The role of extra and intra vascular cells and their molecules in modulating glomerular inflammation during health and disease

By

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Abstract:

Endothelium forms an interface between vasculature and cellular sub endothelial environment. It sits in a vulnerable position responding to the changes in the immediate environment in health and disease. During vasculitic glomerulonephritis (VGN), autoantibodies raised against neutrophil serine proteases (SP) activate the neutrophils resulting in the release of neutrophilic products onto the endothelial surface changing its ability to support neutrophil recruitment and leading to its damage. We have hypothesized that endothelial functions are modulated by intra and extracellular environments of the vasculature and that the glomerular epithelial cells (podocytes) regulate glomerular endothelial cell (GEnC) functions.

To address this, firstly we have investigated the effects of SP on EnC phenotype and demonstrated that endothelium presents proadhesive phenotype prior to the development of injury. Proteinase-3 (PR3) and human neutrophil elastase (HNE) induced neutrophil recruitment to viable endothelial cells under static conditions at 1µg/ml, whilst PR3 also increased neutrophil recruitment to HUVEC under flow. At 1µg/ml, neither PR3 nor HNE altered endothelial viability, whilst high concentrations (10µg/ml) and prolonged exposure (24hours) of endothelium to either protease caused detectable damage. Most of the effects were inhibitable by the presence of α1-antitrypsin (α1-AT).

Further, using an *in vitro* static co-culture system, the role of podocytes in modulating neutrophil recruitment by GEnCs was

investigated in health and disease with anti-neutrophil cytoplasmic autoantibodies (ANCA) and puromycin aminonucleoside (PAN) injured podocytes.

Co-culture of GEnCs and podocytes stimulated with higher concentrations of TNF-α (100U/ml), demonstrated a protective role by significantly reducing neutrophil recruitment by up to 50% when compared to GEnC mono-cultures. The inhibition of neutrophil recruitment was mediated by inteleukin-6 (IL-6), demonstrated by neutralising IL-6 in co-cultures and addition of exogenous recombinant IL-6 in GEnC mono-cultures. The neutrophil interactions in co-culture seem to be mediated by CXCR2 and ENA-78. The ability of ANCA to enhance neutrophil recruitment by minimally activated GEnC mono-cultures (TNF-α 2U/ml), was mitigated in co-cultures and the effect was reversed in highly activated endothelium (TNF-α, 10 & 100 U/ml). PAN injured podocytes in co-culture with GEnCs, lost their protective role and therefore resulting in elevated neutrophil recruitment with TNF-α stimulation, both in the presence and absence of ANCA. PAN injured podocytes demonstrated the consequences of a disease state.

Overall, these studies elucidate that GEnC presents an adhesive phenotype attracting neutrophils under the influence of inflammatory cytokines. During disease conditions as in VGN, exposure of endothelium to SPs results in a pre-activation stage leading to damage with high and/or prolonged exposures. Podocytes modulate glomerular endothelial responses to cytokine stimulation in health and disease.

Thus, endothelial functions are tightly regulated by interacting cells and their molecules and could be an important phenomenon in the disease process of VGN where neutrophil recruitment plays a central pathogenic role.

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ABBREVIATIONS

A-

- Antineutrophil Cytoplasmic Autoantibodies (ANCA)
- ANCA Associated Vasculitides (AAV)
- ANCA Associated Small Vessel Vasculitidis (ASVV)
- Angiopoietin (ang)
- Alpha 1- anti trypsin (α1-AT)
- A Proliferation Inducing Ligand (APRIL)
- Anti-endothelial Cell Antibodies (AECA)
- Ammonium Per Sulphate (APS)
- Acid Citrate Dextrose (ACD)
- Analysis of Varience (ANOVA)
-

B-

- Bovine Serum Albumin (BSA)
- BLyS (B lymphocyte stimulator)

C-

- Conditionally Immortalized Glomerular Endothelial Cells (ciGEnC)
- Conditionally Immortalized Glomerular Epithelial Cells or Podocytes(ciGEpC)
- Cell Adhesion Molecules (CAM)
- Cytokine Induced Neutrophil Chemoattractant (CINC)
- CXC motif Receptors (CXCR)
- Cytoplasmic ANCA (cANCA)
- C-C chemokine Receptor type 4 (CCR4)
- Chemokine (C-C motif) ligand (CCL)
- Cluster of Differentiation (CD)
- Cyclohexamide (CHX)
- Cytokine Synthesis Inhibitory Factor (CSIF)

D-

- Dimethyl Sulphoxide (DMSO)
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
- 4'-6-Diamidino-2-phenylindole (DAPI)
- Dulbecco's Modified Eagle Medium (DMEM)

- Deoxy Ribo Nucleic acid (DNA)

E-

- Ethylene Diamine Tetra Acetic acid (EDTA)
- Ethylene Glycol Tetra Acetic acid (EGTA)
- Electrical Resistance (ER)
- Endothelial cells (EnCs)
- Epithelial cells (EpCs)
- Epithelial-like liver cell line (AKN)
- Endothelial basal medium (EBM)
- Epithelial cell deived neutrophil activating protein (ENA-78)
- E-selctin ligand -1 (ESL-1)

F-

- Formyl-Methionyl-Leucyl-Phenylalanine (FMLP)
- Foetal calf serum (FCS)
- Flow cytometry (FACS)
- Foetal bovine serum (FBS)

G-

- Glomerular Endothelial Cell (GEnC)
- Glomerular Epithelial Cell (Podocyte)
- Glomerulonephritis (GN)
- Granulocyte macrophage colony-stimulating factor (GM-CSF)
- Glomerular Basement membrane (GBM)
- Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1)
- Glutamic acid-leucine-arginine sequence motif (ELR motif)
- Growth related Oncogene – α (GRO-α or CXCL1)

H-

- Human Umbilical Vein Endothelial Cells (HUVEC)
- Human Neutrophil Elastase (HNE)
- Hydrogen Peroxide (H_2O_2)
- Human retinal pigment epithelial cell line (ARPE-19 Cells)
- Human kidney cellline (HKC)

I-

- Immunoglobulin (Ig)
- Intercellular adhesion molecule-1 (ICAM-1)
- Interleukin (IL)
- IL-1 receptor type (IL-1 R)
- Interferon-inducible protein-1 (IP-10)

J-

- Junctional adhesion molecules (JAM)

L-

- Lymphocyte function-associated antigen-1 (LFA-1)
- Lysosomal membrane protein-2 (LAMP-2)
- Liopolysaccharide (LPS)

M-

- Myeloperoxidase (MPO)
- Monocyte Chemoattractant Protein-1 (MCP-1)
- Macrophage inflammatory protein-2 (MIP-2)
- Macrophage antigen 1 (Mac-1)
- Microscopic Polyangiitis (MPA)
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

N-

- Neutrophil extracellular traps (NETs)
- Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)
- Nicotinamide adenine dinucleotide phosphate (NADP)

O-

- Optical density (OD)

P-

- Proteinase – 3 (PR3)
- Phosphate buffered saline (PBS)
- Polymorphonuclear cell (PMN)
- Propidium Iodide (PI)
- Platelet-endothelial cell adhesion molecule-1 [PECAM-1/CD31]
- Platelet activating factor (PAF)
- Polyethylene terepthalate (PET)
- Protease activated receptors (PARs)
- Pascal (Pa)

- Puromycin aminonucleoside (PAN)
- Polymerase chain reaction (PCR)
- Plasma exchange fluid (PEX)
- P-selectin glycoprotein ligand-1(PSGL-1)
- Phenylmethanesulfonylfluoride (PMSF)

R-

- Roswell Park Memorial Institute (RPMI)
- Reactive oxygen species (ROS)
- Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES)
- Reactive oxygen intermediates (ROI)

S-

- Small Vessel Vasculitidis (SVV)
- Serine Proteases (SP)
- Simian virus 40 large tumour antigen (SV40LT)

T-

- Tumour Necrosis Factor - α (TNF-α)
- Toll like receptor (TLR)

V-

- Vasculitic Glomerulonephritis (VGN)
- von Willebrand factor (vWF)
- Vascular cell adhesion molecule-1 (VCAM-1)
- Vascular endothelial growth factor (VEGF)

Papers arising from this thesis

Podocytes regulate neutrophil recruitment by glomerular endothelial cells via IL-6 mediated cross talk – Resubmitting to Journal of American Society of Nephrology.

Kuravi, S J, Satchell, S C, Saleem, M A, Harper, L, Williams J M, McGettrick H M, Rainger, G E, Savage C[1].

Podocyte and its injury in the pathogenesis of ANCA associated vasculitis.

Kuravi, S J, Satchell, S C, Saleem, M A, Harper, L, Williams J M, Rainger, G E, Savage C.

Neutrophil serine protease-mediate inflammatory cell recruitment by glomerular endothelium and progression towards dysfunction – Accepted by Nephrology Dialysis and Transplantation.

[1]S. J. Kuravi, [1]A. Bevins, S.C. Satchell, L. Harper, J. M. Williams, G.E. Rainger, C. O. S. Savage and S.P. Tull

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Abstracts

Sahithi J Kuravi (formerly Sahithi J Panchagnula)

Panchagnula, S, Satchell, S C, Saleem, M A, Harper, L, McGettrick H M, Rainger, G E, Williams J M, Savage C. **Modulation of recruitment of neutrophils on cytokine stimulated glomerular endothelial cells by podocytes in the presence and absence of ANCA.**

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Panchagnula, S, Satchell, S C, Saleem, M A, Harper, L, McGettrick H M, Rainger, G E, Williams J M, Savage C.

Synergistic relation of podocytes and glomerular endothelium during inflammatory episodes of vasculitic glomerulonephritis - recapitulation of glomerular micro environment

15th International Vasculitis & ANCA Workshop 2011, Chapel Hill, North Carolina, U.S.A.

Panchagnula, S, Satchell, S C, Saleem, M A, Harper, L, McGettrick H M, Rainger, G E, Savage C. **The co-ordinated role of podocytes and glomerular endothelial cells in glomerular inflammation.**

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The role of IL-6 in neutrophil recruitment to glomerular endothelial and epithelial co-cultures, a model of the glomerular micro-environment

American Society of Nephrology Renal week 2010, Denver Colorado.

Panchagnula, S, Satchell, S C, Saleem, M A, Harper, L, McGettrick H M, Rainger, G E, Williams J M, Savage C. **The co-ordinated role of glomerular epithelial cells and endothelial cells in neutrophil recruitment during glomerular inflammation**

13th Imperial College Symposium 2010, London, UK

Anne Bevins, **Sahithi Panchagnula**, Samantha Tull, Matthew Morgan, Simon C. Satchell, Lorraine Harper, G. Ed Rainger, Caroline Savage **Treatment of endothelial cells with serine proteases induces exocytosis of weibel-palade bodies.**

Renal Association Conference, Manchester, May 2010

Anne Bevins, **Sahithi Panchagnula**, Simon C. Satchell, Lorraine Harper, G. Ed Rainger, Caroline Savage, Samantha Tull. **Phenotypic changes of endothelial cells resulting in increased neutrophil adhesion in ANCA-associated vasculitis**

15th International Vasculitis & ANCA Workshop 2009, Sweden.

Samantha Tull, **Sahithi J Panchagnula**, Anne Bevins, Simon C. Satchell, Lorraine Harper, G. Ed Rainger, Caroline Savage. **Effect of serine proteases, elastase and proteinase 3, on glomerular endothelial cell-neutrophil interactions**

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Chapter-1: The role of neutrophil serine proteases and podocytes in glomerular inflammation

1.1 Introduction

Vasculitic diseases are characterised by the presence of inflammation in blood vessels. Primary causes in disease occurrence may be intrinsic to the organ, genetic abnormalities or autoimmune conditions whilst secondary causes are associated with certain infections such as Hepatitis B, Hepatitis C or HIV, drugs, connective tissue diseases or cancers . Several subgroups have emerged under vasculitis which were predominantly classified at the Chapel Hill Consensus Conference, on the basis of site or size of the inflamed blood vessels or distinct clinical pathologies (Jennette, 1994; Scott et al., 1994). These diseases share a common pathology with the development of focal necrotizing lesions, which affect many different vessels and organs.

