAs GEC1 was also tested by stimulating with TNFα. The GEC1 line did not express E-selectin on unstimulated cells, this could not be up-regulated and permeabilsing the cells did not lead to detection of any E-selectin (Figure 39).
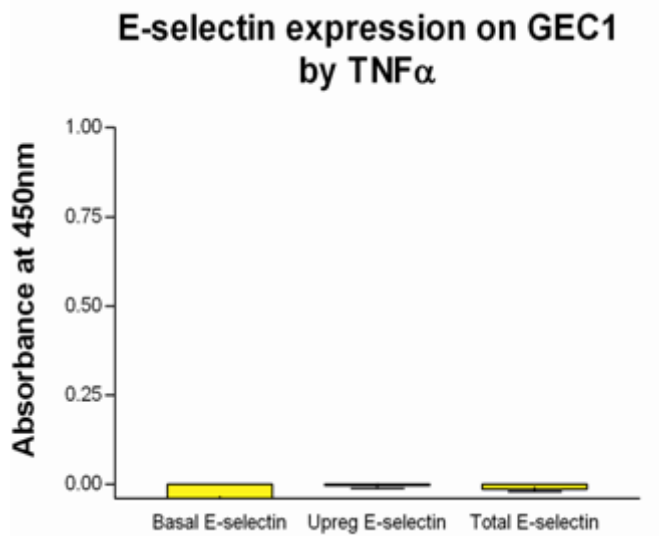
Figure 39: E-selectin is not expressed on GEC1.

E-selectin was not detected by ELISA (primary antibody, anti-E-selectin; secondary antibody, HRP-conjugated sheep anti-mouse IgG). Upreg E-selectin = after treatment with cytokines; Total E-selectin = permeabilised cells enabling detection of intracellular E-selectin. n=3. Comparison with T test.

7.3.2   ICAM-1

ICAM-1 was expressed on all three cell types after 12 hours treatment with TNFα or

IL-1β as demonstrated by flow cytometry (unpermeabilsed cells) (Figure 40), and

ELISA, (Figure 41).
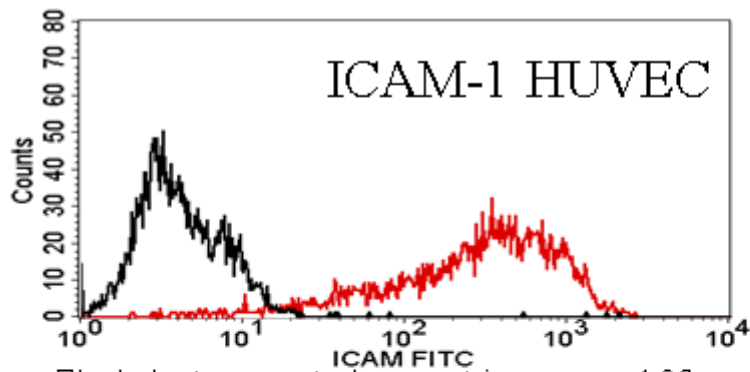
Black: Isotype control geometric mean = 4.32
Red: ICAM-1 after 12 hours TNFα geometric mean = 253.46
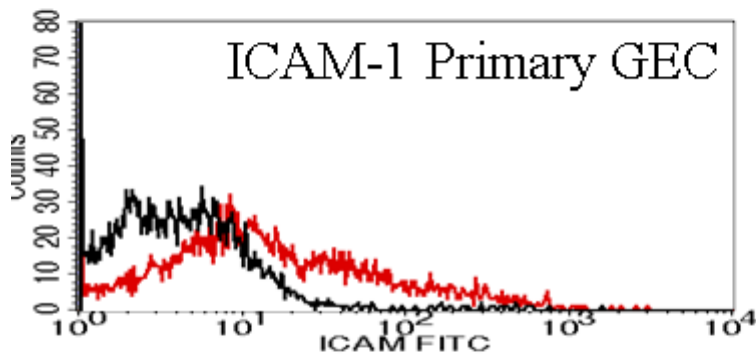
Black: Isotype control geometric mean = 3.88
Red: ICAM-1 after 12 hours TNFα geometric mean = 16.68
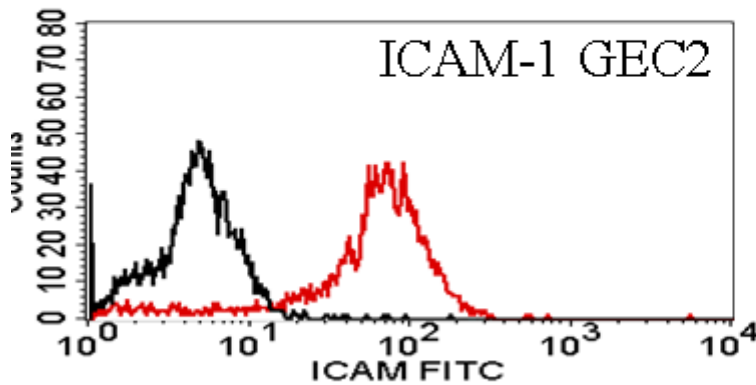
Black: Isotype control geometric mean = 4.41
Red: ICAM-1 after 12 hours TNFα geometric mean = 48.82

Figure 40: ICAM-1 expression on HUVEC, primary GEC and GEC2 by flow cytometry.

ICAM-1 was detected by flow cytometry (primary antibody, anti-ICAM-1 and secondary anti-mouse immunoglobulins-FITC) compared to an isotype control (mouse IgG1κ murine myeloma) on HUVEC and GEC2 but less convincingly on PGEC. Expression of ICAM-1 was increased by TNFα.

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ICAM-1 (Figure 41) was up-regulated on HUVEC by TNFα (surface p=<0.001; permeablised 0.002); on PGEC (surface p=0.002; permeablised p=<0.002); and on GEC2 (surface p=0.002; permeablised <0.001). IL-1β also up-regulated ICAM-1 on all three cell types (HUVEC, surface p=<0.001; permeablised =<0.001; PGEC surface p=0.003; permeablised 0.002; GEC2 surface p=0.002; permeablised p=<0.001)
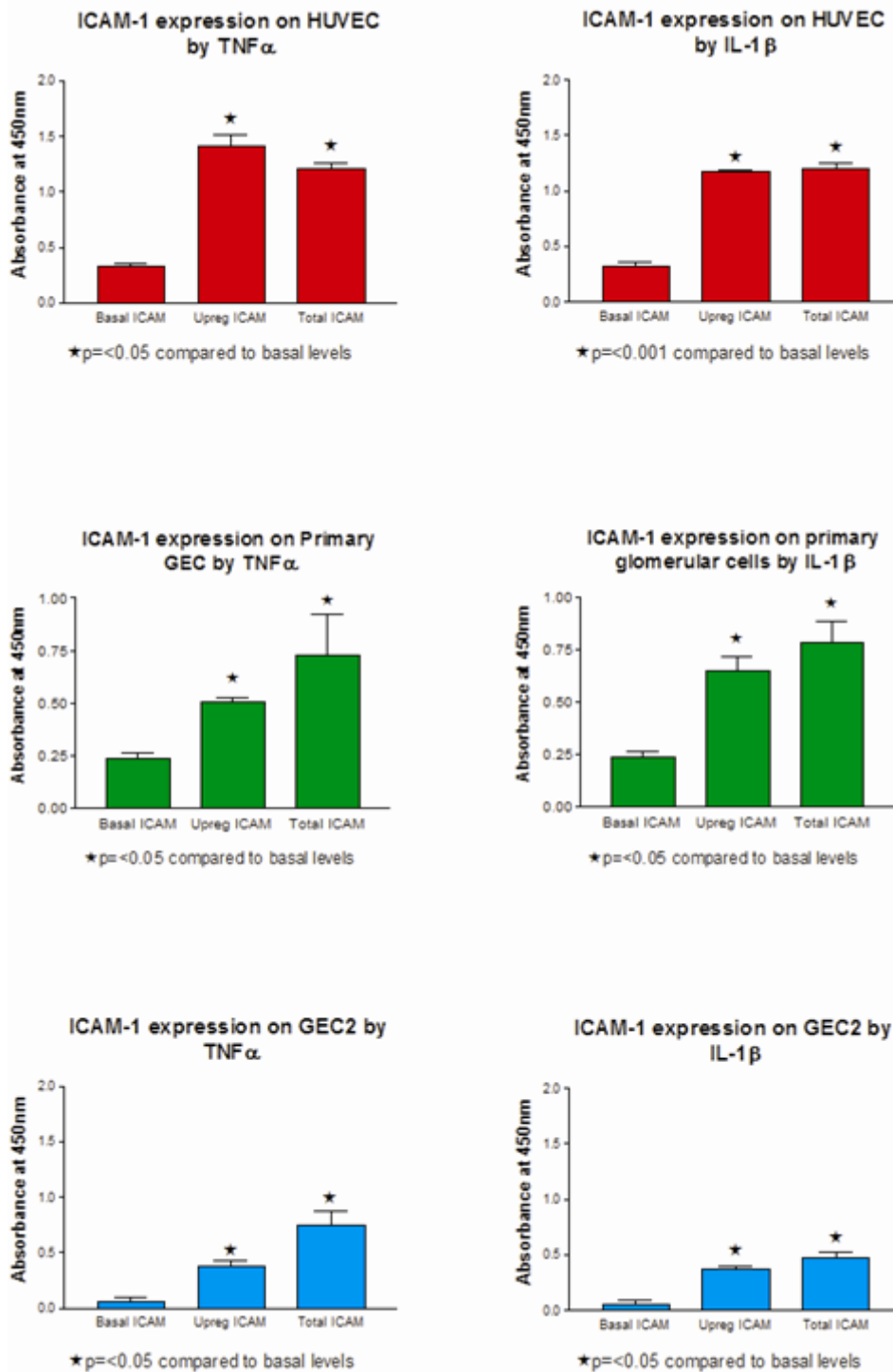
Figure 41: ICAM-1 is expressed on HUVEC, primary GEC and GEC2 by ELISA.

ICAM-1 was detected by ELISA (primary antibody, anti-ICAM-1; secondary antibody, HRP-conjugated sheep anti-mouse IgG) and was up-regulated by TNFα and IL1-β. Upreg ICAM = after treatment with cytokines; Total ICAM = permeabilised cells enabling detection of intracellular ICAM-1 (n=3). Comparison with T test.

VCAM-1 expression was tested by flow cytometry, immunocytochemistry (Figure 42)

and ELISA (Figure 43) after stimulation with TNFα or IL-1β for 24 hours.
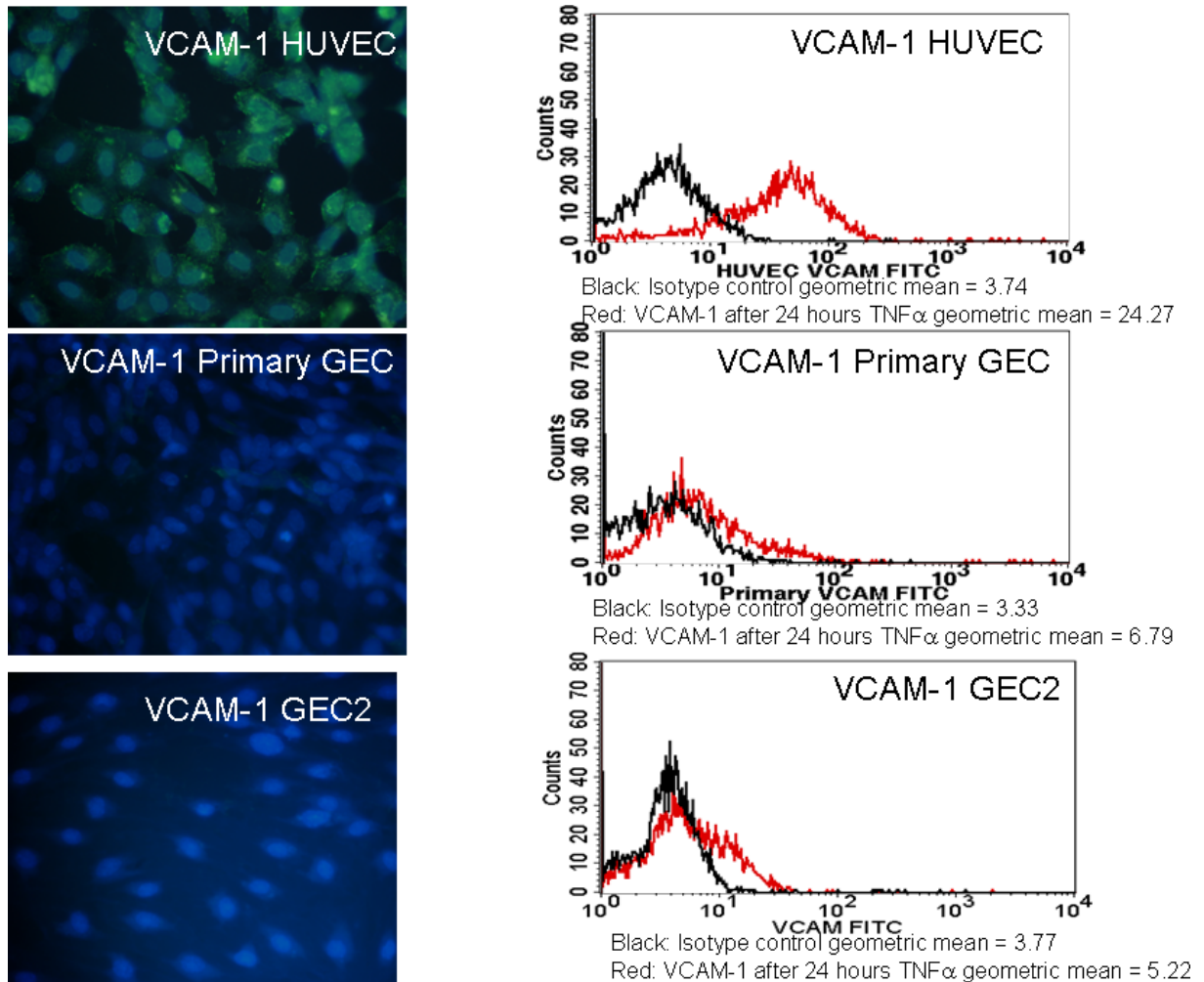
Figure 42: VCAM-1 is expressed on HUVEC but was not detected on primary GEC and GEC2.