The small-vessel vasculitides (SVV) that affect vessels smaller than arteries (i.e., arterioles, venules, and capillaries), are the focus of the present studies and in particular anti-neutrophil cytoplasmic antibodies (ANCA) -associated SVV (ASVV), which includes Granulomatosis Polyangiitis (formerly known as Wegener's Granulomatosis) 2011(Jennette), Microscopic Polyangitis and Churg-Strauss Syndrome. ANCA are mostly immunoglobulin (Ig) G antibodies directed against molecules of human neutrophils and monocytes (Davies et al., 1982; Van Der Woude, 1985). Evidence have also demonstrated the presence of IgM ANCA (without IgG) alone or in conjunction with IgG ANCA presenting pulmonary haemorrhage (Esnault et al., 1992). There is a frequent

involvement of kidneys targeting the glomerular, tubular and vascular structures and thus damage leading to renal failure. Glomerulonephritis (GN) is often a prominent component of vasculitis, causing inflammation of the glomeruli. Glomerular crescent formation is a feature where recruited inflammatory cells and proliferating epithelial cells form a 'crescent' in Bowman's space and surround the glomerulus leading to impaired glomerular function (Huang et al., 1994). The disease incidence is increasing, with more than 20 per million and is most often seen in elderly population (peak age, 55 to 70 yr). Survey figures in the United Kingdom in 2008 showed an estimated incidence of 2 cases per 100,000 persons (Watts et al., 2008).

My thesis concentrates on the VGN associated with ANCA and the role of ANCA and their antigens in damaging the vascular endothelium. The role played by the extra vascular cells in supporting or provoking the endothelium during the process is also described.

In the introduction, I aim to explore the autoimmunity associated with ANCA, the inflammatory process in the presence of ANCA, the role of SP that are released by neutrophils in activating or damaging the endothelium, natural inhibitors of SP and synergistic relationship of glomerular endothelial and epithelial cells in neutrophil recruitment during inflammation, including mechanisms and the mediators involved.

1.1.1 ANCA in vasculitis and its antigenic targets

ANCA vasculitis is an autoimmune disease where ANCA IgG are directed against proteinases 3 (PR3) (Gross et al., 1986) or myeloperoxidase (MPO) (Falk and Jennette, 1988a) and uncommonly human neutrophil elastase (HNE) (Lüdemann J, 1990 ; Tervaert et al., 1993) contained in cytoplasmic granules of neutrophils and monocytes. ANCA were first reported by Davies *et al.,* 1982 (Davies et al., 1982), although the first report on the importance of these autoantibodies as a diagnostic marker of granulomatosis polyangiitis was by Van der Woude *et al.*, (Van Der Woude, 1985). Since then, two major ANCA patterns have been recognized by Falk *et al.,* in 1988 (Falk and Jennette, 1988b) using indirect immunofluorescence of ethanol-fixed neutrophils: cytoplasmic staining (c-ANCA) with 90% specificity to PR3 (Goldschmeding et al., 1989; Jennette et al., 1990; Niles et al., 1989) and a perinuclear staining pattern (p-ANCA), 90% specific for MPO (Falk and Jennette, 1988b). In addition, atypical c- and p-ANCA were also described in inflammatory bowel disease and rheumatoid arthritis patients (Savige et al., 2003).

Figure 1: Immunofluorescence staining patterns of ANCA on neutrophils (pictures taken from colleague Hamad Al Nuami)

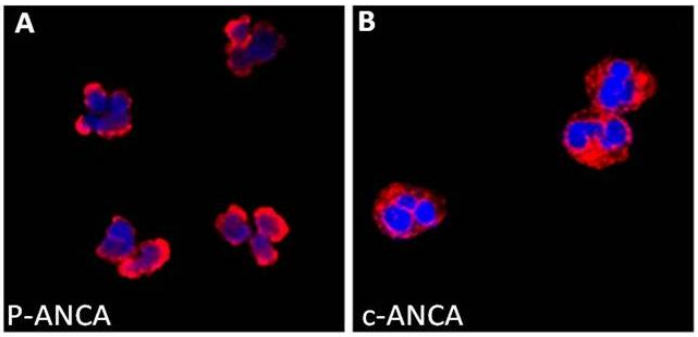


Figure 1: ANCA binding pattern on neutrophils: (A) Cytoplasmic staining pattern, C-ANCA targets proteinases 3 and (B) perinuclear pattern, p-ANCA targets myeloperoxidase.

The occurrence and severity of ANCA IgG differ within the IgG sub-classes. The most frequently represented are IgG1 and IgG4, whereas the IgG2 sub-class is infrequent (Locke et al., 1999). IgG3 or IgG4 are associated with disease severity or relapse and there are controversies with some investigators finding no association at all (Brouwer E, 1991b; Esnault VL, 1991; Jayne DR, 1991; Nowack et al., 2001). Pankhurst et al recently published about the variability in the pathogenicity of IgG subclasses and reported that IgG3 is the most pathogenic than other IgG followed by IgG1 and IgG2 and last IgG4 in regulation of neutrophil recruitment on endothelium under flow conditions (Pankhurst et al.).

The interaction of ANCA with the neutrophil may occur in the circulation or after the margination of a primed neutrophil to the endothelium, causing it to degranulate and transmigrate through the vessel wall. ANCA stimulated neutrophils may undergo premature degranulation of lytic enzymes and toxic metabolites which may promote the recruitment and activation of other leukocytes. ANCA also interferes with the resolution of inflammation through dysregulated neutrophil apoptosis and preventing apoptotic cell removal leading to secondary necrosis (Harper et al., 2001a) . ANCA is a pathological mediator and thus projected as an important diagnostic tool and marker in systemic vasculitis. ANCA is also found in non-vasculitic conditions including infection and inflammatory bowel disease where the antibody specificities may be directed towards a range of antigens other than PR3 or MPO (Jayne et al., 1995; Tervaert et al., 1989).

1.1.1.1 ANCA binding to antigens expressed on neutrophils

Falk et al in 1990 described the capability of anti- PR3/MPO antibodies in activating TNF-α primed neutrophils in vitro. Reumaux et al., demonstrated additional effects of TNF priming on signal transduction by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and involvement of β2 integrins (Reumaux et al., 2006). Studies from our lab have shown that the $F(ab)_2$ portion of ANCA IgG engages with target antigens on the cell surface of neutrophils and that the Fc portion interacts with Fc receptors (FcγRIIIb (CD16) and FcγRIIa (CD32)) (Ben-Smith et al., 2001). This engagement of both Fab and Fc portions of the antibody is a prerequisite for full ANCA-induced neutrophil activation inducing a respiratory burst as measured by superoxide assay (Ben-Smith et al., 2001; Kettritz et al., 1997; Porges et al., 1994; Williams et al., 2003). Even though the signaling process is not fully understood, signaling through Fcγ receptors is followed by tyrosine kinase dependent activation of phospholipase C, independent of G-protein activation (Santana et al., 1996), and an increase in calcium levels are reported (Hundt and Schmidt, 1992; Vossebeld, 1995). ANCA also bind to endothelial-bound antigens, inducing antibody dependent cellular cytotoxicity (Ewert et al., 1991). The promoted neutrophil adherence to EnCs and neutrophil migration into the tissues increase the likelihood of injury to endothelium during the process. The leukocyte recruitment to the glomerular capillary bed is thought to be a crucial step in the development of disease process (Huang et al., 1994; Huang et al., 1997).

1.1.1.2 Possible reasons for the development of ANCA:

It still remains unclear why autoimmunity occurs and why ANCA develop in the first place. There are several theories:

First, under normal conditions, PR3 and MPO that are released will be quickly scavenged or neutralised by naturally occurring inhibitors eg. α1-antitrypsin (α1-AT). However the molecules that are expressed on the cell surface cannot be neutralised by the inhibitors due to the steric hindrance (Campbell et al., 2000; Charles et al., 1991; Csernok et al., 1994a), and therefore could be exposed to the non-tolerized immune system resulting in the production of autoimmune antibodies. In patients with ANCA associated vasculitides (AVV), α1-AT can be inactivated by ROS which would lead to an acquired localized protease/antiprotease imbalance which could be further enhanced by PR3-ANCA interactions. The deficient PIZ α1-AT phenotype is also associated with AAV as well as a poorer prognosis for ANCA-positive systemic vasculitis (Segelmark et al., 1995). Recently published findings from our lab indicated that the Z but not the S allele is associated with AAV and also found polymers of α1-AT in the patient's serum and glomeruli of at least some patients with the Z allele, which may promote inflammation through priming of neutrophils (Morris et al.), 2010).

The other factors that contribute to the initiation of the ANCA autoimmune response include environmental pressures, including silica exposure (Hogan et al., 2001). Genetic susceptibility of certain individuals to develop autoimmune diseases along with infection becomes sufficient stimuli in the development of widespread vasculitis in a patient with ANCA (Lawyer et al., 1994; Stegeman et al., 1994).

Other possible explanations include:

There is the concept of molecular mimicry which suggests that there are similarities between human proteins and bacterial or viral proteins that result in the development of autoantibodies. Studies by the Chapel Hill group have provided evidence that vasculitis patients with ANCA specificity for PR3, also develop antibodies to a peptide translated from the antisense DNA strand of PR3 (complementary PR3) or to a mimic of this peptide. This theory of formation of antibodies against antigens which are complementary in their peptide sequence to autoantigens is referred as inverted hydropathy (Pendergraft et al., 2004).

Another possible contributing factor to the development of autoimmune responses is the improper deposition of PR3 and MPO due to dysregulated apoptosis of neutrophils (Harper et al., 2001a; Utz, 1998) on the EnC and subsequent presentation to immune cells. Further, the Witko-Sarsat group suggested that the bimodal expression of membrane PR3 on the neutrophil surfaces and the proportion of expression within a given donor is a possible key in the development of ANCA and may provide a risk factor for vasculitis, as well as being associated with relapse (Halbwachs-Mecarelli et al., 1995). Work by the Csernok group demonstrated occurrence of PR3 on the surface of the neutrophil in addition to an intracellular pool on resting neutrophils (Csernok et al., 1994b).

At the cellular level, neutrophil activation also results in the formation and release of neutrophil extracellular traps (NETs) that contain chromatin, MPO and SP, including PR3 and HNE (Brinkmann et al., 2004), which have been detected in affected human glomeruli. Kessenbrock *et al.* demonstrated that NETs perpetuate the autoimmune response against neutrophil components in patients with AAV (Kessenbrock et al., 2009).

1.1.1.3 *In vitro* and *In vivo* studies – a proof that ANCA are pathogenic

Evidence that ANCA are pathogenic is provided from both *in vitro* and *in vivo* studies. There are many *in vitro* studies which have provided strong evidence that both MPO and PR3-ANCA are directly involved in causing the glomerular and vascular inflammation in ASVV. Important *in vitro* evidences from the studies by Savage group and Jennette group showed that ANCA binds to cytokine primed neutrophils and monocytes resulting in functional activation with elevated respiratory burst and degranulation leading to the endothelial damage (Ewert et al., 1995; Ewert BH, 1992; Kettritz et al., 1997; Lu et al., 2006; Lu et al., 2004; Mulder et al., 1994; Radford et al., 1999). Falk *et al*., first observed that ANCA could stimulate leukocytes to undergo degranulation and respiratory burst (Falk et al., 1990b). *In vitro* findings lead to the ANCA-cytokine sequence theory of inflammatory injury to the vessel wall which is triggered by neutrophil recruitment to the endothelium and release of auto-aggressive neutrophil constituents (Gross et al., 1986).

The argument that all the *in vitro* studies were laboratory artefacts was addressed by advancements with *in vivo* models that provided compelling evidence that ANCA are pathogenic. During the 1990s, various rodent models were developed to investigate the effect of anti-MPO antibodies in renal injury. Qasim et al., (Qasim et al., 1993) reported the development of necrotizing vasculitis in Brown Norway rats treated with MPO-ANCA along with lower doses of mercuric chloride. Brouwer *et al.,* (Brouwer et al., 1993) immunized Brown Norway and Lewis rats with human MPO and reported development of anti-human-MPO antibodies that cross-react with rat MPO. They also induced crescentic GN in Brown Norway rats by infusing a lysosomal extract directly into the renal artery of animals immunized with human MPO and replicated human autoimmune vasculitis (Brouwer et al., 1993). Sub-nephritogenic doses of antiglomerular basement membrane (anti-GBM) and

anti-MPO antibodies given to animals developed necrotizing and crescentic glomerulonephritis, whereas animals that received only anti-GBM antibodies developed only minor disease (Heeringa et al., 1996). However, murine models developed by Xiao and colleagues in 2002 documented a definitive evidence for pathogenicity of MPO-ANCA (Xiao et al., 2002). They passively infused high-affinity anti-MPO antibodies raised in MPO knockout mice, into immune-deficient or immune- competent mice which resulted in the development of crescentic GN. This facilitated further studies looking at the role of neutrophils as key effector cells (Xiao et al., 2005), TNF-α (Huugen et al., 2005) and complement (Xiao et al., 2007) in the pathogenesis of vasculitis.

Little group induced anti-MPO antibodies in WKY rats by immunizing with human MPO and developed systemic anti-MPO-associated vasculitis resembling human ASVV by exogenously administering human MPO adjuvant (Little et al., 2005b). They also demonstrated the cross reactivity of induced anti-MPO antibodies with rat neutrophils and augmenting leukocyte-endothelial interaction, inducing microvascular injury *in vivo* (Little et al., 2005b). The same model formed the basis of intra-vital microscopy where interaction of MPO-ANCA with leukocytes is directly observed in mesenteric venules along with the development of micro haemorrhages (Little et al., 2005a).

All these models convincingly demonstrate the pathogenic potential of MPO-ANCA with clear limitations. These cannot be considered as autoimmune models due to the reliability on active immunization depending on use of adjuvant for disease induction. The renal phenotype in these models is mild and thus limits the testing for novel therapies.

In vivo evidence on the pathogenicity of PR3 ANCA is very limited as rodents lack PR3 expression on murine neutrophil surface and with little homology for the ANCA epitopes between human and murine PR3. Pfister *et al* in 2004 developed ANCAs against

recombinant murine PR3 in PR3/HNE double deficient mice, but the raised PR3-ANCA did not induce vasculitis when transferred into wild type mice due to the lack of expression of antigen on the surface of mouse neutrophils unlike human neutrophils. However these antibodies did induce a local inflammatory response following the administration of subcutaneous TNF-α in supporting PR3-mediated tissue damage (Pfister et al., 2004). Van der Geld and colleagues immunized mice and rats with chimeric human-mouse PR3 which elicited an antibody response to mouse PR3 and rat granulocytes with no signs of developing vasculitis (van der Geld and 2007). Recently published work from Little group was successful in inducing PR3-ANCA associated vasculitis in mice with a human immune system (Little et al 2011). MPO-ANCA SVV animal models have laid road in understanding the unique pathogenic mechanisms involved in ANCA-mediated vasculitis which will lead to new routes in developing therapeutic strategies. Yet the questions remain concerning the pathogenesis and immunogenesis of ANCA associated diseases. The development of PR3- ANCA SVV *in vivo* model elucidating more facts of that end is still awaited.

1.1.1.4 Other Antibodies involved

In addition to ANCA, there are reports about involvement of other autoantibodies in ASVV, including antibodies directed against EnC specific antigens called anti-endothelial cell antibodies (AECA) (Frampton, 1990; Lauren et al., 1989). AECA are a heterogeneous family of autoantibodies reacting with several antigen determinants in various diseases. In 1990, Frampton et al.,reported that 50% of ANCA positive patients with WG and MPA showed positivity for AECA IgG (Frampton, 1990). These antibodies are detected *in vitro* using cellular ELISA with cultured HUVEC (Belizna et al., 2006; Rosenbaum J, 1988). AECA can activate the EnC and induce apoptosis (Bordron, 2001). *In vitro* and *in vivo*

studies have shown direct and indirect cytotoxicity of these antibodies in promoting vascular damage (Matsuda, 1988; Tannenbaum et al., 1986). There is clinical evidence that these antibodies have possible pathogenic role in the disease (Belizna et al., 2006; Dulce et al., 1999; Leung et al., 1986; Rosenbaum J, 1988). However, it is possible that AECA may not be the cause of disease rather may follow EnC damage. Altogether, these antibodies could be directed against constitutively expressed or cytokine induced cryptic antigens along with adhesion molecules, extracellular matrix components (Belizna C, 1997; Direskeneli et al., 1994).

Anti glomerular basement membrane antibodies are another group of auto antibodies, often seen coexisting with ANCA (Harris AA, 1998), more often accompanied by MPO-ANCA than by PR3-ANCA. These antibodies react with non collagenous domain of type IV collagen and nearly all the patients with these antibodies have shown necrotizing lesions and crescents associated with GN, alveolar necrosis and accumulation of immunoglobulin accumulation along the GBM (Hellmark et al., 1997; Wilson CB, 1973). The studies by Hellmark et al., showed the specificity of anti GBM antibodies was same in patients with or without ANCA (Hellmark et al., 1997). *In vivo* studies have documented the pathogenic potential of these antibodies (bolton, 1996). The pathogenic mechanism of anti-GBM antibodies involves binding to its antigenic target, fixation of complement leading to neutrophil and monocyte recruitment which results in destruction of glomerular capillary walls thereby initiating crescent formation.

Anti laminin antibodies are raised against laminin which is a connective tissue matrix component of glomerular basement membrane. These antibodies are found in various diseases including ASVV, especially those with MPO specificity (Hellmark et al., 1997;

Vecchi et al., 2000). Circulating anti-entactin antibodies were also found in patients with GN and SLE (Saxena, 1994; Saxena et al., 1991).

Other antibodies which have taken importance on the GN stage recently are a new subtype of ANCA against lysosomal membrane protein-2 (LAMP-2) (Kain, 1995). The anti LAMP-2 antibodies were found in the majority of patients with focal necrotizing GN. LAMP-2 is an abundant protein on neutrophil and endothelial cell surfaces and shuttles between lysosomes and the cell membrane. *In vivo* evidence has shown that antibodies to LAMP-2 cause pauci-immune FNGN when injected into rats, and apoptosis of human microvascular endothelium *in vitro (Kain et al., 2008)*. A human LAMP-2 epitope (designated $P_{41–49}$) has been shown to have 100% homology to the bacterial adhesion, FimH, and rats immunized with this protein developed pauci-immune FNGN and also develop antibodies to rat and human LAMP-2. These studies suggest that infection with fimbriated pathogens could be an aetiological factor in the development of focal necrotizing GN. The presence of anti-LAMP-2 antibodies in ASVV awaits confirmation by other groups.

1.1.2 Neutrophils and their role in ASVV:

Neutrophils form part of the polymorphonuclear cell family (PMN) together with basophils and eosinophils, and are most abundant leukocytes in the bloodstream. They act as the first line of defense at sites of bacterial infection or injury. Neutrophils phagocytose and eliminate foreign microorganisms by undergoing activation and release of oxygen dependent and independent antimicrobial cytotoxic molecules from secretory granules. Neutophilic granules undergo exocytosis and release their constituents cruicial for

neutrophilic functions (Faurschou and Borregaard, 2003). The process of Exocytosis is Ca^{2+} dependent and involves fusion of vesicular membranes to the plasma membrane resulting in the expression of the vesicular constituents on the surface of the cell (Miller LJ, 1987). The controlled mobilisation of the cytoplasmic organelles of the neutrophil transforms it from a passively circulating cell to an effector cell of innate immunity. Neutrophils respond quickly to tissue injury and are the hallmark of acute inflammation.

1.1.2.1 Neutrophil activation by ANCA: Degranulation and Respiratory burst.

Neutrophilic granules consist of SP, components of the respiratory burst, a wide range of membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products and soluble mediators of inflammation. Specific granules are suggested to be involved in inflammatory responses and azurophilic granules with the antimicrobial functions of neutrophils (Faurschou and Borregaard, 2003). Neutrophil antimicrobial actions include the generation and release of reactive oxygen species (ROS) and release of SP from the intracellular granules. In ASVV, neutrophil activation by ANCA results in the generation of release of ROS and is important in triggering neutrophil apoptosis (Charles et al., 1992; Falk et al., 1990b; Jacobson, 1996). During inflammatory episodes, activated leukocytes are recruited from the blood stream onto the endothelium and migrate into the tissue. The adhesive interaction of neutrophils with endothelium triggers the mobilisation of secretory vesicles and subsequent exocytosis of the granules from neutrophils (Borregaard et al., 1994) resulting in the expression of receptors and release of additional matrix-degrading enzymes, including collagenase and SP, which facilitate migration.

The respiratory burst of neutrophils involves release of ROS (superoxide radicals and hydrogen peroxide) and hypochloric acid. NADPH oxidase produces superoxide, which spontaneously recombines with other molecules to produce reactive free radicals.

Oxygen burst: $2O_2 \rightarrow 2O_2^- + NAD^+ + H^+$ (Catalysed by NADPH oxidase)

Superoxide production: $2O_2 + 2H^+ \rightarrow 2O^-$ (superoxide) + H_2O_2 (Catalysed by superoxide dismutase)

Respiratory burst and degranulation occurs simultaneously from the leukocytes. Under normal conditions these processes occur in a controlled fashion, but under unusual circumstances they may be uncontrolled leading to adverse effects.

1.1.2.2 Neutrophil SP (PR3 and HNE), role in endothelial activation and injury in ASVV

PR3 and HNE are two SP stored in azurophilic granules in the cytoplasm of neutrophils, as well as monocytes. PR3 is a 29-kd SP and was first reported in 1978 by Baggiolini *et al.,* . HNE is of ≈25-kd SP (Navia et al., 1989). PR3 shares 60% sequence homology with HNE in the first 40 residues and identical first four N-terminal residues. HNE, unlike PR3, is strictly localized in azurophilic granules and does not have an extra granular localization in neutrophils. PR3 was discovered as a major antigen of c-ANCA in WG and detection of these autoantibodies has been helpful in diagnosis and follow up of the disease (Goldschmeding et al., 1989; Gross et al., 1986; Niles et al., 1989). ANCA against HNE have also been discovered in patients with a p- ANCA pattern (Tervaert et al., 1993).

The physiological concentrations of these enzymes in a healthy individuals are, 3.24 ± SD0.24 pg/neutrophil (Campbell et al., 2000) and 1.59 pg/neutrophil (Campbell et al., 1989; V. V. Damiano, 1988) for PR3 and HNE respectively. It is shown by Campbell et al., that brief priming and activation of neutrophils by proinflammatory mediators alone induces a rapid and persistant 5 to 6 fold increase in surface expression of PR3 and minimal free release. Significantly raised circulating levels of PR3 have been observed in plasma of SVV patients, in the range of 110-3940, median 560 µg/l, (n=59) compared to healthy donors 350 µg/l, 110-580 range, (n=30) (Ohlsson et al., 2003).

SP help the migration of neutrophils by activating endothelium and further leads to its detachment by degrading several constituents of the extracellular matrix proteins like elastin, fibrinogen, collagen type IV, fibronectin, heparan sulfate proteoglycans, laminin, vitronectin (Campanelli et al., 1990; Rao et al., 1991b; Rao et al., 1991c). Yang et al have shown that internalisation of PR3 and MPO may occur but only PR3 leads EnCs towards apoptosis. Other *in vitro* studies also demonstrated the direct detachment and cytolysis of EnCs by SP (Brouwer et al., 1994; Yang et al., 1996; Yang et al., 2001). The C- terminal domain of PR3 has an apoptotic function. Active PR3 induces apoptosis via direct cleavage of p65 NF-kB, resulting in its reduced transcriptional activity (Preston et al., 2002). PR3 also has functional similarities to caspase-1 and processes inactive cytokine precursors to their active forms. In fact PR3 has been shown to mimic functions of caspases 1, 2 and 3. PR3 acts like caspase-2 and -3 to cleave the Sp1 transcription factor (Piedrafita and Pfahl, 1997) and like caspase-1 to process IL-1β (Coeshott et al., 1999) which is involved in leukocyte recruitment.