All cells were treated with 24 hours of TNFα before immuncytochemistry for VCAM-1. VCAM-1 is seen by immunohistochemistry staining (primary antibody anti-VCAM-1; secondary rabbit anti-mouse immunoglobulins-FITC conjugated) on HUVEC but not on the other EC. VCAM-1 was detected by flow cytometry (primary antibody, anti-VCAM-1 and secondary anti-mouse immunoglobulins-FITC) compared to an isotype control (mouse IgG1κ murine myeloma) on HUVEC but was only detected in very small amounts on primary GEC and GEC2 after stimulation by TNFα.

VCAM-1 was found on HUVEC by immunohistochemistry, flow cytometry and ELISA (Figure 43) when stimulated with TNFα (up-regulated p=0.028; permeabilised cells p=0.046) but was not detected above basal levels with IL-1β in ELISA. VCAM-1 was not detected on either of the glomerular EC by immunohistochemistry and only in very small amounts by flow cytometry. It was not detected in ELISA on TNFα or IL-1β stimulated cells above basal levels (Figure 43). It was also not detected when the cells were permeabilsed.
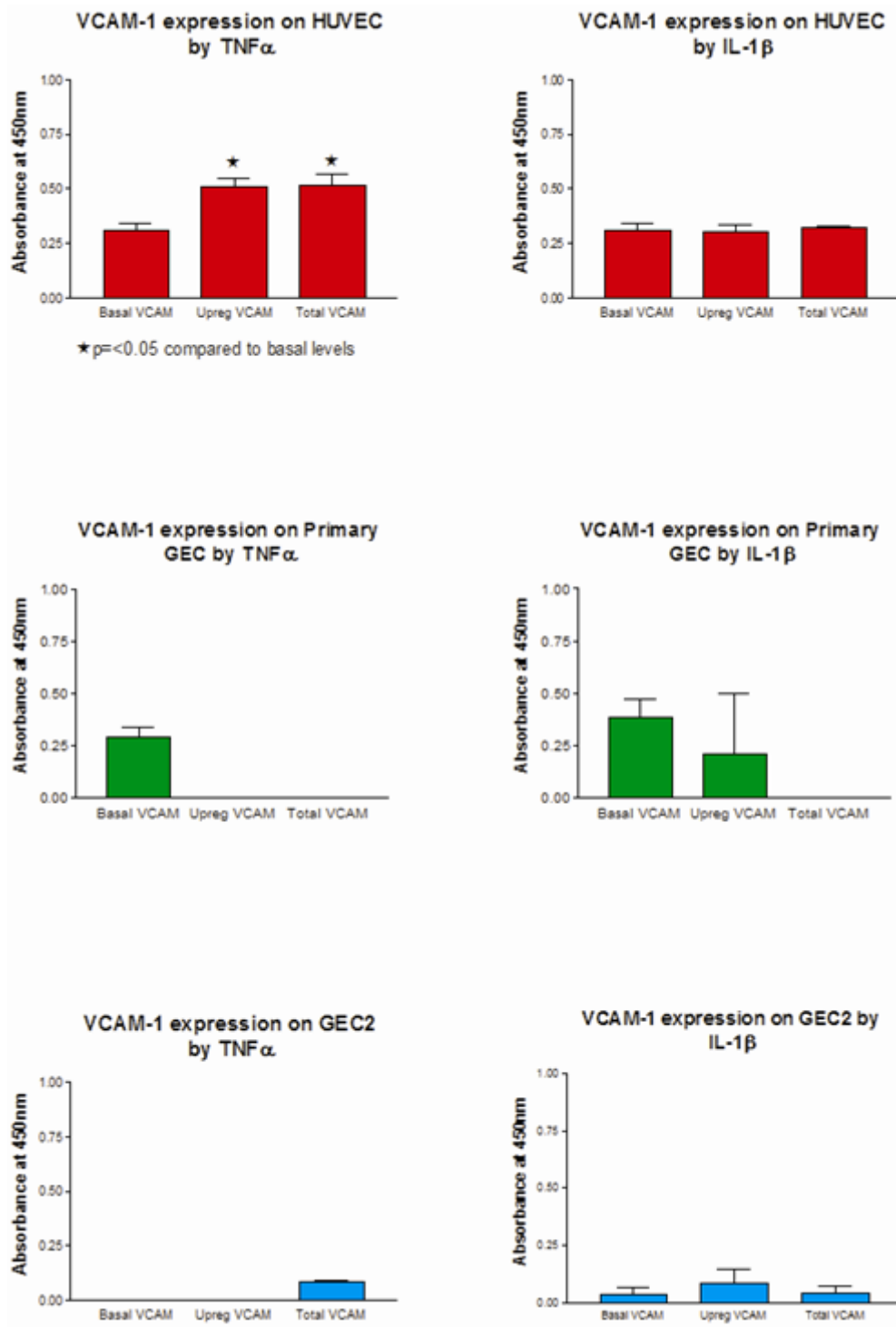
Figure 43: VCAM-1 is detected by ELISA in HUVEC but not glomerular EC.

VCAM-1 was detected by ELISA (primary antibody, anti-VCAM-1; secondary antibody, HRP-conjugated sheep anti-mouse IgG) on HUVEC and was up-regulated by TNFα. Intracellular VCAM-1 was detected after cell permeabilisation. There was no difference from basal levels after

stimulation by IL1-β. On primary GEC and GEC2, VCAM-1 was not reliably detected and was not up-regulated by cytokines (n=3). Comparison with T test. Upreg VCAM-1 = after treatment with cytokines; Total VCAM-1 = permeabilised cells enabling detection of intracellular VCAM-1.

7.3.3.1 Western blotting for VCAM-1

Having found VCAM-1 on the surface of HUVEC but not on glomerular EC we performed Western Blotting on all the cell types to check if the protein was present in the cells. We were able to find bands at the correct molecular weight for VCAM-1 between 90-100kDa in all three cell types (Figure 44). In a non-reduced Western blot on HUVEC, VCAM-1 was demonstrated at a molecular weight of 90kD. In primary GEC and GEC2 the VCAM-1 bands appear at 100kD. This may represent different isoforms of the protein, discussed further below. In a reduced sample all three endothelial types demonstrate bands at 90kD, HUVEC and PGEC demonstrate 2 bands, while GEC2 has only one. We concluded that although the VCAM-1 protein was therefore being synthesised, it was only expressed in low levels on the cell surface of glomerular cells, perhaps because of different isoforms. The observation that banding was similar between primary GEC and GEC2, adds confidence to the interpretation that VCAM isoforms are different in glomerular cells.
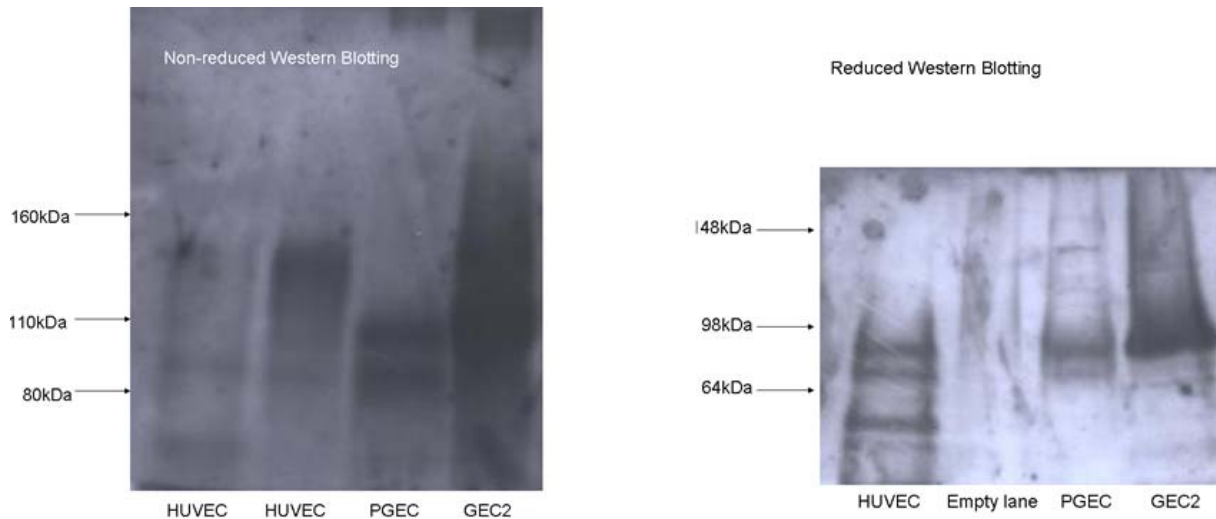
Figure 44: Western Blotting for VCAM-1.

In a non-reduced sample, on HUVEC, VCAM-1 is demonstrated at a molecular weight of 90kD. In primary GEC and GEC2 the VCAM-1 bands appear at 100kD. This may represent different isoforms of the protein. In a reduced sample all three endothelial types demonstrate bands at 90kD, HUVEC and PGEC demonstrate 2 bands, while GEC2 has only one.

7.4   Functional studies in flow

Having established the presence or absence of adhesion molecules on the various EC types, we wished to explore the function of these adhesion molecules in flow.

Selectins and ICAM-1 are needed for capture of neutrophils in the adhesion cascade. Selectins engage fast flowing neutrophils from the circulation and cause them to roll on an EC surface. Firm adhesion is mediated through neutrophil integrins binding to ICAM-1. VCAM-1 is used by PBL for capture onto EC monolayers. VCAM-1 can support capture from flow and rolling adhesion through VLA-4 integrin, but if the integrin is activated the adhesion becomes stable and stationary.

Initial experiments established that GEC1 did not support adhesion of neutrophils (which was expected since this cell line did not express selectins). Further use of this line was therefore abandoned in favour of GEC2. We also checked that the medium the EC were grown in did not affect their function. Finally we compared the EC in supporting neutrophil and subsequently PBL binding.

7.4.1   GEC1 behaviour in flow

The cell line was tested in the flow model to ascertain whether neutrophils could be captured from flow. In the absence of any other stimulus no cells could be captured on GEC1 in a TNFα titration. Levels of TNFα were increased to 1000units/ml on GEC1 but at 15 minutes after inflow no neutrophils remained adhered compared to 700-800 neutrophils adhering to HUVEC at 100units/ml TNFα (data not shown). Experiments were also repeated with a second stimulus of patient ANCA (with normal IgG as a control). Very small numbers of cells could be captured onto GEC1 with very high levels of TNFα and halving the wall shear stress to 0.05Pa. Even under these conditions the number of cells captured was low when compared to HUVEC which had been stimulated with only 2units/ml of TNFα and where the wall shear stress was higher at 0.1Pa (Figure 45). It is interesting that in the presence of ANCA, and even of control IgG some capture could be induced. The behaviour of the captured neutrophils on GEC1 as compared to HUVEC is difficult to comment on due to the very low number of neutrophils captured on GEC1, but in general any captured neutrophils were stationary rather than rolling.

Stimulation with IL-1β and histamine did not induce capture of neutrophils on the GEC1. Histamine was also used to stimulate the cells continuously for 20 minutes by infusion with the neutrophils. Histamine causes P-selectin to be expressed on the cell surface of ECs and thus would be expected to increase neutrophil capture. This affect is seen within 20 minutes, but did not cause any neutrophil capture with this cell line.
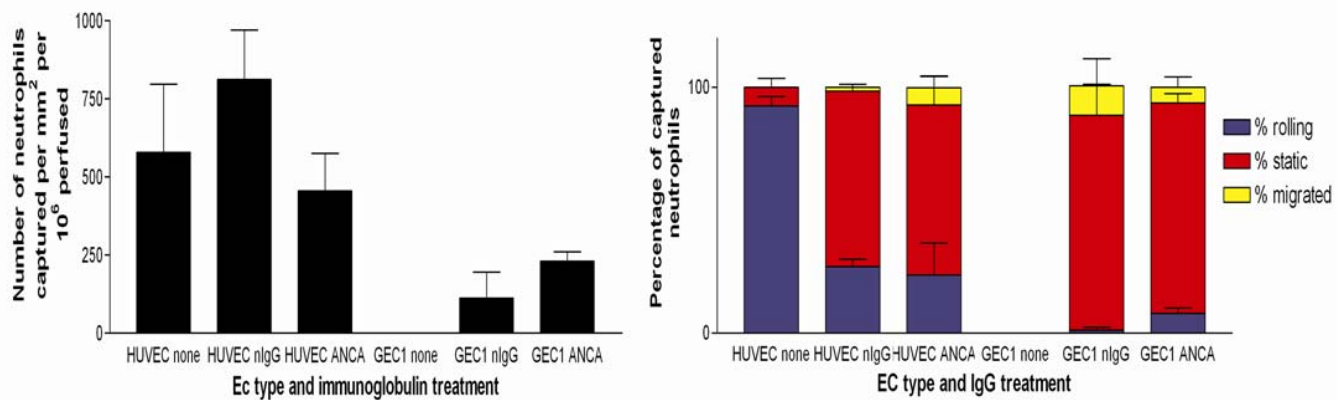
Figure 45: Comparing neutrophil capture and behaviour on GEC1 and HUVEC.