These enzymes are potent and play a key role in digesting phagocytosed microorganisms and cell debri (Campbell et al., 2000). The microbicidal property of SP is shown to be

independent of their enzyme activity and operates depending on charged interactions between SP and bacterial cell membrane (Odeberg and Olsson, 1976; van der Geld et al., 2001). Similarly, the cationic nature of the enzymes also allows them to bind non-covalently to the anionic endothelium to which ANCA then binds, and triggers antibody-dependent cellular cytotoxicity (Ballieux et al., 1994). Their physiological roles explain their toxicity when these enzymes are released in excessive concentrations during disease conditions. PR3 may also play a role in enhancing IL-8 production by EnCs (Berger et al., 1996a), which further acts as a strong chemotactic factor and activates neutrophils.

Sibelius *et al.,* reported presence of PR3 messenger RNA in HUVEC detected by the PCR method. Contrary to these findings, others were unable to detect PR3 in resting or cytokine activated HUVEC (King et al., 1995; Pendergraft et al., 2000). Mayet *et al.,* reported the PR3 expression by cytokine activated human EnC's in the cytoplasm which increase the pathological consequences of WG (Mayet et al., 1993). Deposition of PR3 has been detected on the glomerular basement membrane and endothelium in kidney biopsies from patients with WG (Ballieux et al., 1994; Berger et al., 1996b).

The mechanism by which PR3 interacts specifically with EnC is not clearly understood. *In vitro* studies by Taekema *et al*., suggested that PR3 binds to 111kD membrane protein on EnC's. EnC's also express protease-activated receptors (PAR) (Cupit et al., 1999), which are believed to be the receptors for binding of PR3. PR3 is presented by CD177 (NB1 glycoprotein) on circulating neutrophils which has been shown to co-localise with membrane bound PR3 and was proposed to be a receptor for PR3. In patients with ASVV, elevated levels of CD177 was detected on circulating neutrophils (Hu et al., 2009). However, it is to be noted that neutrophils lacking CD177 have been shown to express membrane PR3 and are susceptible to ANCA induced activation. Thus the interactions

between CD177 and PR3 and its importance in disease pathogenesis have not been fully characterized.

1.1.2.3 Inhibitors of SP:

Under normal conditions, the proteolytic action of PR3 and HNE is always precisely regulated by the presence of endogenous antiproteases also called serpins (serine protease inhibitors). The most important of protease inhibitor is α 1-anti trypsin which physiologically acts on both PR3 and HNE (Bergenfeldt et al., 1992; Rao et al., 1991a). There are other inhibitors which include elafin (Wiedow et al., 1991), α2-macroglobulin (Rao et al., 1991a), glycosaminoglycans (Bergenfeldt et al., 1992).

PR3 complexed with α1-AT, as well as free circulating PR3, has been detected in plasma of WG patients and healthy controls (Baslund et al., 1994; Timothy et al., 1994). Under some pathological conditions, an imbalance of protease and inhibitor may occur at the site of inflammation due to deficiency inhibitors or abnormal α1-AT phenotypes or phenotype variants (Callea 2003; Segelmark et al., 1995). There is evidence that PR3- ANCA-positive vasculitis patients are more likely to have a Z allele that is associated with a deficient phenotype (Esnault et al., 1993; Griffith et al., 1996; Savige JA, 1995). The release of oxygen radicals can reduce the association of α1-AT with SP and thereby increasing the local activity of SP (Esnault, 1997). Thus, it may be that deficiency of serpins may contribute to the autoreactivity of SP by determining the local ratio of SP and their inhibitors and the final proteolytic damage by neutrophils and monocytes.

1.1.3 Other Leukocytes in ASVV:

Monocytes and macrophages are the predominant infiltrating cells during the development of GN (Ferrario F, 1999; Rastaldi MP, 2000, 1996). Previous human and experimental models have revealed the involvement of monocytes and macrophages in the formation of glomerular crescents and lesions at interstitial level and playing a vital role in inflammatory process leading to renal injury. They participate in crescent formation by infiltrating into Bowman's space, interstitium and periglomerular space causing their rupture with further accumulation. They also synthesize and secrete a variety of chemokines, chemoattractants and growth factors. PR3 and MPO as constituents of monocytes may have direct effect of ANCA on monocytes/macrophages along with neutrophils. (Kobold et al., 1999; Sven et al., 2001; Tay SP, 1998) In vitro studies also reported that ANCA promotes monocyte recruitment (Casselman et al., 1995; Ralston et al., 1997).

T and B lymphocytes are also shown to be strongly involved in the disease process of ASVV. T lymphocytes are a large component of infiltrating leukocytes in vasculitic lesions and granulomas, thus may have a role in the pathogenesis (Griffith ME, 1996). Circulating T cells with Th1- type cytokine profile were evident in ASVV patients during active disease and remission. T cells are particularly necessary in production of epitope specific responses and class switching of ANCA IgG. Renal biopsies from vasculitic dependent crescentic GN patients shows the presence of CD4+ T cells (Bolton WK, 1987; Muller, 1988). CD4+ T cells, specific for ANCA antigens, are shown to proliferate in response to exposure to PR3 or MPO in patients with AAV (Brouwer E, 1994; Griffith ME, 1996; King WJ, 1998). Increased levels of soluble IL-2R is seen in active disease (Brouwer E, 1991a;

Schmitt W, 1992). A study by Popa et al., demonstrated that activation of T-cell persists during disease remission (Popa ER, 2002). Giscombe described a high prevalence of CD28 cells within the activated T-cell subset in vasculitis patients and suggested that they could be the effector arm of the T-cell response in ASVV (Giscombe et al., 1998). An increased percentage of activated T cells measured by the expression of CD25 and D-related human leukocyte antigen on CD4+ and CD8+ cells are found in patients in remission (Ludviksson et al., 1998). The role of T cells is also suggested by the partial success of anti T cell therapy. Treatment with rabbit anti-lymphocyte globulin showed some efficacy in patients by depleting autoreactive T cells (Hagen EC, 1995).

B-cells occupy centre stage in vasculitis as precursors of plasma cells that produce ANCA. They interact with and act as antigen presenting cells themselves providing co-stimulatory support to T-cells. Abnormal B cell function is evident in autoimmunity and particularly in the pathogenesis of T-cell auto reactivity (Takemura et al., 2001). Also abnormal T cell regulation is dependent on B cells in AAV and B cell proliferation may be amplified by T cell derived cytokines like BLyS (B lymphocyte stimulator) and APRIL (a proliferation inducing ligand) . Nasal biopsies of AAV patients have shown abnormal B cell clusters adjacent to PR3 ANCA positive cells (Voswinkel J, 2006). Renal biopsies have also been shown to have B cell infiltrates. Low surface expression of CD19 by B cells results in reduced transmembrane signaling and may allow auto reactive B-cells into peripheral circulation as seen in ASVV and be associated with the breakdown of tolerance to ANCA antigens (Culton DA, 2007). The effective use of rituximab, the B cell depleting agent, has provided a valuable insight into the importance of B cells in vasculitis.

1.1.4 Role of cytokines and chemokines in neutrophil recruitment

Endothelium is the source and the target to inflammatory mediators called cytokines that play a vital role in inflammation and immunity. EnCs stimulated by inflammatory cytokines, produce chemo attractants or chemokines to recruit and activate leukocytes. Different cytokines have different functions in mediating inflammation and immunity.

1.1.4.1 Tumor necrosis factor (TNF-α) in neutrophil priming:

In inflammatory diseases, TNF-α is a principle mediator and functions primarily in regulating immune cells. It is produced by a variety of cells such as neutrophils, macrophages and T-cells. All cells involved in inflammation have TNF-α receptors, and are activated by it to synthesize more of their own TNF-α. Dysregulated TNF-α production has been implicated in variety of diseases such as ischemic renal disease, WG and MPA (Ludviksson et al., 1998; Noronha et al., 1993; Tomer et al., 1999). Interaction of TNF-α with vascular endothelium renders it hyper-adhesive by increased expression of adhesion molecules which attract neutrophils (Briscoe et al., 1992; Pober et al., 1986). It plays a crucial role in cell-cell interactions both in vitro and in vivo (Falk et al., 1990b, c; Kunkel et al., 1991). During active ASVV, upregulation of TNF-α mRNA expression and cytokine release results in neutrophil priming which facilitates an ANCA-induced respiratory burst, degranulation and dysregulated neutrophil apoptosis (Falk et al., 1990a, b; Mulder et al., 1994; Porges et al., 1994).

1.1.4.2 Interleukin -6:

Cytokines such as IL-6 are vital modulators of immune and inflammatory responses. IL-6 is secreted by different cells including fibroblasts, endothelial cells, monocytes and macrophages, lymphocytes, podocytes and other different cell types (Weissenbach, 1980); (Corbel and Melchers, 1984);(Aarden et al., 1987; Vansnick et al., 1989). IL-6 is not produced constitutively by normal cells, but can be induced by infections and by other cytokines such as TNF-α and/or lL-1 or in the presence of IFN-γ (Shalaby et al., 1989; Vandamme et al., 1987). IL-6 interacts with its ligand-binding receptor IL-6R (CD126) and the signal transducing transmembrane receptor subunit gp130 (Hirano T, 1992; L Syners, 1989) promoting the initiation of intracellular signalling (Rose-John et al., 2001; Taga et al., 1989). IL-6R expression is limited to hepatocytes, neutrophils, monocytes/macrophages and certain other leukocytes and gp130 is expressed on almost all cells of the body. Trans-signalling of IL-6 occurs by the interaction between soluble form of IL-6 receptor (sIL-6R) and IL-6 stimulating the cells by binding to gp130 ligand (Mackiewicz et al., 1992; Taga et al., 1989). IL-6 affects T and B lymphocytes and the production of acute-phase proteins by liver. IL-6 switches innate immunity to acquired immunity, being responsible for the transition from neutrophil recruitment to mononuclear cells by trans-signalling (Topley et al., 1996). IL-6 deficient mice have shown that neutrophil accumulation is blocked by IL-6 at inflammatory sites (Xing et al., 1998). In the resolution of innate immunity, trans-signalling of IL-6, not only blocks pro-inflammatory cytokines and suppresses IL-8 and GRO, but also promotes release of ENA-78 and CXCL-6 (Hurst et al., 2001; McLoughlin et al., 2004; McLoughlin et al., 2003; Modur et al., 1997). IL-6 is a resolution factor balancing pro- and anti inflammatory outcomes and thus acts as an immunological switch. IL-6 interacts with other inflammatory mediators and there is

interplay between IL-6 mediated signals. The raised levels of IL-6 were shown to correlate with the disease activity in diseases such as rheumatoid arthritis and sepsis (Hack et al., 1989; Houssiau et al., 1988). The high titers of IL-6 can result in amplification of the inflammatory process by involving IL-1 and TNF-α (Brouckaert et al., 1989). In chronic diseases, IL-6 blocking is therapeutic (Mihara et al., 1998; Shinkura et al., 1998; Yoshizaki et al., 1998). Over production of IL-6 is also linked to variety of diseases such as proliferative mesangial GN, acting in an autocrine fashion (Kishimoto, 1989).

1.1.4.3 Chemokines:

The quiescent circulating neutrophils are actively recruited onto the endothelium by chemotactic agents. CXC chemokines mainly act on neutrophils and CC chemokines are inactive on neutrophils and stimulate monocytes, basophils, eosinophils and T lymphocytes. Chemokines act by signalling leukocyte migration, working in association with selectins and integrins (Reviewed by (Baggiolini, 1998)).