HUVEC captured more neutrophils even when the wall shear stress was halved on GEC1 (0.05Pa) and the TNFα concentration was increased to 1000units/ml (compared to 2units/ml on HUVEC). Data shown at 15 minutes. n=3

7.4.2   Comparison of HUVEC, primary GEC and GEC2 in flow

7.4.2.1 Comparing mediums

In order to compare the three types of EC in flow, we wished to make sure that as much as possible, all factors were equal. Until this point, HUVEC had been grown in medium as described in the methods, which is cheaper than the EBM2 medium used

for the primary GEC and cell lines, which will not grow in the HUVEC medium. An initial set of experiments was therefore carried out in order to check that HUVEC grown in these two media behaved similarly in flow.

HUVEC were stimulated with 0, 2 and 100 units of TNFα. At 11 minutes, total numbers of neutrophils captured were the same in the two media, however, the percentage of cells migrating varied (p=0.032). Due to this difference, all further comparisons between EC types were conducted with HUVEC which had been grown in the same media (i.e.EBM2) to the primary and immortalised cells.
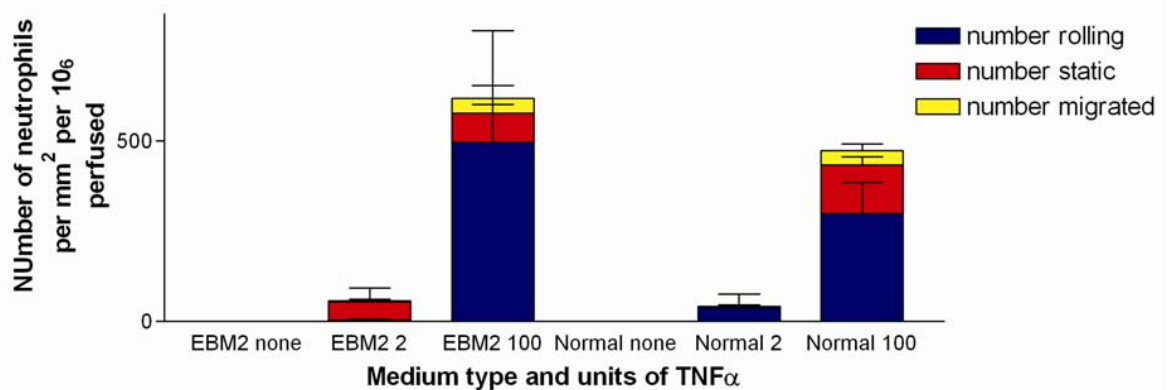
Figure 46: Capture and behaviour of neutrophils on HUVEC grown in EBM2 or normal medium.

In the flow model, HUVEC were stimulated with 0, 2, 100 units of TNFα. Data was collected at 2 minute intervals and is shown at 11 minutes. Total capture is similar between the two media, but migration behaviour is significantly higher on EBM2 (p=0.032). Wall shear stress =0.1Pa, n=5.

7.4.2.2 Comparison of HUVEC, Primary GEC and GEC2: neutrophil capture

The EC cell types were compared for neutrophils captured after being treated with no cytokine, TNFα and IL-1β at various concentrations and hydrogen peroxide. These flow experiments were performed taking multiple data comparisons at 2 minute time intervals and all this data was compared in ANOVA. To present this data in graph form with all time points is very complex, so for simplicity, data are shown at 5 minutes only (although all the data exists for all these comparisons). In some instances although the five minute comparative data doesn't look as if it matches the statistics, the ANOVA is taken from the entire data set.

Comparison after TNFα stimulation

The three cell types were compared to see if unstimulated ECs captured neutrophils and whether the behavior of the neutrophils differed. As with HUVEC experiments previously, very small numbers of neutrophils were captured by any ECs (less than 35 per mm^2 per 10^6 cells perfused) and there was no significant difference in capture of behavior between the EC types (data not shown).

The three endothelial types were then compared after stimulation with 2units/ml of TNFα. When all three cell types were compared together there were significant differences only in the number of neutrophils captured. When this was further dissected, HUVEC was not different from either of the other cell types, but the numbers of neutrophils captured was different when comparing primary and GEC2 (p=0.017) (Figure 47). The significance of these findings are however difficult to

interpret as perhaps with a small increase in TNFα, capture would have been greater on GEC2 particularly since at higher doses of TNFα the three cell types behaved the same.

In a highly stimulated system, when the EC had been stimulated with 100units/ml TNFα, the three EC demonstrated similar numbers of cells captured, and similar behavior, only different in the amount of migration which was increased on GEC2 compared to the two primary cell types (Figure 47).
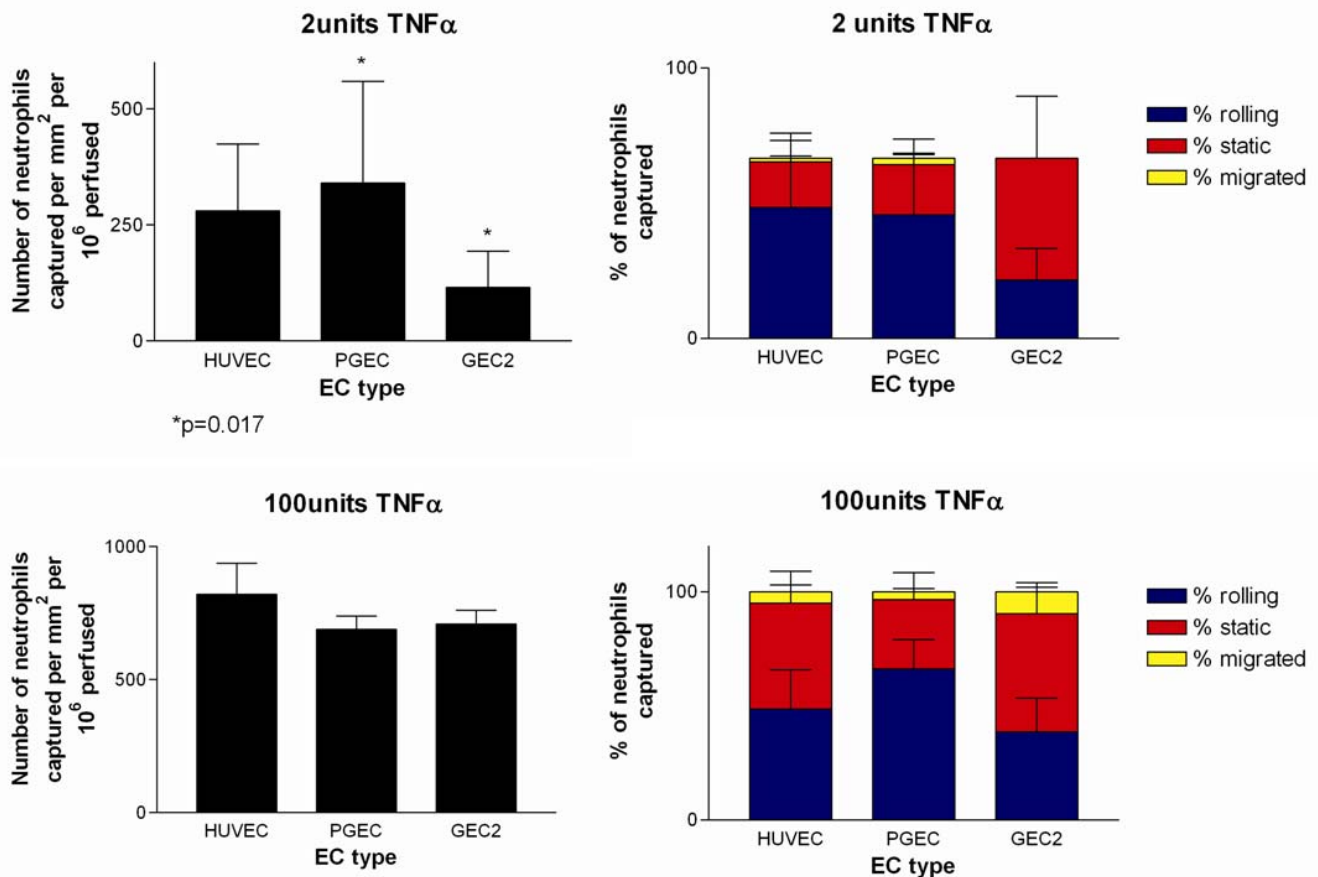
Figure 47: Comparing endothelial types capturing neutrophils in flow after stimulation with TNFα.

HUVEC, PGEC and GEC2 were compared for capture and behaviour of neutrophils in the flow model after stimulation with 2units/ml TNFα or 100units/ml TNFα. Less neutrophils were captured on GEC2 compared to PGEC with 2units/ml TNFα (p=0.017) but behaviour was similar. Capture was the same on all EC with 100units/ml TNFα and behaviour was similar. Wall shear stress =0.1Pa, n=5.

Comparison after IL-1β stimulation and hydrogen peroxide treatment

These experiments were performed comparing HUVEC with the primary glomerular cells only. Under the influence of a low level of IL-1β (5 x 10^{-10}g/ml), there were

significantly less cells captured by PGEC (p=0.001) than HUVEC, and the proportion of static cells on PGEC were also less (p=0.005). Thus the cells captured on PGEC were either rolling or had migrated (Figure 48).

Comparing PGEC with HUVEC under the influence of high levels of IL-1β there was significantly more capture on HUVEC (p=0.015). As previously, there was a significantly greater proportion of neutrophils static on HUVEC (p=<0.001) and more rolling on HUVEC (p=0.006) (Figure 48).

When HUVEC and PGEC were compared under the influence of H_2O_2 (1 millimolar) the resulting neutrophil capture was very different. HUVEC captured substantial numbers of neutrophils whereas PGEC did not (p=<0.001). The neutrophils captured on HUVEC rolled or were static, very few migrated (Figure 48).
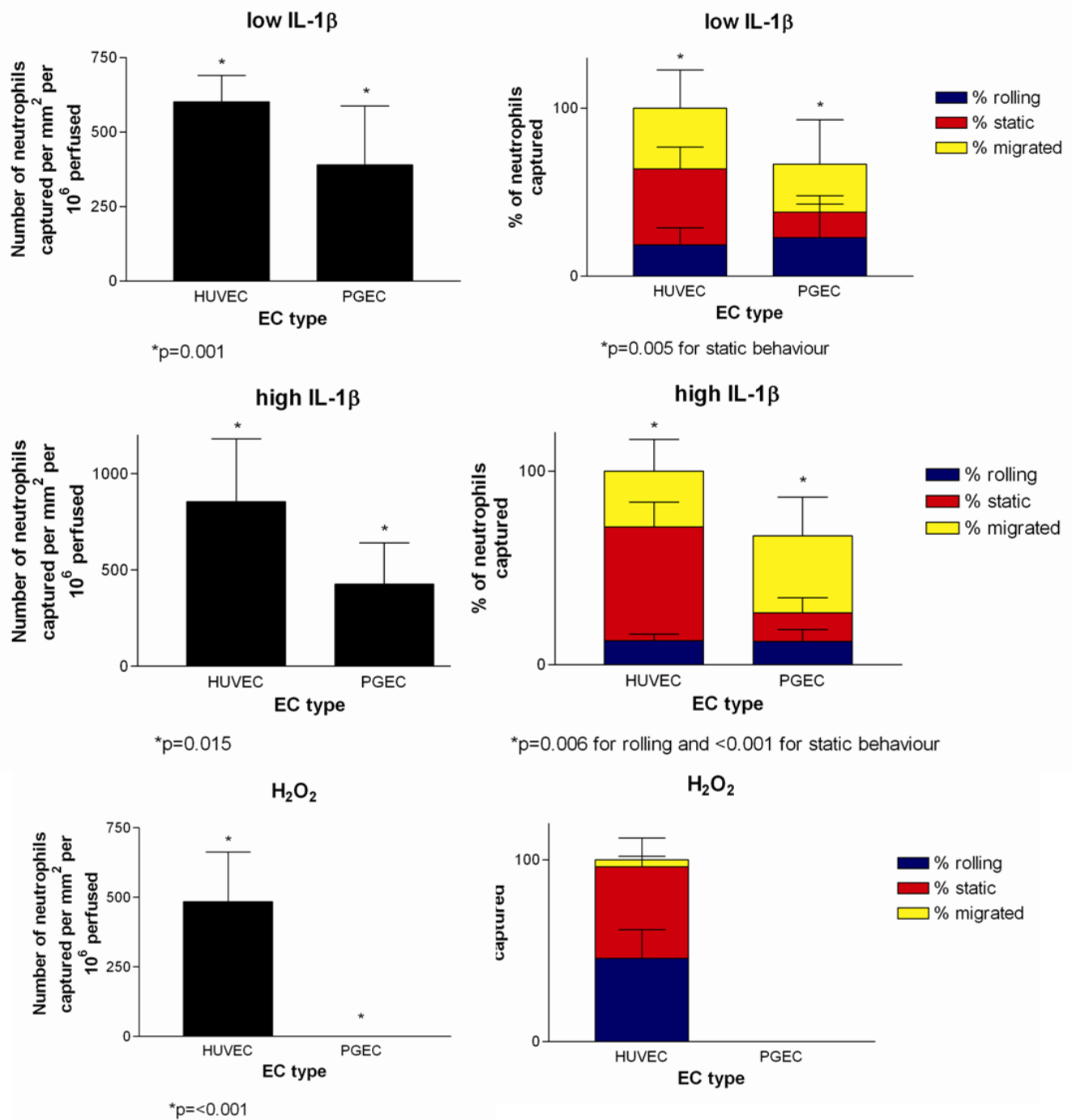
Figure 48: Neutrophils on HUVEC and PGEC after stimulation with IL-1β or H₂O₂.