To induce stable attachment of neutrophils, EnCs synthesize, secrete and display CXC chemokines, like interleukin-8 (IL-8 /CXCL8) and several analogs GRO, epithelial cell-derived neutrophil-activating protein (ENA-78/CXCL5), neutrophil-activating protein-2 (NAP-2/CXCL7) and platelet activating factor (PAF) on luminal surface induced by inflammatory cytokines (IL-1 or TNF or both) and by LPS (Breviario et al., 1988; Prescott et al., 1984; Warner et al., 1987; Zimmerman et al., 1990). All these chemokines share properties of IL-8 as neutrophil chemoattractants and the effects were also tested during *in vivo* studies (Colditz et al., 1989; Vanzee et al., 1991). Interleukin-8 and GRO-α (CXCL1) are active on neutrophils and IL-8 rendered neutrophils adhere to endothelium and, in contrast, there are findings which show that EnC derived IL-8 inhibits binding of

neutrophils to activated EnC. The transmigration of neutrophils is partially dependent on the local production of IL-8. It was shown that the timing in the presentation of these chemotactic agents is very important for the successful neutrophil transmigration (Luu et al., 2000). CXC chemokines were shown to have high affinity for two receptors: CXCR2 with high affinity for all CXC chemokines and CXCR1 with high affinity for IL-8 only (Ahuja and Murphy, 1996; Moser et al., 1991; Schumacher et al., 1992). It was shown that both receptors function independently by mediating similar responses to different CXC chemokines (Loetscher et al., 1994).

1.1.5 Glomerular Endothelium and its role in the course of a disease process:

Endothelium forms the inner cell lining of blood vessels and has many functions. EnC phenotype is considered heterogeneous between different sites of origin. The specialized characteristics, the unique structure and functions of endothelium in the glomerular microcirculation and are also considered to be some of the contributing factors of its damage in ASVV. The ultra-structural variation of renal endothelium is non-diaphragmed fenestrations which potentially increase exposure of the basement membrane and may give rise to a propensity for damage. GEnCs are exposed to high numbers of circulating cells and high shear stress, thus express specific EnC adhesion molecules in response to injury (Maria Pia et al., 1996). The special features of glomerular endothelium may be accounting in placing kidneys as major target of vasculitic diseases (reviewed by (Little MA, 2008; Pankhurst et al., 2009)).

1.1.5.1 Endothelial Cell activation and damage

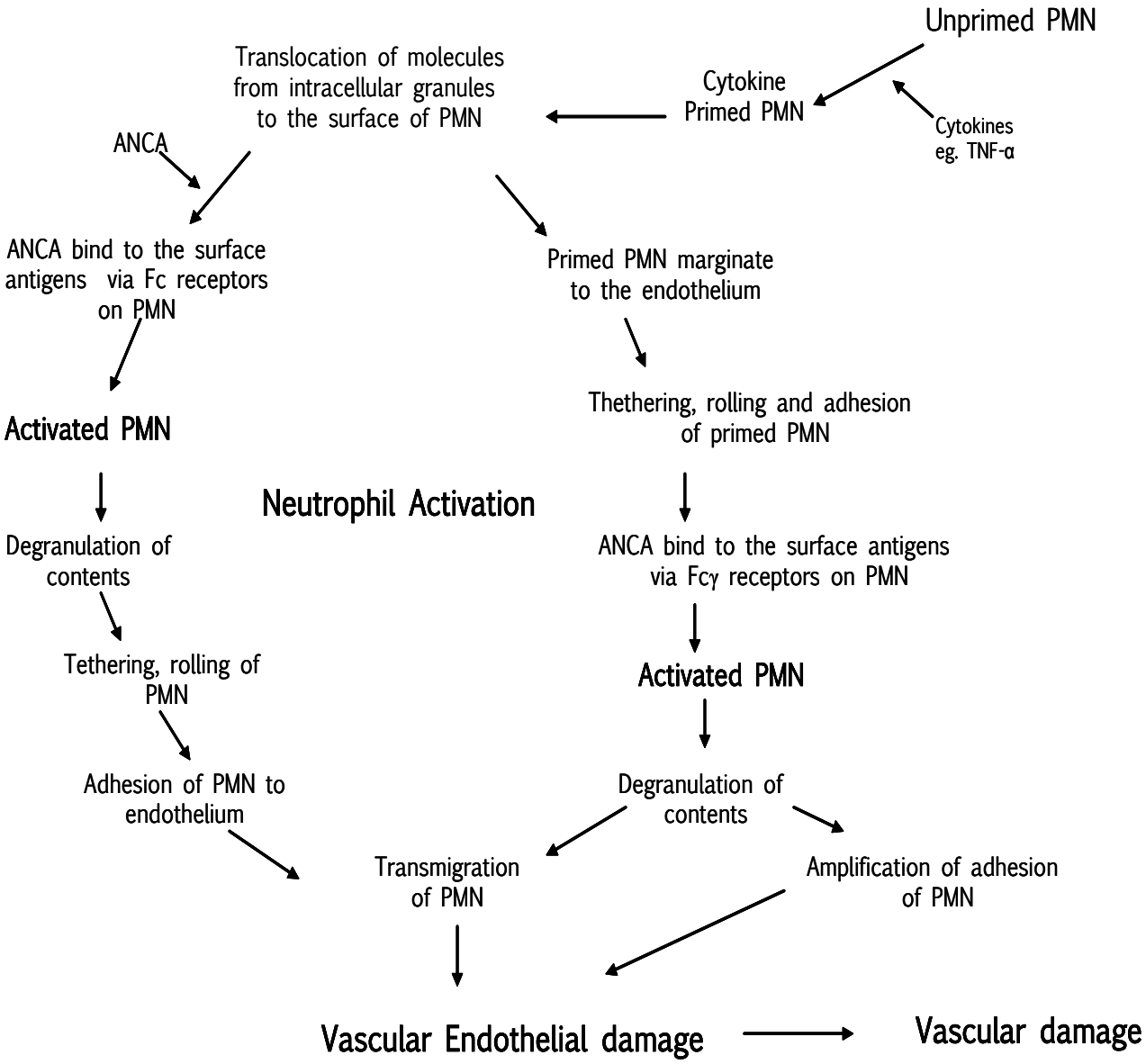
Activation of endothelium in response to cytokines (e.g. TNF, IL-1), mechanical stress, circulating autoantibodies, local inflammatory mediators (e.g. histamine, thrombin) and bacterial products (e.g. endoxin) are pivotal in acute and chronic inflammation. Endothelial activation induce release of proinflammatory cytokines and upregulation of cell adhesion molecules promoting leukocyte endothelial interactions. At some inflammatory sites, apart from the involvement of known classical adhesion molecules, a novel molecule like VAP-1 also seems to play a role in different conditions (Holmen et al., 2005) and strong expression of VCAM-1 induced by IL-4 is specific for glomerular endothelium in vasculitis patients (Maria Pia et al., 1996). ANCA stimulated neutrophils are also shown to increase glycoprotein Von-Willebrand factor (vWF) release, a circulatory cofactor for factor VIII on endothelium which is a measure of endothelial cell activation / stress (Lu et al., 2006). Recent studies by Bevins *et al*., from our lab, also demonstrated the increased release of vWF by SP treated EnC showing the possibility that vWF release also represents endothelial activation.

Primed neutrophils associating with ANCA become activated resulting in antigen presentation, free radical generation, cytokine production, adhesion with resident glomerular cells. Many *in vitro* studies have demonstrated potential for the endothelial injury in course of the disease process (Büschenfelde, 1993 ; Ewert et al., 1995; Ewert BH, 1992; Keogan MT, 1993; Savage et al., 1992). To prevent inappropriate tissue damage during neutrophil extravasation, EnC has been shown to be protected by initial integrin-mediated downregulation of ROS through temporary suppression of NADPH oxidase activity (Zhao, 2003). However, Lu et al., have shown that superoxide generation from

fMLP and ANCA-activated neutrophils is inhibited in the presence of EnC, but SP release was not inhibited (Lu et al., 2006).

The highly cationic neutrophilic ANCA antigens bind non-covalently to EnC and ANCA can bind to them resulting in endothelial injury. In vitro studies were logically extended by use of physiological flow studies to more closely mimic the in vivo environment in vitro. Our group has successfully used flow chambers to enumerate different aspects of endothelial recruitment of neutrophils following ANCA stimulation (David et al., 2000; Little et al., 2005b; Nolan et al., 2008; Radford DJ, 2001). Thus, endothelial dysfunction is integrally coupled with the activation of endothelium during inflammatory events which conclude in vascular injury. However, EnC activation controlled by local chemokine expression is characterized by enhanced leukocyte adhesion and extravasation as part of a co-ordinated multi-step process.

Figure 2: Hypothetical scheme of neutrophil activation by ANCA resulting in the endothelial damage and vascular injury



1.1.5.2 Role of adhesion molecules and the multistep process of neutrophil extravasation

The basis for neutrophil-endothelial interactions during inflammation involves three main steps. Step one involves selectin dependent tethering and rolling, step two involves chemoattractants to activate integrin adhesiveness and put on the brakes, and in step three, the immunoglobulin (Ig) family member binds integrins and causes cells to arrest from rolling.

Selectin mediated leukocyte rolling

Most leukocytes express L- selectin. E-selectin and P-selectin are expressed by activated EnCs while platelets also express P-selectin, which mediate early rolling events in the adhesion process. Ligands for L-selectin are CD34 and GLYCAM-1; E-selectin binds to ESL-1 and glycosylated CD44 (Hidalgo et al., 2007) and P-selectin binds to PSGL-1 (Kalogeris et al., 1999). E-selectin, unlike P-selectin, has no preformed storage units in EnCs. Increased levels of cell surface expression occur by transcription-dependent protein synthesis in response to inflammatory cytokines. P-selectin is stored specifically in α-granules in platelets and Weibel-Palade bodies in EnCs and is rapidly translocated to the cell surface in response to mediators of acute inflammation. The selectins interact with their ligands to enable leukocytes to adhere to inflamed endothelium under conditions of blood flow.

Under inflammatory conditions VAP-1 is also translocated from Weibel-Palade bodies of EnCs and supports leukocyte rolling, firm adhesion, and transmigration under shear stress (Koskinen et al., 2004). In 1996, Salmi *et al.,*elucidated that mature VAP-1 mediates carbohydrate-dependent, selectin-independent lymphocyte binding under shear

stress (Salmi and Jalkanen, 1996). VAP-1 regulates the selectin bond dissociation, mediating tethering and rolling (Smith et al., 1998) and shares functional similarity to PECAM-1 (CD31) in regulating transmigration.

Integrin-mediated firm adhesion

Integrins mediates firm leukocyte adhesion, triggered by chemokines and chemoattractants that cause conformational changes within integrin molecules on the neutrophil surface. Leukocyte integrins bind to the immunoglobulin super family members ICAM-1 and VCAM-1, expressed by EnCs (Campbell et al., 1996; Henninger et al., 1997) . The α2 and β2-integrins are most relevant to leukocyte adhesion and arrest and are implicated in ANCA induced neutrophil recruitment (De Vriese et al., 1999a). Blocking of α2 integrin has been shown to prevent an adhesion response (De Vriese et al., 1999b) and blocking of β2-integrin completely inhibited ANCA-induced neutrophil-endothelial adhesion (David et al., 2000). β2-integrins, CD11a/CD18 (LFA-1) and CD11b/CD18 (macrophage antigen 1 [Mac-1]) bind to ICAM-1 on the endothelium and are reported to be involved in neutrophil crawling. Elevated levels of expression of ICAM-1 and VCAM-1 are observed on renal capillaries and small renal vessels in WG patients (Ledford, 1997).

Crawling and trans-endothelial cell migration

The final step in leukocyte emigration into inflamed tissue is the transmigration through venular walls. An adherent neutrophil crawls through the tissue towards chemoattractants by engaging the junctional adhesion molecules (JAM). Leukocyte membrane protrusions extend into the endothelial-cell body and cell junctions which are triggered by ligation of ICAM-1 by MAC-1 phillipson (Phillipson et al., 2006). Ligation is associated with enhanced

endothelial cell contraction and opening of inter-endothelial contacts promoting leukocyte migration through endothelial junctions (paracellular pathway) or through the body of the endothelium (transcellular pathway). The transmigration is mediated by CD31 and JAM's A-C and may involve CD99 (Lou et al., 2007).

Figure 3: Neutrophil recruitment cascade

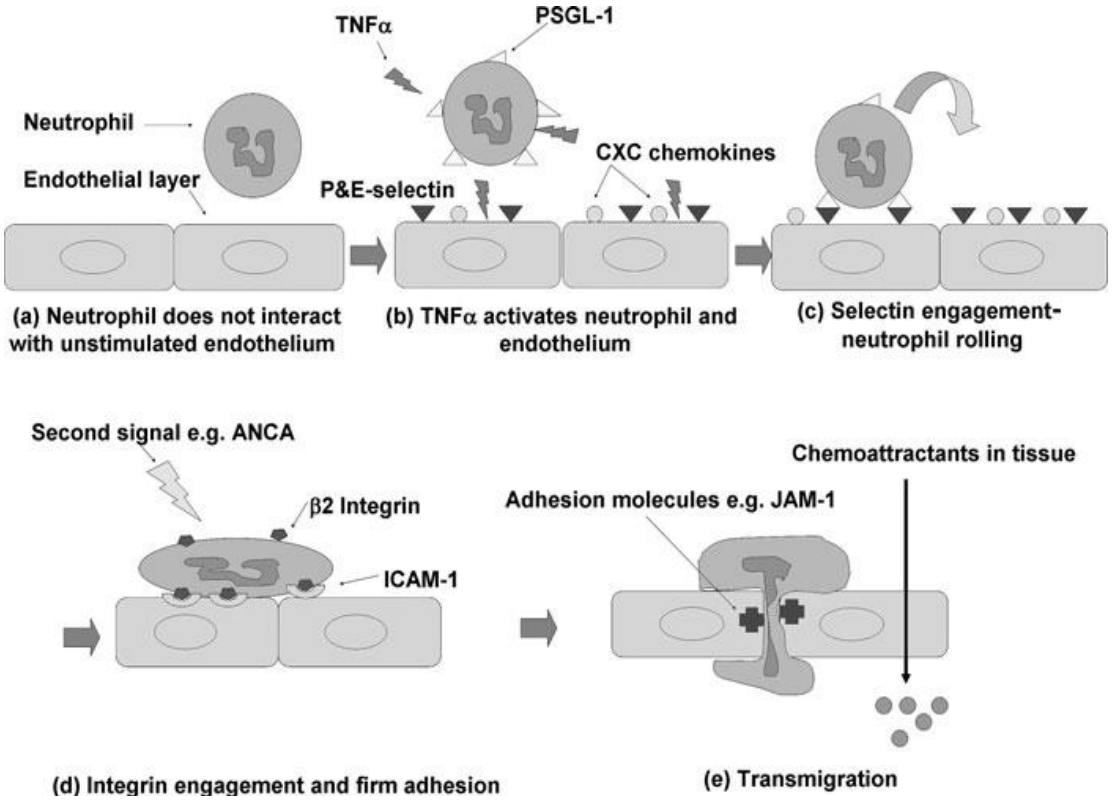


Figure 3: Neutrophil recruitment cascade: Demonstration of interactions between the neutrophil and cytokine activated endothelium. Reviewed by (Pankhurst et al., 2009)

1.1.5.3 Choice of glomerular endothelial cells

GEnC were used for the experiments due to their specialized role in glomerular function and the contribution that this may make to the pathophysiology of GN. As maintaining primary cultures of GEnC are difficult, conditionally immortalized human GEnC (ciGEnC) were used for these studies (Gift from Dr S Satchell, Bristol). ciGEnC were prepared from isolated primary cultures from glomeruli. GEnC were transduced with temperature-sensitive simian virus 40 large tumour antigen (SV40LT) and telomerase using retroviral vectors. ciGEnC proliferate at the permissive temperature (33^{o}C with 5% CO_2) and become growth arrested at the non-permissive temperature (37^{o}C with 5% CO_2). ciGEnC have been shown to retain early passage primary culture morphological features up to at least passage 41 (Satchell et al., 2006). Messenger RNA (mRNA) analysis confirmed expression of the endothelial markers platelet endothelial cell adhesion molecule-1 (PECAM-1), ICAM-2, vascular endothelial growth factor (VEGF) receptor molecule-2 and vWF, validated by immunofluorescence and Western blotting (work by Pankhurst).

1.1.6 Glomerular visceral epithelial cells

Glomerular visceral epithelial cells or podocytes (GEpC) are complex, specialized, unique cells and play a critical role in health and disease of kidney. They have a cell body, major processes, and foot processes. The podocyte foot processes interdigitate and forms the slit diaphragm. Podocytes and GEnCs have a mutual glomerular basement membrane (GBM) and form the glomerular filtration barrier in the kidney which maintains filtration. Many *in vitro* studies support the notion that podocytes provide physical support for the GBM to counteract the hydrostatic pressure of the blood. Any small interference in this

complex system will lead to escape of protein, leading to proteinuria, a hallmark of glomerular disease, and in severe cases leading to morbidity and mortality of children and adults (Bernard et al., 1988). Functionally, podocytes are responsible for GBM turnover and regulation of glomerular filtration. In addition they also mediate some immunological functions and are injured in many immune mediated glomerular diseases (Mundel and Kriz, 1995). Podocytes were also shown to express cytokine and cytokine receptors making them fragile and vulnerable during inflammatory episodes (Huber et al., 2002; Moutabarrik et al., 1994a; Xing et al., 2006);(Moutabarrik et al., 1994c).

Figure 4: Scanning electron micrograph of portion of glomerulus (Arakawa, 1971)

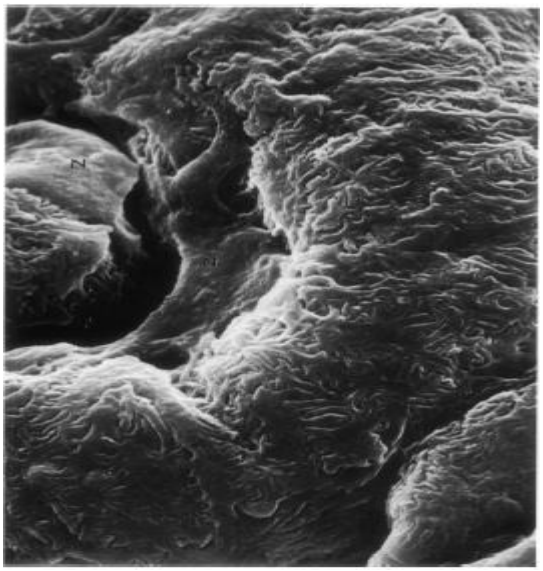


Figure 4: Scanning electron micrograph illustrating Glomeular capillaries covered by visceral epithelial cell with its cell body and foot processes embracing capillary wall of glomerulus.

1.1.6.1 Podocytes as GEnC supporting cells:

In glomerular capillaries, podocytes extend their foot process around the GEnCs, thus supporting through juxtacrine mechanisms (Vicki and David, 2005). Both the cells are shown to play a major role in the progression and regression of disease states such as glomerulosclerosis. Glomerular podocytes, despite having limited regenerative capacity, influence the growth, proliferation and regeneration of GEnCs (Liang et al., 2006). Podocytes abundantly produce and secrete VEGF A and angiopoietin-1 (ang-1) necessary survival factors required for GEnC (Hirschberg et al., 2008; Kitamoto et al., 1997). Hirschberg et al have shown that podocytes provide angiogenic support to GEnCs and regulate their maintenance and angiogenesis by secreting mediators (Hirschberg et al., 2008). Podocytes lack VEGFR2 and Tie-2 receptors, but in contrast, GEnCs express high levels of the receptors (Fan L, 2002; Satchell et al., 2002; Simon et al., 1998). The barrier properties of glomerular endothelium were also shown to be mediated by mediators, including VEGF, from podocytes (Sugimoto et al., 2003). The requirement of podocyte derived VEGF in maintenance and organization of GEnC was studied in homozygous and heterozygous podocyte specific knockout mice (Eremina et al., 2003).

1.1.6.2 New approaches to studying the integrated role of podocytes and GEnCs in glomerular inflammation

The co-ordinated role of glomerular cells such as GEnC, mesangial cells and podocytes, and their roles in different processes such as inflammation and angiogenesis have been a subject of discussion since many years. With the latest advancements in the technology of

culturing the cells in vitro, and with the availability of propagative lines of glomerular cells which retain their morphological features and immunological properties (Saleem et al., 2002; Satchell et al., 2006), *in vitro* co-culture systems can enable the glomerular microenvironment to be mimicked and enables the influence of different glomerular cells on each other to be studied. This encouraged us to study the intricate communication between podocytes and GEnCs and their role in neutrophil recruitment in response to cytokine stimulation during inflammation and the mechanisms involved.

1.1.6.3 Influence of podocytes on the ability of endothelium to support leukocyte recruitment

EnCs exist in close contact with organ specific stromal cells, such as pericytes, and the interactions with underlying stromal cells are known to modify responses of endothelial cells. During inflammation, stromal cells have a crucial role in regulation of neutrophil recruitment by EnCs as they exist in very close proximity (reviewed by (Ahmad et al., 2002; Hirschi and D'Amore, 1996; Nash et al., 2004)). Previous studies have shown that EnCs co-cultured with alveolar EpCs showed increased recruitment of mononuclear leukocytes upon cytokine stimulation. Others have found that co-culture alone caused upregulation of mRNA for adhesion receptors ICAM-1, E-selectin and VCAM-1 (Hsiai, 2008). Furthermore, endothelial co-culturing with smooth muscle cells and fibroblasts has shown a direct influence on leukocyte recruitment leading to EnC dysfunction. Recent studies examining the cross-talk between stromal fibroblasts and EpC have shown a defined site specificity (McGettrick, 2010). Similarly, it is possible that the podocytes in the kidney regulate inflammation and may play a role in the initiation and propagation of

glomerular damage, although most underlying cell biological mechanisms are not completely understood (Eremina et al., 2003; Patrakka and Tryggvason, 2009). It is clear that *in vitro* co-culture of EnCs with stromal cells is known to modify a range of endothelial responses to neutrophil recruitment, so the same may be true with GEnCs and podocytes.

Figure 5: Schematic representation of neutrophil recruitment in the glomerular vasculature

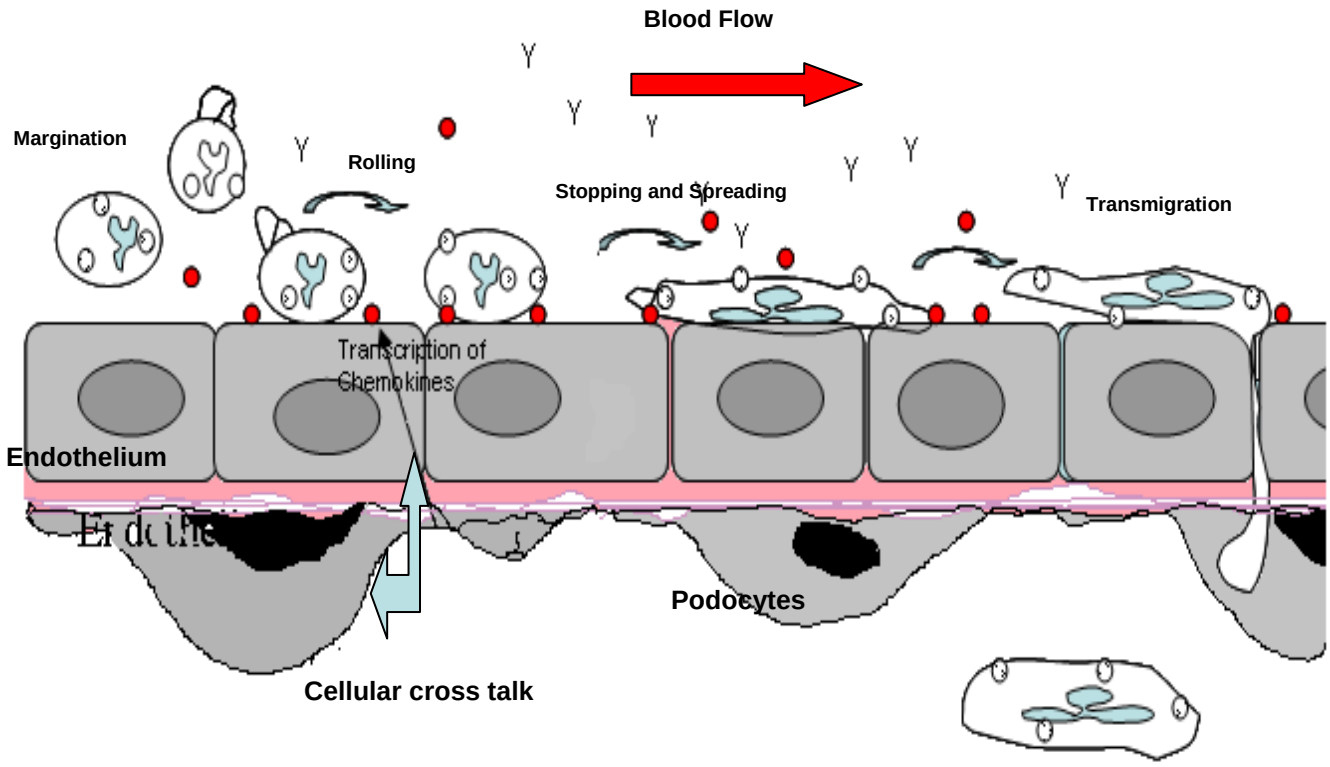


Figure 5: Neutrophil recruitment by the endothelium and their transmigration through the epithelial cells from blood into inflamed tissue.