Using the flow model, neutrophils were captured less onto PGEC than HUVEC when stimulated with high IL-1β (5 x 10⁻⁹g/L) (p=0.001), low IL-1β (5 x 10⁻¹⁰g/L) (p=0.015) or H₂O₂ (p=<0.001) where PGEC did not capture neutrophils. A greater proportion of neutrophils were static on HUVEC compared to PGEC with low (p=0.005) and high (p=<0.001) IL-1β, and rolling was also less on PGEC (p=<0.001). Data shown only at 5 minutes

7.4.2.3 Comparison of HUVEC and Primary GEC: peripheral blood lymphocyte capture

When EC were treated with TNFα there was very little difference in neutrophil capture on primary GEC compared to HUVEC, and this finding was consistent with the previous findings that selectins and ICAM-1 were present on all three cell types. However because of the lack of VCAM-1 on the glomerular cells, we postulated that this would affect lymphocyte capture. We therefore repeated the flow experiments with HUVEC and PGEC and stimulated these with 100units TNFα for 24 hours in order to upregulate VCAM-1. We flowed human peripheral lymphocytes over the monolayers and observed capture and behaviour. PBLs adhered to both HUVEC and PGEC but significantly less to PGEC (p=0.036) in keeping with the reduced /absent surface expression of VCAM-1 found in the initial studies (Figure 49). There was no difference in the proportion of PBL rolling, undergoing static adhesion or migrating on the different EC types once they had been captured.
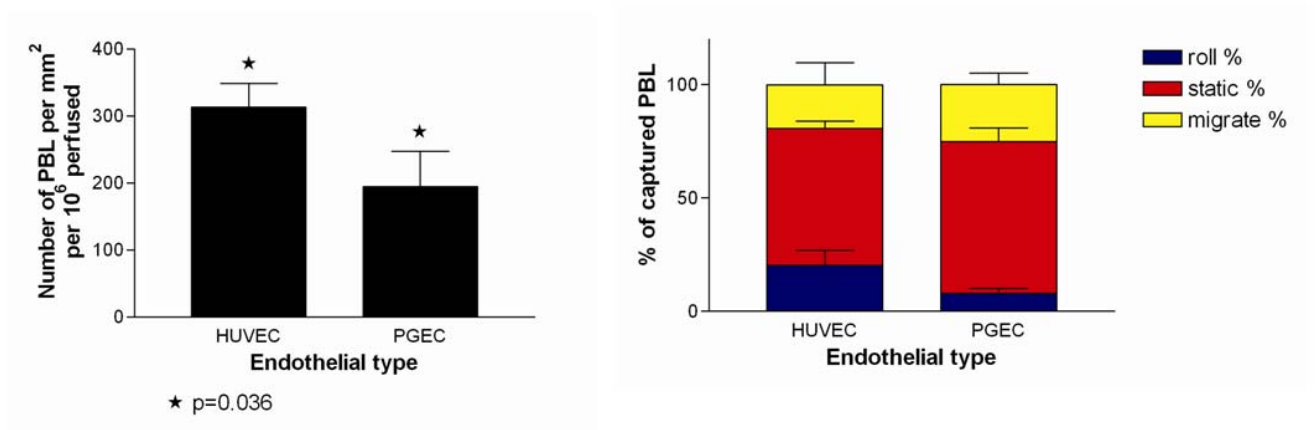
Figure 49: Capture and behaviour of peripheral blood lymphocytes (PBLs) on HUVEC and PGEC.

PBL are captured significantly less onto PGEC than HUVEC (p=0.036) in the flow model. The behaviour of PBL is similar on the two EC types (blue, rolling cells; red, static cells and yellow, migrated cells). Wall shear stress = 0.1Pa, n=3.

Blockade of VCAM-1 and its ligand VLA4

In order to check that the binding we were observing was VCAM-1 dependent we initially blocked VCAM-1 on the surface of the EC, and subsequently blocked its ligand VLA4 on the PBL. Blocking VCAM-1 on the endothelial surface (Figure 50) reduced binding on both HUVEC and PGEC (HUVEC p=0.007; PGEC p=0.037).

Blockade of the VCAM-1 ligand VLA-4 on the PBL reduced binding but this did not reach significance (Figure 50). There was no difference between control treated and untreated cells in either experiment. Thus the PGEC, although expressing reduced amounts of VCAM-1, must be expressing enough to support minimal adhesion of PBL.
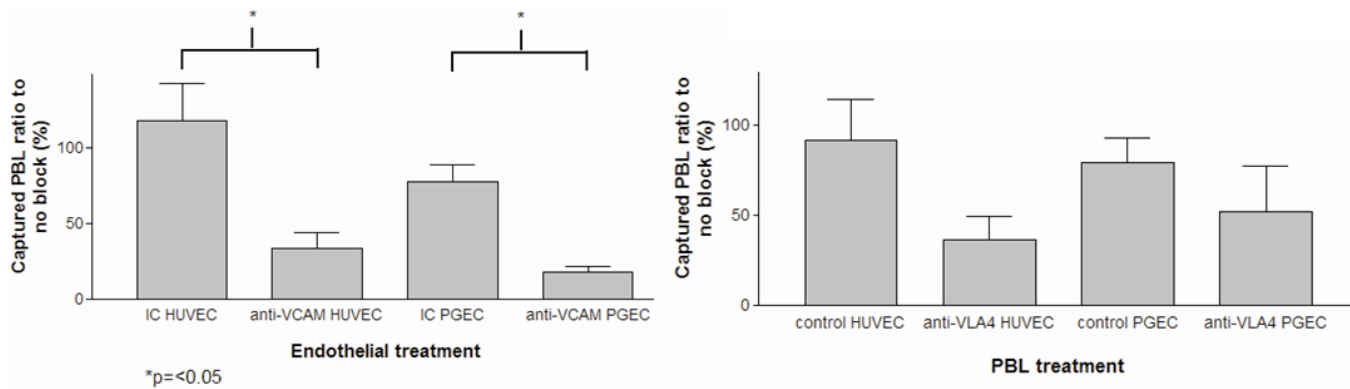
Figure 50: Peripheral blood lymphocyte capture after blockade of VCAM-1 and VLA-4.

Using the flow model, peripheral blood lymphocytes (PBLs) capture onto human umbilical vein ECs (HUVEC) and PGECs (PGEC) were compared without treatment of the EC, to EC treatment with CD18 (control) or anti-VCAM-1 antibodies. Blockade of VCAM-1 results in a significant reduction in adhesion on both cell types compared to control (HUVEC p=0.007; PGEC p=0.037). n=3; wall shear stress = 0.1Pa. Subsequently PBLs were untreated or treated with control (anti CD18) or anti-VLA-4 antibodies. Blockade of VLA-4 results in a non-significant reduction in adhesion on both cell types compared to control. All the data has been compared to untreated cells and expressed as a ratio. IC = isotype control.

7.5   Immunohistochemistry of whole kidney for VCAM-1

From the above studies we had established that VCAM-1 appeared to have reduced expression on glomerular endothelium and this had functional consequences in terms of PBL capture. We postulated that this may be a protective mechanism in the kidney to prevent cell capture and inflammation. We wished to explore VCAM-1 expression in vasculitis, to establish if the expression of VCAM-1 is up-regulated here. Using immunohistochemistry we stained kidney sections of a patient with vasculitis and looked at glomeruli which were histologically relatively unaffected by the disease, those that were actively affected and those that had been inflamed and were sclerosed, or scarred, and no longer functional. We observed that the VCAM-1

expression was minimal in glomeruli unaffected by vasculitis, was up-regulated by the process of inflammation and was almost absent again after scarring and shrinkage of the glomerulus, where there was little if any functional endothelium present to express VCAM-1 (Figure 51).
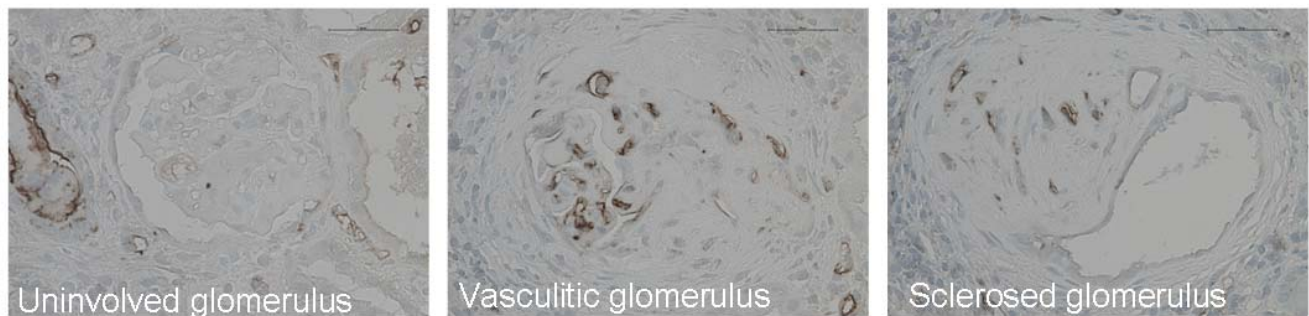
Figure 51: VCAM-1 staining on human kidney.

In a patient with Wegener's Granulomatosis VCAM-1 is not expressed on the endothelium of an unaffected glomerulus. In the same kidney section glomeruli with vasculitic lesions have up-regulated expression of VCAM-1. Once the glomeruli are scarred and sclerotic the expression of VCAM-1 is reduced as functional endothelium dies.

7.6   RNA arrays

After comparison of the three endothelial types as described and functional studies, we wished to explore gene expression. The three cell types were compared by RNA array analysis using the whole human genome. Firstly by predictive analysis microarrays (PAM) the cells grouped according to their derivation as three distinct groups, PGEC, HUVEC and GEC2 (Figure 52). There were six samples in each group. The PGEC and the GEC2 grouped together while the HUVEC divided into two groups within the population (Figure 52). The groups were compared to each other for the number of genes that were significantly different from each other, PGEC compared to HUVEC had 3% of different genes; GEC2 compared to HUVEC 3%,

and PGEC compared to GEC2 11%. The immortalised and primary GEC were more different from each other by these analyses than to HUVEC.

Using significance analysis microarrays (SAM) set to a 0% false positive discovery rate, comparing all three populations together, 322 genes were different. For PGEC versus HUVEC 22 genes were different; for PGEC versus GEC2 793 genes were different and for GEC2 versus HUVEC 39 genes differed. The reason for the difference in these numbers is the variability seen within the HUVEC genes, thus genes that might well be significant have to be discounted due to too high a variability within the HUVEC population. The GEC populations are more consistent internally, and hence significant differences are more easily discerned when these are compared.

We next looked for specific gene expression. vWF has 2 genes and in comparing the two primary cells, vWF was expressed four times more in HUVEC than PGEC. Expression in PGEC compared to GEC2 was similar, that is, there was less than a 2 fold difference in expression of these genes. Specifically looking at adhesion molecule gene expression we found that E-selectin was similarly expressed in HUVEC and PGEC but ICAM-1 was expressed less (0.23) in PGEC than HUVEC. This may be part of the explanation as to why in some of our assays neutrophil capture was reduced on glomerular cells.

VCAM-1, which has 2 genes, was expressed similarly in HUVEC and PGEC, but more highly in PGEC compared to GEC2 (5.7 and 2 times for each gene).
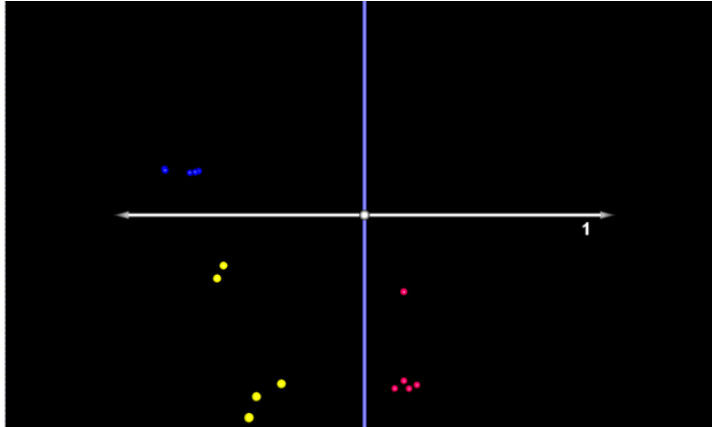
Figure 52: RNA arrays, predictive analysis microarrays (PAM).

The ECs tested fall into three distinct populations (PGECs, blue; human umbilical vein ECs (HUVEC), yellow; immortalised glomerular ECs (GEC2), red). The HUVEC divide into two populations. 5 samples in each group.

We were particularly interested in the difference in adhesion molecule surface expression and particularly the pathways governing expression of VCAM-1 on the cell surface. Post-translational modification of VCAM-1 in the endoplasmic reticulum and Golgi adds an $\alpha2$ 6-linked sialic acid, which may affect adhesion (Abe, Smith et al. 1999). We therefore looked to see if the gene encoding the enzyme Galß1-4GlcNAcα2,6-sialyltransferase (ST6N) responsible for the addition of this sialic acid was different between HUVEC and GEC.

We found that the gene was similarly expressed in PGEC and GEC2 in the 6 samples tested, but was more highly expressed in HUVEC (comparing PGEC to HUVEC the gene was expressed four times more in HUVEC when the samples were averaged), and importantly this difference was consistent within the sample groups (Figure 53).
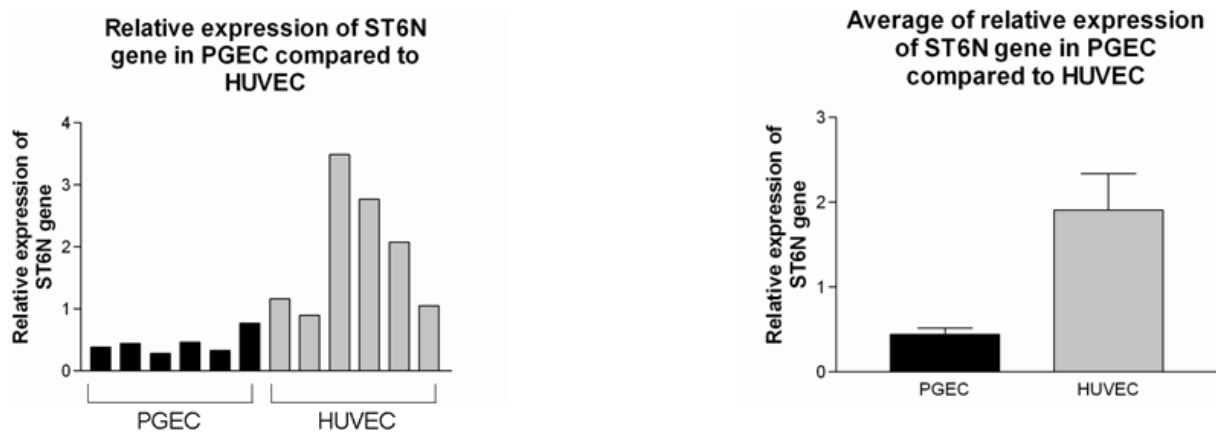
Figure 53: Expression of ST6N gene in PGEC compared to HUVEC.

Using RNA array analysis, the ST6N gene is expressed 4x more highly in HUVEC compared to PGEC and this is relatively consistent between the 6 samples in each group.

7.7   Discussion

ECs have different functions depending on their location. Macrovascular endothelia such as HUVEC are functionally required to carry blood from the mother to the foetus and are not required to undertake the complex filtering function of glomerular microvascular endothelium. Glomerular endothelium is distinct in its structure and function.

7.7.1   Appearance of EC under phase contrast and electron microscopy

The EC grew in monolayers and appeared similar to each other. There was no bunching or overlap of cells and they retained a characteristic 'cobblestone' appearance under phase contrast microscopy. Under electron microscopy the primary glomerular cells demonstrated fenestrations which were increased in the presence of high concentrations of VEGF, fenestrations were also demonstrated in

GEC2 under conditions of high VEGF, but were not induced in HUVEC. This is in agreement with physiological appearances of the cells and confirms their EC derivation. It is interesting that HUVEC could not be induced to fenestrate in these studies and demonstrates a further important difference between these and microvascular cells.

7.7.2   <u>GEC1</u>

The GEC cell line 1 did not efficiently capture neutrophils from flow, and there was very little capture even when treated with high concentrations of TNFα. These cells could be induced to capture more neutrophils if the neutrophils had been exposed to ANCA, but this capture remained inefficient when compared to HUVEC. ILβ-1 and histamine did not induce neutrophil capture on this cell line.

It is likely that during passage this cell line has been corrupted. From the ELISA data it did not express E-selectin and therefore cannot capture cells in flow. This problem has been encountered before in previous experiments in our laboratory on another immortalised dermal cell line (unpublished data) and may reflect the complex cardohydrate-based post-translational modifications that have to occur for functional selectin molecules to be synthesised. vWF was also very minimal on immunohistochemistry and since vWF exists with P-selectin in Weibel-Palade bodies, we postulate that the mechanism transporting these molecules to the cell surface may have become disrupted in these cells, which would add another reason for poor expression of selectins overall. The appearance of the cells under phase contrast

microscopy and the presence of CD31 on immunohistochemistry makes it likely that these cells are endothelial, and that there had not been an inadvertent selection for another cell type (such as fibroblasts) during passage.

Other workers have reported such problems with EC lines. In one study four different immortalised endothelial lines were used to study interactions with lymphocytes, and compared to HUVEC or human dermal microvascular ECs (HDMEC). When stimulated with TNFα, the HUVEC and HDMEC supported rolling and adhesion of lymphocytes, but none of the immortalised lines did. Cell surface expression of E-selectin was only seen on HUVEC and HDMEC. Immunohistochemistry showed some of the cell lines to express E-selectin intracellularly, suggesting impaired post translational processing or transport, and some did not even express E-selectin mRNA, suggesting impaired transcription (Oostingh, Schlickum et al. 2007).

In light of these experiments, further work with the cell line GEC1 was abandoned, and the full reasons for these findings were not further explored.

7.7.3 CD31 and vWF

Initial studies verified the phenotype of PGEC, GEC2 and HUVEC as endothelial using CD31 and vWF.

7.7.3.1 Lymphatic cell contamination

There is no specific marker for endothelial cells, and it should be noted that lymphatic endothelial cells also express CD31 (but not vWF) and have previously been derived from vascular EC *in vitro* (Groger, Loewe et al. 2004) raising the possibility of transformation of vascular EC to lymphatic EC in our experiments. However since our cells also stained for vWF we are confident this was not the case.

7.7.4 <u>E-selectin, ICAM-1 and VCAM-1</u>

To explore the potential functionality of glomerular endothelium in immune and inflammatory responses, the expression of E-selectin, ICAM-1 and VCAM-1 were assessed on resting and cytokine-activated, as well as on permeabilised cells. Although the results of the ELISA confirm that all three adhesion molecules could be detected above basal levels after permeabilisation, the levels were not consistently higher than levels after cytokine stimulation. This could indicate that all the protein is expressed on the cell surface, with none within the cytoplasm, which seems unlikely, or that the permeablisation was not very effective. It is known that cells can close holes made in their surface, and it is possible that because detergent was not used with application of the secondary antibody, complete permeabilsation was not achieved.

Our data demonstrated that E-selectin was present and surface expression was up-regulated by TNFα and IL-1β on all three cell types by ELISA. Flow cytometry on HUVEC and GEC2, and immunohistochemistry supported these findings. Flow

cytometry on PGEC was less convincing. This may be due to problems with the fragility of the PGEC cells as discussed elsewhere, the low numbers of cells used or due to technical problems with the methodology.

Factors governing paucity of glomerular endothelial expression of E-selectin *in vivo* are clearly complex given that these cells have the potential to express this molecule, as evidenced by both this and previous studies where E-selectin expression was induced in cultured glomeruli by exposure to exogenous IL-1β, TNFα and Interferon-γ (Savage, Brooks et al. 1997). E-selectin was induced on cultured human GEC and HUVEC by TNFα (Murakami, Morioka et al. 2001).

H_2O_2 was used to upregulate selectins on the EC surface, so it is surprising that this could not be demonstrated in these studies with HUVEC. This substance is however known to be toxic to cells and it may be that in these concentrations, in static conditions, EC are being damaged.

ICAM-1 was similarly expressed and up-regulated on all EC tested although as with the E-selectin the flow cytometry data for PGEC was of limited value for similar reasons as discussed for E-selectin expression. Unfortunately the antibodies available did not allow for immunohistochemistry so this data is not available for ICAM-1. There is a disparity between the expression of ICAM-1 demonstrated by ELISA and that by flow cytometry, likely to be due to limited numbers of poor quality cells used in the flow cytometry experiments. However, although VCAM-1 was clearly detected on resting HUVEC and expression was up-regulated by TNFα, it was

detected in very low amounts on glomerular EC using flow cytometry and not detected using immunocytochemistry. Thus, although VCAM-1 was detected on resting PGEC by ELISA, there was little expression on cytokine-stimulated PGEC and on resting or cytokine-stimulated GEC2. This contrasts with previous reports suggesting that VCAM-1 was induced on cultured human GEC after 6 hours exposure to IL1β or TNFα (Park, Chang et al. 1998; Murakami, Morioka et al. 2001). The present studies did show that VCAM-1 was detected by Western blotting on all three EC types, although there was evidence of possible variability of isoform expression between HUVEC and glomerular cells from the molecular weigh profiles. HUVEC and primary GEC demonstrated 2 bands between 90-100kD (Woodside, Kram et al. 2006) under reducing conditions. In HUVEC the bands were equally dark, but on primary GEC the higher molecular weight band was heavier. On GEC2 there appeared to be only one band at the higher molecular weight. VCAM-1 is known to exist in three alternative spliced forms on ECs, the more frequent form has 7 domains and the other widely recognized form has 6 domains, both have been found in HUVEC (Cybulsky, Fries et al. 1991; Hession, Tizard et al. 1991; Osborn, Vassallo et al. 1994). A further form with 8 domains has also been described (Kilger, Needham et al. 1995). All these isoforms are said to support PBL binding. VLA-4 binds to domains 1 and 4. In a study of VCAM-1 binding ability, the 7 domain isoform was found to bind with higher affinity and was a more efficient agonist of VLA-4 function than the 6 domain form (Woodside, Kram et al. 2006). If the different isoforms have differing abilities to be transported to the cell surface, this could explain the variation in VCAM-1 expression between the EC types. The domain 6 isoform of VCAM-1 lacks domain 4, but binding of PBL to VCAM-1 has been reported to be dependent

on domain 1 (Abe, Ballantyne et al. 1996; Konstantopoulos, Kukreti et al. 1997). Domain 4 has been suggested to be important for strengthening adhesion (Needham, Van Dijk et al. 1994).

The low levels of VCAM-1 expression on normal renal endothelium in this study may be relevant to disease processes. It is possible that under normal circumstances the kidney is protected from leukocyte interaction by expressing low levels of VCAM-1 and when this becomes disrupted, inflammation ensues. For example, in AAV, neutrophil-endothelial interaction increased by the presence of ANCA may in part be driven by aberrant VCAM-1/ neutrophil interaction (Reinhardt, Elliott et al. 1997). In mice, neutrophils appear in some situations, to bind to MPO via the ligand VLA-4 which is also the ligand binding VCAM-1 (Kuligowski, Kwan et al. 2009). Additionally, in renal biopsies from patients with vasculitis, VCAM-1 was found in glomerular endocapillary cells and was associated with more severe lesions (Pall, Howie et al. 1996). In a subsequent study VCAM-1 was again found in vasculitis in human renal tissue (Arrizabalaga, Sole et al. 2008) where abnormal VCAM-1 and ICAM-1 were seen in the tubules of more than 80% of patient samples and in the glomerular tuft in more than 60%. VCAM-1 was associated with leukocyte infiltration, suggesting that VLA-4 bearing leukocytes are contributing to glomerular injury. This association was also found where VCAM-1 was expressed in tubular and vascular areas in biopsy specimens of patients with vasculitis. VCAM-1 was a good marker of necrotising extra-capillary damage and was thought to be crucial for macrophage recruitment to these areas (Rastaldi, Ferrario et al. 1996).

7.7.5   <u>Functional comparison of HUVEC, PGEC and GEC2</u>

The choice of media is important as the environment in which the cells are grown will change their behaviour. Not only does this have implications for experiments where comparisons are made in the behaviour of neutrophils on the EC surface, but also has implications about how closely these cells are reflecting the behaviour of their counterparts *in vivo*. Clearly, if the environment in which the cells develop has a huge impact on their ability to capture cells, it may be rather futile to look at these cells when they have been grown in standard *in vitro* conditions. In other words, the glomerulus may indeed demonstrate very different properties in cell capture to the umbilical cord, but this may be influenced by the environment and particular cytokines found there, rather than the type of EC *per se*.

When unstimulated, the three EC types were comparable in that they did not capture neutrophils. This is an important finding because if neutrophils were captured on unstimulated glomerular ECs this would have implications for disease processes within the glomerulus.

The only difference between the three cell types when stimulated with small amounts of TNFα was the difference between the numbers of cells captured on primary GEC and GEC2. The behaviour of neutrophils on the three cell types was similar once the cells were captured, and there were no significant differences between HUVEC and the glomerular cells.

Unlike in the system where only a low level of TNFα was used, the highly stimulated system did not demonstrate differences in capture or rolling, but only in migration. Under the influence of TNFα neutrophils undergo more migration on any EC but this was demonstrated to be increased on GEC2 more than HUVEC or primary cells. A more detailed discussion of cell lines and the possible problems involved with them is undertaken later in this thesis, and because of this complicating factor, further studies were concentrated on the comparison of the two primary cells, HUVEC and PGEC.

In these experiments with TNFα on PGEC compared to HUVEC we therefore concluded that neutrophil capture and behaviour was similar between the cell types. These results were expected in light of the findings that E-selectin and ICAM-1 was similarly expressed on both cell types.