1.1.6.4 Possible mediators released by podocytes and their pathophysiological role in inflammation.

Podocytes and GEnCs are involved in many pathological processes of the kidney and the damage to these cells is a common occurrence during disease processes in several forms of GN. It is also evident that podocytes contribute to the initiation and propagation of disease by direct stimulation and inhibition of endothelial functions (Xing et al., 2006). Endothelium has been shown to produce potential mediators of inflammation some of which have autocrine factors and some paracrine and these are thought to stimulate the endothelium and other cells. There is increasing evidence that these factors are present in various forms of GN, regulating endothelial activation *in vitro* and *in vivo*. However, resident neighboring podocytes are also capable of producing soluble factors, thereby potentially stimulating endothelium in a paracrine manner.

Several proinflammatory mediators play crucial roles in cell-cell interactions both *in vitro* and *in vivo* (Abe et al., 1990) and also mediate injury, for example, PAF augments glomerular injury by mediating inflammation and increasing vascular permeability (Camussi, 1986; Camussi et al., 1983; Camussi et al., 1984; Stewart et al., 1990; Zimmerman et al., 1990). Podocytes can respond to a variety of soluble mediators as they express cytokine and chemokine receptors which could alter the functions of neighboring cells during glomerular inflammation. Huber et al., (Huber et al., 2002) detected the expression of functional active chemokine receptors CCR4, CCR8, CCR9, CCR10, CXCR1, CXCR3, CXCR4, and CXCR5 in human differentiated podocytes . Podocytes were also shown to produce the proinflammatory cytokines TNF-α, IL-1 and IL-6 *in vitro* (Hughes et al., 2001; Neale et al., 1995; Niemir et al., 1997; Timoshanko et al., 2003). They also demonstrated the ability of podocytes to produce and respond to chemokines

such as IL-8, since they express CXCR1/CXCR2 receptors, thereby participating in an autocrine loop. Podocytes were shown to respond to exogenous chemokines by producing ROS *in vitro* (Huber et al., 2002). Pavenstadt's group demonstrated that ROS released exogenously from cultured differentiated mouse podocytes resulted in increased induction of GM-CSF mRNA as well as protein release (Greiber et al., 2002). GMCSF induced by ROS may activate mature leukocytes and play a role in prolonged inflammatory episodes. GMCSF also modulates cellular properties of GEnCs in a paracrine fashion. The release of ROS that accompanies the activation of CCRs and CXCRs may contribute to podocyte injury. Besides IL-8, undifferentiated rat podocytes in culture were demonstrated to release monocyte chemoattractant protein-1 (MCP-1), suggesting that they may actively participate in local inflammatory events during GN (Alon et al., 2003; Natori et al., 1997). Previous studies have demonstrated the active role of podocytes in inflammatory cell recruitment by up-regulating toll like receptor (TLR-4) expression (Rollins, 1997).

Another important mediator involved in inflammation and in pathogenesis of the disease is IL-6. IL-6 is reported to be released by neutrophils and EnCs and has been shown to play a significant role in neutrophil retention. Renal over expression of IL-6 has been described in experimental GN. Xing *et al.,* and Moutabarrik *et al.,* reported production of IL-6 in both unstimulated and TNF-α stimulated podocyte culture supernatants (Moutabarrik et al., 1994b; Xing et al., 2006). The expression of non-ubiquitous lL-6 receptor by cultured podocytes was also reported with inflammatory stimulation but the interaction of IL-6 with its receptor is not well documented. IL-6 may play a substantive role in the regulation of processes which appear critical to the initiation of progressive glomerular disease.

Podocytes release angiopoetin-1 which can bind to its receptor Tie2 on endothelium and thereby regulate some of the actions of VEGF in stabilizing the endothelium and resist

increased permeability, inflammation, and angiogenesis in the mature endothelium (Thurston et al., 1999a; Thurston et al., 1999b). Podocytes are also capable of producing TGF-β and fibroblast growth factor 2 in various experimental models of GN, with potential for deleterious effects on podocytes themselves and on other intrinsic glomerular cells (Floege et al., 1995; Lee and Song). However, the functional significance needs to be established. Even though the effects of the cytokines and/or chemokines are undescribed, soluble mediators of GEnC and podocytes clearly have the potential to play a part and respond to various soluble products during inflammation in the glomerulus either by autocrine or paracrine processes and play a major role in the pathophysiology of Vasculitic GN.

1.1.6.5 Choice of Glomerular epithelial cells

Culture of primary podocytes proved complex with regard to repetitive isolation and maintenance, so, conditionally immortalised podocytes were derived from primary podocytes by Saleem and his group in University of Bristol. The cell line was conditionally immortalised with a temperature sensitive mutant of immortalised SV40 T-antigen (Saleem et al., 2002). At the "permissive" temperature of 33^{o} C (with 5% CO_2) the SV40 T antigen is active, and allows the cells to proliferate rapidly. Thermo switching the cells to the "non-permissive" temperature of 37^{o} C (with 5% CO_2) silences the transgene and the cells become growth arrested and differentiated. Cells were grown for a period of 14 days to ensure growth arrest and differentiation.

1.1.7 Hypothesis

The hypothesis of this project is that the SP provoke the endothelium by initial activation and thereby alter neutrophil endothelial interactions leading to EnC damage and that the cross talking of podocytes with endothelium regulate its response in neutrophil recruitment to the inflammatory stimulus.

1.1.7.1 Aims of the study

The aim of the project is to address the following questions:

1. Whether the SP activates the endothelium and promotes neutrophil recruitment.
2. Determine whether SP mediate damage to the endothelium.
3. Determine whether intercellular cross-talk occurs between the glomerular EnCs and podocytes and whether it affects the ability of activated endothelium to recruit neutrophils or other functional responses of endothelium.
4. If cross-talk occurs, to determine whether it is cell-cell contact dependent or due to soluble mediators that are released.
5. To determine the ANCA activated neutrophil response on GEnC in the presence of podocytes.
6. To determine whether injury to podocytes disturb the symbiosis between the glomerular cells.

Chapter-2: Experimental procedures

2.1 Cell culturing

2.1.1 Propagation of ciGEnC

ciGEnC were grown in endothelial basal medium 2 – microvascular (EBM 2-MV, Cambrex, Wokingham, UK) containing foetal calf serum (5%) (Sigma Aldrich) along with growth factors supplied (Table 1.1). Cells proliferate at the permissive temperature of 33°C (with 5% CO_2) and enter growth arrest and fully differentiate when transferred to 37°C.

Confluent T75 cm^2 flasks of cells were selected by phase-contrast microscopy (AXIOVERT 40C), washed with 0.02% EDTA (Sigma Aldrich), trypsinized with 0.05% trypsin (Sigma Aldrich), centrifuged at 1500rpm for 5mins, and reseeded in fresh flasks at a dilution of 1 into 3. Cells grown to confluence were switched to 37^oC (with 5% CO_2) incubator and could be used for experimental purposes.

2.1.2 Culture of primary GEnC

Primary cultures of GEnC (Cell Systems, USA) were grown using EBM-2 medium (as above) in tissue culture flasks pre-coated with fibronectin and placed in 37^oC CO_2 (5%) incubator. They were grown to confluence and are reseeded into fresh flasks at a dilution of 1 into 3 or grown in 96 well plates (20,000 cells / well) for use in experiments. The medium was changed every 2-3 days.

2.1.3 Isolation of HUVEC

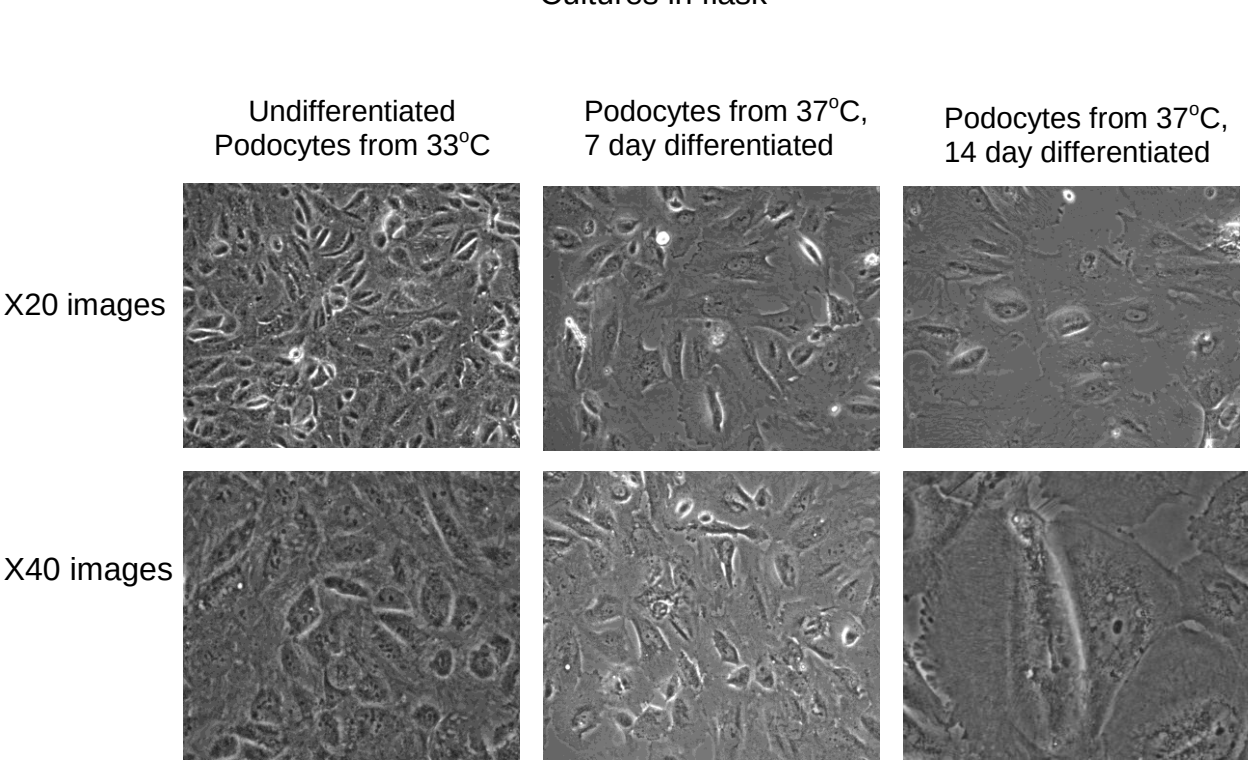
HUVECs were isolated from umbilical veins. Fresh human umbilical cords were collected with ethically approved permission from Birmingham Women's Hospital (Ethics code 5779). EnCs were isolated by collagenase 1mg/ml (Sigma Aldrich) digestion. Following isolation, HUVEC were seeded on 2% gelatin (Sigma Aldrich) coated T25 cm^2 tissue-culture flasks in medium 199 (Gibco) with supplements (shown in table 1.2). Confluent primary cultures were dispersed using 0.02% EDTA and 2X trypsin (Sigma Aldrich) and were seeded into 96-well flat-bottomed microtiter plates or T25 cm^2 flasks at a concentration of $2x10^4$cells/well and allowed to grow to confluence at 37^oC. Each experiment was conducted with HUVEC's from a different primary culture that was freshly isolated.