We subsequently explored this theory in more detail by looking at the EC under the influence of IL-1β and H_2O_2. Under the influence of IL-1β at two concentrations, a cytokine elevated in AAV, fewer cells were captured on the glomerular cells and within the population of captured neutrophils, there was proportionally more rolling or migrating behavior. Cells in the glomerulus influenced by IL-1β therefore seem to either roll or migrate suggesting that they can pass through the EC layer more easily than on HUVEC. H_2O_2 induces selectins on the surface of EC but is also toxic to the cells. The differences seen in cell capture between HUVEC and PGEC could therefore be explained either by the fact that HUVEC is less sensitive to toxicity or that HUVEC more readily up-regulates selectins. If the later is the case, this may explain the increased rolling and static behaviour seen in the previous experiments,

and the ability of HUVEC to capture more cells than glomerular EC. If there were actually less expressed selectin in the glomerulus this could be a protective mechanism for the kidney, ensuring that neutrophils are only captured to migrate if the conditions are highly inflamed. The neutrophils captured however migrated more across the EC which may also be an advantage, as neutrophils remaining in the circulation would block small capillaries. If they are moved effectively through the EC layer to the tissues where they are needed for an effector response, any problems deriving from infective organisms would be cleared quickly. Such a theory would not be applicable to the umbilical cord which is a conduit vessel and would not need neutrophils to move across it's endothelium for a tissue response. In conclusion, although initial experiments suggested that glomerular EC and HUVEC were similar in supporting neutrophil adhesion and behaviour, subsequent work suggests that it may not be reliable to use HUVEC to extrapolate about the behavior of other types of EC, which despite being grown in *in vitro* environments, nevertheless seem to retain distinct properties of their own.

7.7.6   Functional aspects of PBL

We examined the functionality of glomerular endothelial VCAM-1 expression using PBLs, since previous studies have shown that VCAM-1 and E-selectin, but not ICAM-1, are capable of supporting the adhesion of T-cells to endothelium under flow conditions (Konstantopoulos, Kukreti et al. 1997). Peripheral blood lymphocytes utilise VLA-4 to bind to endothelial VCAM-1 (Carlos, Schwartz et al. 1990; Carlos, Kovach et al. 1991). Interestingly we found that recruitment of PBLs to glomerular

EC was reduced compared to HUVEC, although the cells that were recruited behaved similarly on each EC tested. This recruitment was blocked on all types of EC by blocking VCAM-1 or its ligand VLA-4, indicating that although there is reduced recruitment on glomerular EC, it remains VCAM-1 dependent. This is strong evidence that, despite low levels of VCAM-1 expression on glomerular cells, its presence is of functional relevance, in accord with up-regulation of VCAM-1 within diseased glomeruli.

7.7.7 RNA arrays

RNA arrays demonstrated importantly that the cells were grouped into their three distinct groups and were different from each other. However, PGEC and GEC2 were more different from each other than from HUVEC in some of these analyses, and this influenced further studies where the comparison was focused on the two primary cell lines.

In comparing specific genes we looked at gene expression for adhesion molecules and found that E-selectin and ICAM-1 were similarly expressed in the two primary cell lines. Interestingly ICAM-1 expression was reduced in PGEC and although this did not seem to affect neutrophil adhesion at high levels of TNFα, this may be part of the explanation as to why there were some differences with reduction of capture onto PGEC with IL-1β and low levels of TNFα. We did not detect differing levels of ICAM-1 on the surface by flow cytometry or ELISA, but these methods may not be sensitive enough to detect subtle differences.

We attempted to look for reasons why VCAM-1 was not as highly expressed on the cell surface of GEC, and looked at the gene responsible for the enzyme Galß1-4GlcNAcα2,6-sialyltransferase (ST6N) responsible for the addition of sialic acid to the protein. This is a post-translational modification which may affect adhesion (Abe, Smith et al. 1999). The gene was expressed less in PGEC compared to HUVEC. We postulate that this may contribute to the reduction in VCAM-1 expression on the GEC surface.

Given more time the microarray data would ideally be validated by quantitative polymerase chain reaction.

In summary we identify significant differences between GEC and HUVEC and suggest that it is not always acceptable to extrapolate from HUVEC to microvascular EC. In particular we found that VCAM-1 surface expression is reduced on glomerular cells and this resulted in reduced capture of PBLs onto this EC. Given that the kidney handles a large volume of blood it is important that it is protected from unnecessary leukocyte recruitment, down-regulation of adhesion molecules on the endothelial surface may be crucial in this role. Altered expression in disease states may contribute to glomerular damage during vasculitis and other inflammatory glomerular diseases.

8. DISCUSSION

8.1 Early experiments and optimisation of models

8.1.1 The flow model: applicability and reproducibility

The flow model was first set up in 1993 and has been used to study a wide variety of interactions between flowing cells on various surfaces including selectins and ECs (Buttrum, Hatton et al. 1993).

8.1.1.1 How good is it as a model?

The flow model enables interactions of neutrophils, ECs and ANCA to be studied in real time and for interactions to be directly observed. It also enables interactions to be dissected, so that only interactions between the neutrophil and selectins can be studied by coating the slides with P-selectin rather than an EC monolayer. Different EC types may also be studied in this model.

The model is limited as the ECs have not been grown under normal environmental conditions. They are not subject to the same chemokine/cytokine influences as occur in their native cellular beds, nor usually, to the influence of specific fluid shear. They are also detached from the cells making up the vessel walls such as smooth muscle cells and perictyes. This is likely to alter the adhesion cell profile of the cells *in vitro*.

Results from the model are reproducible providing there are no changes in the reagents used. Different batches of P-selectin may bind differently to the surface of

glass microslides and different batches of cytokines have varying potencies. There is also variation among neutrophils, ECs and antibodies used in the experiments, so some variation between different experiments is likely.

The flow model is a relatively difficult technique to master and is restricted in the number of treatments that can be used in any one experiment. Slides cannot be left out of medium for long, and preparations of neutrophils especially have to be used within a short time frame before apoptosis takes place. In addition, each slide must be grown in specialised plates, usually with 3 or 6 slides at a time. This limits the size of experiments usually to six slides per experiment.

8.1.1.2 Capture of neutrophils from flow

Neutrophils are captured from flow in humans by selectin molecules expressed on the surface of ECs which bind to their ligands on neutrophils. Selectins are expressed on HUVEC and primary GEC, and up-regulated by cytokines such as TNFα as demonstrated, and if they are not present, as on the GEC cell line 1, neutrophils are not efficiently captured.

The captured neutrophils roll on activated endothelium and in the presence of a secondary stimulus convert to stationary adhesion due to engagement of integrin receptors on the neutrophil surface, which bind ligands such as ICAM-1.

This secondary stimulus can be provided by bacterial agents such as fMLP, and also by patient ANCA, although not by all patient ANCA as demonstrated. Chimeric anti-

PR3, of a single subclass and directed against one epitope can also provide this stimulus.

8.1.1.3 Receptor expression on HUVEC compared to the P-selectin model

In order to be able to study interactions of ECs and neutrophils, a simplified model was used where P-selectin was coated onto the microslides. In this model, neutrophils could not transmigrate as there was no EC layer, and any interactions between the neutrophils and the selectin surface were due to the interaction of neutrophils with selectins. Because the model is simple, variation is reduced.

8.1.1.4 Previous work with ANCA in the flow model

There have been three previous papers examining the influence of ANCA on neutrophils in flow. In the first paper microslides were covered with a layer of activated platelets, and neutrophils which were treated with patient ANCA or normal IgG immediately prior to infusion, were perfused over the platelets. ANCA, but not normal IgG stopped the neutrophils rolling and this effect was transient, returning to baseline at 15 minutes. Antibodies blocking the FcγRIIa and the integrin CD11b/CD18 stopped this effect (Radford, Savage et al. 2000).

In the second paper, neutrophils were treated with ANCA or normal IgG and perfused over ECs activated with 100units/ml (highly activated) or 2units/ml (minimally activated) of TNFα. On highly activated endothelium, most of the neutrophils transmigrated and this remained unchanged with untreated neutrophils, those treated

with normal IgG and those treated with patient ANCA. On minimally activated EC, untreated neutrophils were compared to neutrophils treated with ANCA. There was a significant increase in adhesion and transmigration when ANCA was used. There was no difference between neutrophils treated with ANCA immediately before infusion, 5 minutes or 15 minutes before infusion. Antibodies were used to block CD18, which disrupted adhesion and CD11a, which did not. Treatment of EC with ANCA alone, with subsequent perfusion of neutrophils did not cause neutrophil adherence (Radford, Luu et al. 2001).

In the third paper, antibodies were used to block integrins and CXC receptors. Neutrophils exposed to ANCA were studied on highly and minimally activated endothelium. CD11a/CD18, CD11b/CD18 and CXCR2 were found to be important in adhesion and transmigration in both models, but CD11b/CD18 was more important in the minimally activated model (Calderwood, Williams et al. 2005).

8.1.2 <u>Patient ANCA: variability of pathogenicity</u>

8.1.2.1 Patient ANCA activates neutrophils and varies in pathogenicity

In recent experiments reported above, we confirmed that neutrophils treated with ANCA were arrested on a P-selectin surface to a greater extent than neutrophils treated with normal IgG. In contrast to the experiments reported by Radford et al, pure P-selectin and not layered platelets were used. There was variability between different patient ANCA.

The finding that ANCA promotes adhesion and migration of neutrophils on HUVEC is also supported by recent experiments above. In addition to Radford et al, it was found that there were significant differences between neutrophils treated with normal IgG and those treated with ANCA on highly activated endothelium. This may be due to variation in the TNFα batch used, which allows differences to be seen in the recent experiments which were not obvious in the experiments reported in the Radford et al paper. On minimally activated endothelium, we confirmed that that there is a significant difference between untreated neutrophils and those treated with ANCA. In addition, there was a significant difference between normal IgG and patient ANCA on the behaviour of neutrophils, suggesting that this is not simply a function of IgG on the neutrophil, but is a specific effect of ANCA.

8.1.2.2 ANCA pathogenicity and the relevance of superoxide production

It has been suggested previously that ANCA may act on neutrophils causing them to change in shape and become trapped inside small capillaries where they degranulate and cause damage (Cockwell, Brooks et al. 1999). This theory is not supported by the data in the experiments above which found that ANCA actually promotes adhesion and transmigration of neutrophils across the vessel wall in the presence of cytokines. It may be that ANCA, by causing the neutrophils to activate, simply promotes infiltration into the tissues of activated neutrophils, and these then set up an inflammatory response leading to tissue destruction.

There is additional evidence that the response of neutrophils to ANCA is dysregulated, that abnormal signalling pathways are set up (Williams, Ben-Smith et

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al. 2003) and that the neutrophil undergoes dysregulated apoptosis (Harper, Ren et al. 2000) which may contribute to this pro-inflammatory state.

ANCA activation of the neutrophil leads to two major actions: the superoxide response and the release of proteolytic enzymes. There is some evidence that mediation of endothelial damage may be by proteolytic enzymes (Lu, Garfield et al. 2006) rather than superoxide. In this paper vWF release from damaged EC by fMLP and ANCA, was caused by serine proteases rather than by superoxide. Such findings would be supported by work from this thesis. The superoxide responses were not always related to the findings in the flow model. Patient 220054 elicited a good superoxide response, but was not affective in stopping neutrophils in the flow model, patient 220028 elicited responses in both superoxide and flow and patient 220115 did not illicit a significant superoxide response, but did stop neutrophils in flow.

8.1.2.3 Why patient ANCA varies in pathogenicity

It is not clear why there is variation in responses of patient ANCA in various assays. The amount of ANCA in each sample is not known, only the total concentration of IgG, so that in some samples there could be very little ANCA, and between the various patient ANCA tested the concentration of ANCA is likely to be very variable. It is difficult to quantify or isolate patient ANCA from IgG fractions. The patient samples used in these studies were tested to confirm they were positive by ELISA for ANCA.

All the patient ANCA tested in the flow model induced superoxide responses, but not all ANCA tested in our lab elicit a superoxide response (unpublished data). Co-

workers in our laboratory have demonstrated that all the ANCA used in the flow model increase IL8 release from neutrophils, but again not all ANCA induce this response.

It may be that there is a genuine variation in ANCA activity in different assays, and this is currently a subject of ongoing investigation in our laboratory (unpublished work). All patient samples were isolated during acute, severe disease; patients undergoing plasma exchange have either pulmonary haemorrhage or end-stage renal failure. None of the patient ANCA were from patients in remission or with mild disease. Additionally, patients were all presenting for the first time with vasculitis; there were no patients with relapsed disease. The amount of time that the disease had been present before presentation is unknown. Due to the nature of the disease, many patients will have a long period of non-specific symptoms prior to acute presentation. This would allow the development of IgG subclass switching, and accumulation of IgG4, and could conceivably account for some of the variation in ANCA activity. In addition, subclasses of ANCA may be influencing pathogenicity and it is interesting to note that Patient 220054, whose IgG was the least effective at causing neutrophils to adhere had a very different subclass profile to the other patient samples, with an over representation of IgG2.

Patient ANCA is polyclonal and although generally directed against either PR3 or MPO, is known to be directed against more than one epitope on these molecules. It may be that ANCA directed against some epitopes are more pathogenic than others,

and it has also been proposed that some ANCA may bind to the activate enzymes PR3 and MPO and inactivate them (van der Geld, Tool et al. 2002).

Variability may also be dependent on the neutrophil response to the antibody, and therefore be dependent on neutrophil factors. Neutrophils may express different levels of target antigens (von Vietinghoff, Tunnemann et al. 2007), there may be differences in FcR polymorphisms (Edberg, Wainstein et al. 1997) or polymorphisms between signalling proteins. Differences between neutrophil donors are currently being explored in our laboratory.

8.2 Interactions of immunoglobulin and neutrophils: normal IgG versus ANCA and the effect of IgG subtype

8.2.1 Interaction of neutrophils on solid phase IgG

In order to assess whether the subclass of ANCA IgG was important in activation of the neutrophil, we first wished to explore the interaction between normal IgG and neutrophils. Because neutrophils are not activated by normal IgG in fluid phase, and require crosslinking of many Fc receptors, we designed experiments where IgG was presented in solid phase allowing neutrophils to sample many Fc receptors at once. This was achieved by coating glass microslides with each IgG subclass. Current thinking was supported by our studies where neutrophils bound avidly to IgG3, less to IgG1 and minimally to IgG2 and IgG4 (Jefferis and Kumararatne 1990). The neutrophils adhered to the surface and spread out, extending pseudophilia across

the surface and exhibiting random movement, pulling themselves into odd shapes as they encountered more and more IgG.

IgG3 was the most potent subclass at binding neutrophils. It has also been reported as being pathogenically important in vasculitic disease (Mulder, Stegeman et al. 1995), and to bind Fc receptors more avidly than the other subclasses. This is explained by its structure with a long hinge region allowing the protein flexibility and increased binding (Burton and Woof 1992).

We looked at the roles of the constitutive receptors CD16 and CD32 in neutrophil adhesion in flow conditions, and found that while all subclasses are affected by blocking these, there is a differential utilization of the receptors. Thus, CD16 was more important in IgG3-mediated adhesion under flow, while CD32 was more important for IgG1 and 2 mediated adhesion.

When we examined the role of CD18, part of the integrin complex, blocking resulted in a reduction in the spreading behaviour, neutrophils no longer extended psuedophilia across the cell surface. This behaviour is an active process, requiring cell signalling, because when we abolished the ability of the cell to signal by chelating intracellular calcium with BAPTA-AM, the behaviour was inhibited. This is perhaps unsurprising, but in addition, adhesion to the IgG surface was reduced, indicating that capture is also an active process requiring cell signalling.

8.2.2 Interaction of neutrophils with ANCA subclass

ANCA activity has been detected in all four IgG subclasses and there is evidence that different subclasses of ANCA IgG have different effects on neutrophil activation. One proposed mechanism of ANCA stimulation of neutrophils is the colligation of PR3 antigen with CD32/CD16. Since IgG4 has not been reported to ligate these receptors in primed neutrophils, IgG4 ANCA would not be expected to stimulate a superoxide response. Experiments with fresh human neutrophils (which do not express CD64, only CD32/CD16) and samples of human ANCA IgG1, 3 and 4 from different patients, all were able to elicit a superoxide response. Deglycosylation of ANCA IgG resulted in loss of its activating ability consistent with CD32/CD16 involvement, since deglycosylation impairs binding of IgG to these receptors while deglycosylated IgG may still bind to CD64. IgG4 was also capable of neutrophil activation suggesting that it can activate via CD32/CD16 receptors (Holland, Hewins et al. 2004).

8.2.3 The importance of subclasses in other autoimmune disease

Subclass of IgG has been reported as having a role in influencing the pathogenesis of many other diseases. In Rheumatoid Arthritis, anti-fibrinogen antibodies are predominately IgG1 and IgG4. IgG1 is produced in a predominantly Th1 response (the traditional theory for RA), whereas IgG4 requires IL-4, predominately a Th2 response. Other cells can secrete IL-4 and in the vicinity of B cells this could result in a class switch to IgG4 (Chapuy-Regaud, Nogueira et al. 2005).

In Goodpasture's disease, where the autoimmune immunoglobulin is directed against type IV collagen, experiments in rats have shown that IgG2a and IgG2b antibodies result in severe nephritis, where IgG1 results only in mild nephritis (Kohda, Okada et al. 2004). IgG2a and 2b induce proteinuria where as IgG1 did not, and this effect was due to induction of infiltration of inflammatory cells into the glomerulus (Kado, Kohda et al. 2006).

In Myasthenis Gravis anti-receptor autoantibodies were predominately IgG1 and thus able to activate complement, and IgG4 which is unable to activate complement (Romi, Skeie et al. 2000). The cell mediated response involves both Th1 and Th2 lymphocytes and a Th1 response alone was not necessarily associated with severe disease.

Systemic lupus erythematosis has multiple autoantibodies. The frequent occurrence of a rise in IgG2 anti-nucleohistone and IgG1 anti-dsDNA in patients prior to a renal relapse suggests that, besides IgG1 subclass autoantibodies, IgG2 subclass antibodies to nucleohistone have a particular pathophysiological role in lupus nephritis (Bijl, Dijstelbloem et al. 2002).

While all four IgG anti-beta2-GPI antibody subclasses were represented in patients with anti phospholipid syndrome, there appeared to be a significant overall skewing towards to the IgG2 subclass (Guerin, Casey et al. 1999).

8.2.4   Why we wished to develop chimeric anti-PR3

Patient ANCA is polyclonal, directed against more than one epitope on PR3 or MPO, and sometimes to epitopes on both, and is represented in all four IgG subclasses. It is difficult to isolate from patients, and requires obtaining plasma exchange fluid. Because the disease is treated after this this initial presentation, it is very rare for patients to undergo plasma exchange repeatedly, and thus from each patient there is a limited supply of ANCA.

Total IgG is isolated from plasma exchange fluid and it is difficult to quantify how much ANCA there is in the sample as ELISA is not quantitative. The concentration of ANCA within the total IgG is low, making it impractical to isolate the ANCA fraction alone.

Chimeric anti-PR3 was thus developed in our laboratory in order to provide a standard source of ANCA, whose epitope specificity, subclass and concentration was known.

8.2.5   Evidence that chimeric anti-PR3 behaves in the same way as patient ANCA

In functional studies in our laboratory prior to the current thesis, superoxide studies indicate that responses from IgG1 and IgG3 are similar to each other and to patient ANCA (Colman, Hussain et al. 2007). In this study, inhibition chemiluminesence indicates that chimeric PR3-ANCA of subclass IgG1 and IgG3 bind to the high affinity

receptor FcγR1 similarly to their control. IgG4 has also been confirmed to illicit a superoxide response (Hussain, in press).

8.2.6 Differences between chimeric PR3-ANCA and patient ANCA

Higher concentrations of chimeric PR3-ANCA are needed to cause neutrophil adhesion and migration in the flow model than patient ANCA which probably has a concentration of less than 10μg/ml in these experiments. This may be due to the fact that chimeric PR3-ANCA is directed against a single epitope, whereas in patient ANCA where multiple epitopes could be bound by different ANCA molecules, the Fc regions could then crosslink (this is discussed further below). It is generally accepted that signalling pathways reach thresholds of action and that weak and strong signals through the same pathway can trigger different outcomes. Thus the same pathway triggered at a high threshold may induce differentiation but when triggered at low threshold may induce proliferation. ANCA signalling pathways have been studied within our laboratory and it is already established that superoxide and adhesion pathways are different and engage different transduction molecules (Williams and Savage 2005). Conceivably migration and adhesion are therefore driven by separate signalling pathways, or migration requires stronger signals to reach a threshold. It is possible that with monoclonal ANCA where only one antibody binds to one molecule of PR3 small microdomains of signalling molecules are engaged. In polyclonal ANCA where two ANCA could bind to the same PR3 molecule, 'mats' of antibody could form and cause coalescence of these microdomains into larger lipid platforms triggering stronger/different signalling pathways.

The saturation binding by ANCA to neutrophils is unknown, but is likely to vary depending on neutrophil donor as there is variation in PR3 expression on neutrophils. This is a subject of ongoing investigation in our laboratory.

8.2.7 IgG subclass affects ANCA function and is dependent on Fc receptor type

Previous studies in our laboratory have already established that there is a difference in functional outcomes between different subclasses of ANCA (Table 7) ((Colman, Hussain et al. 2007) and Hussain in press). The affinity of chimeric PR3-ANCA to bind to PR3 (by surface plasma resonance) varied where IgG3 binds more slowly but also dissociates more slowly than IgG1. In IL-8 studies, both IgG1 and IgG3 PR3-ANCA induced IL-8 release from neutrophils, but IgG3 was more potent. IgG4 did not induce IL-8 release. In degranulation studies, all three caused degranulation, but IgG1 and IgG4 were more potent than IgG3. In studies of static adhesion both IgG1 and IgG3 ANCA caused more neutrophil adhesion than their controls and there were similar amounts of binding between the two subclasses. Blocking studies explored the roles of CD32 and CD16. Initially it was established that Fc portions of the antibody were necessary for superoxide response; F(ab')$_2$ fragments did not illicit superoxide production. Whole IgG was also deglycoslylated and again this abolished neutrophil superoxide production suggesting that correct glycosylation of the protein is necessary for function. Pre-incubation of neutrophils with blocking antibody to CD32, but not CD16, inhibited liberation of superoxide by IgG1 and IgG4 PR3-ANCA. In degranulation blocking CD32 prevented IgG1 and IgG4 responses, and blocking

CD16 blocked IgG3 response. In the IL-8 studies CD16, but not CD32 was important in all three subclasses (Hussain et al).

In the present studies, we took these observations forward by studying the effects of ANCA subclass in flow. These studies, on P-selectin and HUVEC established that IgG3 PR3-ANCA is a potent activator of neutrophils, causing them to be converted from rolling to static adhesion in flow. This was blocked by blockade of CD16 and CD32. IgG1 PR3-ANCA was also affective in activating neutrophils and causing an increase in static adhesion, and, although potent, its effect was not as great as IgG3 on HUVEC. Blocking CD16, but not CD32 reduced this activation.

Fc receptor function may be useful to explore in designing therapeutic targets in disease. Taken together, the above studies demonstrate that specific functions of neutrophils, as well as specific subclasses vary in their use of Fc receptors. There is also a difference between the predominant Fc receptors used by neutrophils when engaging normal IgG (CD32 for IgG1 and 2; CD16 for IgG3) and pathogenic ANCA (CD16 more important for IgG3 and IgG1) and causing the neutrophil to adhere under flow conditions.

IgG4 PR3-ANCA was very interesting, as prior to these studies this was thought to be a benign, blocking antibody, but it appears to have pathogenic potential. It was reported to only engage via CD64, which is not present on the non-activated neutrophils in the present studies that only express the constitutive receptors CD32 and CD16. However, in our studies, IgG4 caused a superoxide response and in flow

conditions, although less potent than the other two subclasses tested, was able to cause static adhesion of neutrophils, this must therefore have involved CD32/CD16. This was over a longer time frame and to a lesser extent than either IgG1 or IgG3. In studies blocking CD32 and CD16, no effects were seen on IgG4, but this was due to the early time points used in these experiments (IgG4 did not cause static adhesion until at least 4 minutes in P-selectin experiments), a practical problem due to limited resources left for these studies, making long experiments impossible.

8.3 Comparison of umbilical vein and glomerular endothelial cell types

We wished to examine two questions with these studies; 'are glomerular ECs targeted in AAV because of a distinct phenotype?' and, 'is it relevant to use HUVEC as a substitute for GEC when investigating AAV?' We have established from these studies that glomerular ECs are distinct from HUVEC and display different functional characteristics. Extrapolating from HUVEC to GEC is not reliable and may lead to erroneous conclusions concerning the functionality of these cells. GEC display distinct features, including a distinct adhesion molecule profile and we postulate that the differences that we have found between the EC types may be relevant in explaining the pathogenesis of vasculitis.

8.3.1 Appearance and structure

From our studies we confirm that in *in vitro* culture, PGEC are fenestrated and the GEC2 cell line could be fenestrated with high concentrations of VEGF, but was not

convincingly fenestrated in low concentrations of VEGF. HUVEC could not be fenestrated.

8.3.2 Adhesion molecules

From these studies we showed that HUVEC and GEC both have E-selectins and ICAM-1 on their surface, which support neutrophil rolling and adhesion. Initial studies in flow using neutrophils on a TNFα stimulated surface showed no difference between HUVEC and GEC.

VCAM-1 was not detected or detected only in very small amounts on the surface of GEC, but was present on HUVEC. VCAM-1 supports the binding of lymphocytes to EC and when studied under flow conditions, PBL binding to GEC was reduced compared to HUVEC. By Western Blotting VCAM-1 was present in all the EC and we therefore concluded that either there must be two isoforms of the protein, one more functional than the other; or that there must be a difference in the post translational modification of this protein to the cell surface in GEC reducing its expression. RNA arrays revealed differences between the cell types, and investigation of the specific pathways involving post-translational modification of VCAM-1 revealed a reduction in expression of the ST6N gene in PGEC responsible for the sialylation of VCAM-1, which potentially alters the adhesion capacity of the protein (Abe, Smith et al. 1999).

8.3.3 Why VCAM-1 is reduced on glomerular endothelial cells

The reduction in surface expression of VCAM-1 on GEC may be a protective mechanism to ensure that PBL are not recruited to the normal kidney interstium in large amounts, which would potentially cause stasis within the renal microvasculature.

There are different isoforms of VCAM-1 and although these alternative forms have been found to support adhesion of VLA-4 expressing cell lines (Cybulsky, Fries et al. 1991; Hession, Tizard et al. 1991) there may be different functional characteristics between them. The 6 domain (6D) form lacks domain 4. The 6D isoform in soluble form has also been reported to bind to its integrin $\alpha_4\beta_1$ (VLA-4) with higher relative affinity than the 7D isoform, but in physiological conditions under shear force, cells adhered more avidly to the 7D isoform, which induced cell spreading at lower concentrations than the 6D form (Woodside, Kram et al. 2006).

In vasculitis, VCAM-1, which is not present on undamaged glomeruli was found in necrotizing-extracapillary lesions (Rastaldi, Ferrario et al. 1996). The infiltrating leukocytes were activated in the damaged glomeruli, but where VCAM-1 was positive and the glomerulus undamaged, the infiltrating cells were predominantly macrophages. The authors concluded that VCAM-1 was a good marker of necrotizing extra-capillary damage. In another study, abnormal VCAM-1 expression was seen in more than 60% of patients' glomeruli with PR3-ANCA vasculitis (Arrizabalaga, Sole et al. 2008). These studies were confirmed by our work on whole kidney sections

where we found VCAM-1 was expressed and up-regulated in affected glomeruli in patients with vasculitis.

Although much research in AAV has focused on the neutrophil, the role of the lymphocyte in this disease has also been extensively investigated. T cells and monocytes are the predominant cells in inflammatory vascular infiltrates in AAV (Gephardt, Ahmad et al. 1983) and antibody isotype switching requires T-cell cytokines, such as IL4, so that T cells must be crucial in the disease, but whether they are important in the effector stage of the disease remains under debate. T cell responses are altered in AAV, activated circulating T cells display a Th1 cytokine profile (Gutfleisch, Baumert et al. 1993; Ludviksson, Sneller et al. 1998) as do T cells derived from granuloma of AAV patients (Moosig, Csernok et al. 1998). Whether the T cell responses are ANCA-antigen specific however remains debatable with conflicting evidence. T cells specific for ANCA antigens have been found in normal patients as well as those with AAV (Brouwer, Stegeman et al. 1994; Griffith, Coulthart et al. 1996; Winek, Mueller et al. 2004). The abnormal T cell responses may be driven by other antigens such as bacteria (Mayet, Marker-Hermann et al. 1999).

There is no evidence in the literature pertaining to the interaction of PBL on the endothelium in AAV. Since we have found that VCAM-1 is reduced on normal stimulated glomerular endothelium and that this leads to a reduction in PBL capture, we postulate that this may be a protective mechanism designed to prevent the glomerular vasculature being clogged with inflammatory cells. If this protective

mechanism was to become disrupted, specific targeting of the renal vascular bed may be a consequence.

8.3.4   Why vasculitis targets particular endothelial cell beds

There are some existing theories to explain why AAV targets particular endothelial beds, and these largely centre on the interaction of the neutrophils with ANCA.

Planted antigens (Ruth, Kitching et al. 2006), whereby degranulated neutrophils leave MPO in the glomerulus, which then acts as an antigen for accumulation of ANCA, may explain the continuation of inflammation in one vascular bed. At renal biopsy however, ANCA deposition is not seen, the pathology generally considered to be "pauci-immune" (only scant immune deposits present). Notwithstanding this, immune deposits can be observed in renal vasculitis and, indeed, their presence has been correlated with more severe disease (Haas and Eustace 2004).

If ECs have genuinely different profiles and therefore the potential to recruit neutrophils to a different extent, this may explain the targeting of specific vascular beds. The interaction of ANCA with neutrophils is known to cause abnormal signalling pathways within the neutrophil, and if this causes abnormal activation, local defence mechanisms may be overridden. If the adhesion molecule profile varies within endothelial beds, neutrophils may become trapped and not cleared, setting up a nidus of inflammation.

Anti-EC antibodies have been detected in patients with WG and these have been found to selectively bind to unstimulated nasal, kidney and lung ECs. This binding was lost upon treatment of these cells with IFNγ and TNFα. The authors therefore suggested that these antibodies may be an initiator of vasculitis (Holmen, Christensson et al. 2004).

8.3.5 Development of the cell line, advantages and problems of cell lines

Cell lines can be very useful in the study of endothelium. Primary cells are difficult and expensive to obtain and grow, and are usually only able to be passaged to passage 7 before their phenotype becomes very altered and they are no longer behaving in the same way as early passage cells. Immortalised cell lines can be passaged many times and retain the same phenotype. Our collaborators in Bristol have developed a cell line for GEC and allowed us to study it. Contrary to their original findings however, the first cell line (GEC1) seemed to have been changed in some way so that it did not express E-selectin or vWF on the cell surface, and these findings were supported by its inability to support capture of neutrophils from flow. The second line (GEC2) did express selectins.

Co-workers in our laboratory have studied a human dermal cell line, human microvascular ECs (HMEC-1) and found that this was also unable to support neutrophil adhesion in flow (unpublished data). It may be that the mechanism allowing the expression of selectins within the cell is easily disrupted by transforming these cells.

This is supported by a paper examining four immortalised cell lines compared to HUVEC which found that HUVEC and primary dermal cells supported rolling and binding of lymphocytes but none of the cell lines did (Oostingh, Schlickum et al. 2007). Immunocytochemistry of these cells showed some immortalised lines to have intracellular but not surface E-selectin, suggesting translation but defective post-translational processing. Some of the cell lines had no selectin expression suggesting impaired transcription.

In our studies we found some differences between PGEC and GEC2 which meant that we were concerned about substituting the cell line for primary cells. RNA arrays showed the PGEC and GEC2 to be more different from each other than either was from HUVEC in terms of the numbers of genes that were significantly different. The cells were grouped into their three distinct groups by PAM RNA array analysis but again the two GEC EC were more different to each other than either were from HUVEC. In addition, in flow studies, differences in adhesion between PGEC and GEC2 were detected in studies using neutrophils on minimally TNFα stimulated EC. In culture the GEC2 cells were more robust than either the PGEC or HUVEC. Conclusions comparing GEC and HUVEC were therefore predominantly where PGEC had been compared to HUVEC.

8.3.6 <u>Can HUVEC be used to represent GEC?</u>

From these studies HUVEC cannot be reliably used to represent GEC. From RNA arrays they have different gene profile expressions and GEC express lower amounts of VCAM-1 on the surface. This results in functional differences in leukocyte capture.

9. IMPACT OF RESEARCH STUDIES CARRIED OUT IN THIS THESIS

9.1 Personal development

This thesis has enabled me to greatly increase my understanding of vasculitis, of disease processes and of immunological mechanisms in general. It has facilitated the development of my critical thinking and assessment of exisiting research. I have developed and used many methodologies, gaining skills in scientific processes to address mechanistic questions.

9.2 Understanding disease mechanisms

9.2.1 Patient ANCA

- Polyclonal patient ANCA promote neutrophil activation and increase migration across an endothelial surface rather than frustrating it.
- Different ANCA and different neutrophil donors cause different responses and vary in pathogenicity.

9.2.2 IgG subclass effects on neutrophil activation by normal IgG and ANCA IgG

9.2.2.1 Normal IgG and neutrophils

- IgG subclasses in solid phase can capture neutrophils. Subclass affect has not been previously demonstrated. IgG3 is most efficient at capture, IgG1 is less efficient and IgG2 and IgG4 are inefficient.

- Neutrophil adhesion to IgG3 is dependent on CD32 and CD16, but CD16 is more important.

- IgG1 and IgG2 are dependent on both CD32 and CD16, but CD32 is more important.

- CD18 is important in allowing neutrophil spreading on an IgG surface.

- Adhesion and behaviour of neutrophils are both active processes and require cell signalling.

9.2.2.2 ANCA IgG and neutrophils

- IgG1 and IgG3 PR3-ANCA cause neutrophil activation and static adhesion to P-selectin surfaces and HUVEC. IgG3 is more potent than IgG1 on HUVEC.

- IgG4-PR3 ANCA is also activatory, this must be mediated through CD32/CD16 in contrast to previous literature.

- CD16 is important in adhesion after neutrophil activation by IgG3 and IgG1 PR3-ANCA.

- CD16 influences IgG3 PR3-ANCA activation of neutrophil adhesion.

9.2.3   Endothelial Comparisons

- Cell lines are not entirely reliable as a substitute for primary cells, they have different RNA profiles and different functional outcomes in capture of neutrophils from flow.

- HUVEC and GEC have different adhesion molecule profiles in that GEC has reduced surface expression of VCAM-1.

- Reduced VCAM-1 surface expression results in reduced PBL capture onto GEC compared to HUVEC which may protect the kidney from inflammation.

- Vasculitis may target kidney beds if this protective mechanism is disrupted.

- HUVEC cannot reliably be used as a substitute for GEC.

9.3   Implications for patient therapies

Current strategies for therapy in immune mediated disease tend to be broadly immunosuppressive and result in significant morbidity. Studies which reveal the details of the immune disruption in these diseases could lead to highly specific therapies leaving the majority of the immune response unaffected and therefore vastly reducing the side effects of treatment. In the studies in this thesis pertaining to exploration of IgG subclass, ANCA IgG3 is highly pathogenic and may represent the most significant contributory factor to disease pathogenesis. It may eventually be possible to manipulate specific subclasses in individual patients. Additionally the Fc receptor which is being utilised in order to activate neutrophils may provide a specific therapeutic target.

Regarding the work on endothelial cells, the reason why AAV targets specific endothelial beds may be due to adhesion molecule profiles and their disruption in disease. Inducing or suppressing specific adhesion molecules in these beds may enable blockade of inflammation and subsequent amelioration of damage here. It

may be that blockade of molecules upregulated by the disease process, such as VCAM-1 in the kidney, could therefore change the profile of the disease.

9.4 Further work

This thesis has lead to some interesting findings and it would be useful to explore these further. Firstly, pertaining to the subclasses of ANCA, IgG3 appears to be extremely pathogenic. IgG1 is clearly also an active subclass, and even IgG4 is not benign. It would be useful to study large cohorts of patients and test individual patient ANCA *in vivo* to see if these findings are true of polyclonal ANCA, and also to study whether there is a trend towards more benign disease with chronicity and a switch to IgG4 ANCA.

Manipulation of ANCA subclass, by use of animal models, may also be of therapeutic benefit.

Regarding the work on endothelial cells, it would be useful to look more closely at the role of lymphocytes on diseased glomerular cells, and to see if the recruitment here was different to that in healthy glomeruli. Initially animal models would be helpful. It would be interesting also, to see if the levels of adhesion molecules and adhesion of leukoctyes differed on EC such as lung, which is also targeted by AAV, and compare this to microvasular cells such as intestine, which are not often involved in the vasculitis disease process.

Finally it would be interesting to look at patient neutrophils and compare the interactions on EC in the flow adhesion model of neutrophils from normal donors to those of patients with vasculitis, to asertain if there are specific differences in neutrophils from AAV patients.

10. APPENDIX

10.1 Solutions and Buffers

10.1.1 Immunohistochemistry

10.1.1.1   1M Tris base stock solution

48.44g Tris Base (Sigma)

400ml deionised water

10.1.1.2   Tris-buffered saline (TBS)

100ml Tris base stock solution

1900ml deionised water

17.6g Sodium Chloride (Sigma)

Corrected to pH 7.6 with concentrated HCL

10.1.1.3   10mM Citrate buffer

Tri-sodium citrate (dihydrate) 2.94g (Sigma)

1000 ml distilled water

Corrected to pH 6.0 with concentrated HCl

<u>Western blotting</u>

10.1.2.1 Resolving Gel

% Gel	10%	12.5%	15%	17%
Acrylamide	2.5ml	3.13ml	3.75 ml	4.25 ml
Bis-acrylamide	650 µl	515 µl	400 µl	300 µl
Tris PH 8.8	3.73 ml	3.73 ml	3.73 ml	3.73 ml
10% SDS	100 µl	100 µl	100 µl	100 µl
10% APS	67 µl	100.5 µl	67 µl	67 µl
TEMED	5 µl	7.5 µl	5 µl	5 µl
Water	2.948 ml	2.503 ml	1.998ml	1.548 ml

10.1.2.2 Stacking gel

% Gel	5%
Acrylamide	1 ml
Bis-acrylamide	520 µl
Tris PH 8.8	1 ml
10% SDS	80 µl
10% APS	80 µl
TEMED	8 µl
Water	5.312 ml

10.1.2.3   TRIS PH6.8 (or 8.8)

12.1g trisma base

200mls water

pH to relevant pH

10.1.2.4   10X Running Buffer

30.2G Tris Base

100ml 10% SDS

187.6g Glycine

1L deionised water

10.1.2.5   1X Running Buffer

12.11G Tris Base

2g SDS

57.65g Glycine

2L deionised water

10.1.2.6   10X TBS - PH 7.6

24.2 G Tris Base

80g NaCl

1L deionised water

10.1.2.7   1 X TBS-T

100ml 10X TBS

900ml deionised water

1ml Tween 20

10.1.2.8   5% Blocking buffer

2.5g Marvel

50ml TBS-T

10.1.2.9   Transfer buffer

2.93g Glycine

5.81g Tris

0.375g SDS

200ml Methanol

800ml deionised water

10.1.2.10   6x SDS-Page loading buffer (10mls)

	Stock	Amount needed
0.35M Tris	1 molar	3.5ml
10.28% SDS	powder	1.28g
36% Glycerol	75%	4.8ml
0.6M DTT or 5% B-mercaptoethanol	100ml	0.5ml
Bromophenol blue	powder	Until blue

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