2.1.4 Propagation of podocyte cell line

Podocyte cell lines were grown to confluence at 33^oC (with 5% CO_2) in RPMI 1640 (Sigma Aldrich) with supplements (shown in table 1.3). Confluent T75 cm^2 flasks of cells were selected by phase-contrast microscopy (AXIOVERT 40C), washed with 0.02% EDTA (Sigma Aldrich), trypsinized with 0.05% trypsin (Sigma Aldrich), centrifuged at 1500rpm for 5mins, and reseeded in fresh flasks at a dilution of 1 into 4.

The confluent podocytes were left to differentiate at 37^o C (with 5% CO_2) for 7 or 14 days for use in experiments Figure 6 (Saleem et al., 2002).

Figure 6: Immortalised podocytes in culture undergoing differentiation



2.1.5 Propagation of Human Kidney Cell line -8 (HKC-8)

Cells were grown in DMEM/Ham's F-12 liquid with L-Glutamine (A & E Scientific (PAA)) and 5% FCS (Gibco). Cells were grown to confluence in T75 cm^2 flasks at 37°C (with 5% CO_2). Confluent cultures were dispersed using 0.02% EDTA/0.05% trypsin (Sigma Aldrich) and centrifuged at 1500rpm for 5mins, and reseeded in fresh flasks at a dilution of 1 into 4 or seeded on the outer surface of transwell inserts at a seeding density of 60,000 cells / insert for co-culturing with GEnC.

2.1.6 Propagation of epithelial-like liver cell line (AKN-1 cells)

AKN-1 cells are non-malignant biliary epithelial cells, and were grown in DMEM 1% GPS and 10% FCS (Gibco). Cells were grown to confluence in T75 cm^2 flasks at 37^oC (with 5% CO_2). Confluent cultures were dispersed using 0.02% EDTA/0.05% trypsin (Sigma Aldrich) and centrifuged at 1500rpm for 5mins, and reseeded in fresh flasks at a dilution of 1 into 4 or seeded on the outer surface of transwell inserts at a seeding density of 60,000 cells / insert for co-culturing with GEnC.

2.1.7 Propagation of human retinal pigment epithelial cell line (ARPE-19 Cells)

Cells were grown in DMEM/Ham's F-12 liquid with L-Glutamine (A & E Scientific (PAA)) and 5% FCS (Gibco). Cells were grown to confluence in T75 cm^2 flasks at 37^oC (with 5% CO_2). Confluent cultures were dispersed using 0.02% EDTA/0.05% trypsin (Sigma Aldrich) and centrifuged at 1500rpm for 5mins, and reseeded in fresh flasks at a dilution of 1 into 4 or seeded on the outer surface of transwell inserts at a seeding density of 60,000 cells / insert for co-culturing with GEnC.

2.1.8 Propagation of Hep-2 cells

Cells were grown in DMEM/Ham's F-12 liquid with L-Glutamine (A & E Scientific (PAA)) and 5% FCS (Gibco). Cells were grown to confluence in T75 cm^2 flasks at 37^oC (with 5% CO_2). Confluent cultures were dispersed using 0.02% EDTA/0.05% trypsin (Sigma Aldrich) and centrifuged at 1500rpm for 5mins, and reseeded in fresh flasks at a dilution of 1 into 4 or seeded on the outer surface of transwell inserts at a seeding density of 60,000 cells / insert for co-culturing with GEnC.

Medium Recipes:

Table 1.1 – *Constituents of Endothelial Basal medium(EBM-2) kit supplied by Lonza.*

Medium and Additives	Volume	Source
Endothelial growth medium	500ml	Lonza, Wokingham, UK
hFGF-B - human Fibroblast Growth Factor-B	2ml	Lonza, Wokingham, UK
GA-1000 - (Gentamicin Sulfate, Amphotericin-B)	500µl	Lonza, Wokingham, UK
Foetal Bovine serum (FBS)	25 ml	Lonza, Wokingham, UK
R^3 – IGF – 1 Recombinant-long R-Insulin like growth factor-1	500µl	Lonza, Wokingham, UK
Hydrocortisone	200µl	Lonza, Wokingham, UK
VEGF – Vascular Endothelial Growth Factor	500µl	Lonza, Wokingham, UK
Human Endothelial growth factor	500µl	Lonza, Wokingham, UK

Table 1.2 – *Constituents of Medium199*

Medium and Additives	Volume	Final Concentration	Source
Medium with Phenol red	100ml	-	Gibco
Hydrocortisone	10µl	1 µg/ml	Sigma Aldrich, UK
Penicillin and Streptomycin	1ml	1 mM	Sigma Aldrich, UK
Endothelial growth factor	10µl	1 ng/ml	Sigma Aldrich, UK
L-Glutamine	1 ml	20mM	Sigma Aldrich, UK
Amphotericin	1 ml	2.5 µg/ml	Sigma Aldrich, UK
FBS	20ml	20%	Sigma Aldrich, UK

Table 1.3 – *Constituents of* Roswell Park Memorial Institute (RPMI) *medium*

Medium and Additives	Volume	Final Concentration	Source
RPMI-1640 Medium	100ml	-	Sigma Aldrich, UK
FBS	10ml	10%	Sigma Aldrich, UK
Penicillin and Streptomycin	1ml	1 mM	Sigma Aldrich, UK
ITS (10ug/ml Insulin, 5.5ug/ml Transferrin, 5ng/ml Sodium selenite)	1ml		Sigma Aldrich, UK
L-Glutamine	1 ml	20mM	Sigma Aldrich, UK

2.2 Viability assays

2.2.1 Cell viability measured by a tetrazolium (MTT) assay

Confluent monolayers of HUVEC, ciGEnC, podocytes and primary GEnC were treated for 2 hours with different concentrations (0.5-15µg/ml as indicated) of PR3 (Athens Laboratories) or HNE (Calbiochem) or treated with different concentrations of PAN and incubated at 37^{o}C. Cell viability was assessed using the tetrazolium assay in either 24 well or 96 well plates. Briefly, after treatments, wells were washed and incubated with MTT (3-(4,5-Dimethylthiazol-2,5-diphenyltetrazolium bromide, 5mg/ml, Sigma Aldrich) in a 37^{o}C shaking incubator for an hour. Mitochondrial dehydrogenases converted soluble yellow MTT into blue insoluble formazan. After 1 hour, the MTT was aspirated from the wells and the plate was dried on clean tissue to remove excess liquid. Formazan crystals were then solubilised with neat DMSO (Dimethyl Sulphoxide) (Sigma Aldrich) treated for 30 min at 37^{o}C and the absorbance was read at 540nm.

2.2.2 Endothelial cell detachment measured by crystal violet assay

After incubation of confluent layers of EnC's with PR3 and HNE for 2 hours at 37^{o}C, cells were fixed with 1% formaldehyde (Sigma Aldrich) for 15 minutes and stained with 0.1% crystal violet for 10 minutes to assess cell detachment. The cells were lysed with 1% triton X-100 (Sigma Aldrich) in dH_2O and the absorbance of cell-associated crystal violet was then measured at 550nm.

2.2.3 Endothelial cell membrane integrity was assessed by Propidium iodide (PI)

Confluent layers of EnCs were treated and incubated with 0.5 to 2 µg/ml PR3 (Athens Laboratories) or HNE (Calbiochem) for 2 hours and 1mM H2O2 (Sigma) used as positive control for 1 hour. Following treatments, cells were incubated with 20 µg/ml PI stain (Sigma Aldrich) for 20 minutes. PI staining locates the cells which have lost their membrane integrity. Cells were photographed under fluorescence microscopy on an Olympus inverted microscope. Cells were counted by using Image pro-analysis software (Media Cybernetics UK).

2.2.4 Apoptosis Detection Assays

2.2.4.1 Measurement of Apoptotic Cells by Ultraviolet (UV) Light Microscopy

ciGEnC or HUVEC were seeded at $4x10^4$ /well in 8 well chamber slides and grown to confluence. Wells were washed thrice with warm medium 199 (no phenol red) (GIBCO) containing 0.1% BSA (Sigma Aldrich). Cells were treated with PR3 (Athens Research & Technology Inc., USA) and HNE (Calbiochem, UK) in a dose and time dependent manner and incubated at 37^oC. After treatment, cells were fixed in 100% ethanol (Sigma Aldrich), air dried, and stained with 1µg/ml DNA-specific dye, 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) and incubated for 2mins. DAPI was washed off with PBS with Ca^{+2} (Gibco). Cellular morphology was assessed using a Nikon FXA research light microscope. Apoptotic cells were identified by chromatin condensation and fragmentation.

2.2.4.2 EnC apoptosis detection by flow Cytometry using Annexin V FITC and PI binding

HUVEC or ciGEnC were grown to confluence in T25 flasks. The cells were treated for 2 hours with 1ug/ml of PR3 or HNE at 37^{o}C. A 24 hours treatment with cycloheximide (CHX; 3µg/ml) (Sigma Aldrich) + TNFα (10ng/ml) (NIBSC) was used as a positive control. After incubation, the cells were harvested by gentle trypsinisation and centrifuged for 10min at 1500rpm. Cells were washed with PBS containing calcium and centrifuged for 5min at 1500rpm. The specific binding of annexin V- fluorescein isothiocyanate (FITC) and incorporation of PI (Invitrogen, UK) was performed by incubating the cells for 15 min at room temperature in binding buffer. After incubation, the cells were immediately analyzed using a FACS analyzer (Becton Dickinson, San Jones, CA, USA). Annexin V+/PI+ cells were defined as necrotic (or late apoptotic), while annexin V+/PI- cells were defined as apoptotic. The data obtained is analysed using the Cell Quest software.

2.2.4.3 EnC apoptosis detection by caspase-3 activation using western blotting

Confluent HUVEC or ciGEnC in T25 flasks were treated for 2 hours with 1ug/ml of PR3 or HNE and incubated at 37^{o}C. Cells treated with cycloheximide (CHX) (3ug/ml) +TNF (10ng/ml) for 24 hours were used as positive controls. After incubation the cells were harvested by 0.02% EDTA (Sigma Aldrich) treatment, washed in 1X PBS (Sigma Aldrich) and lysed with 250ul of lysis buffer (recipe table 1.3) PBS with 10ul of Protease inhibitor cocktail (Calbiochem) for 15min on ice. Then the cell extracts were centrifuged for 15min at 1500rpm and the supernatants were collected. The cell lysates were reduced by adding 55 µl of 6X sample buffer which contain 10% SDS and 5% β-mercaptoethanol to 250 µl of

sample and 20 µl loaded onto a 15% Tris-glycine gel (Severn Biotech limited, UK) (Gel receipe, table 1.4).

The proteins were resolved by SDS-PAGE. The proteins were then transferred onto PVDF membrane (Hybond ECL, Amersham Biosciences) using a semi-dry transfer unit at 34 mA, shaking for 1h at 4 ^{o}C. The membrane was then blocked for 3-4 hours at room temperature in Tris Buffered Saline with 0.1% Tween-20, Sigma Aldrich (TBS-T) (24.2g of Tris base and 80g Sodium Chloride in 1L water) containing 5% (W/V) dried milk. This was followed by overnight incubation with polyclonal rabbit anticaspase-3 unconjugated primary antibody (Invitrogen) diluted at 1: 1000 in 5% dried milk, at 4^{o}C. The membrane was washed thrice for 30min each time with TBS-T and incubated with horseradish peroxidise-conjugated goat anti-rabbit (Biosource) at 1:1000, for an hour at room temperature. The membrane was washed with TBS-T as before and the proteins were visualised using chemiluminescence using ECL plus substrate (GE) for 5min.

Table 1.4 – Constituents of Cell lysis buffer

Additives	Final Concentration	Source
Tris HCL pH 7.4	50mM	Sigma Aldrich
Nonidet-P40 (NP-40)	1 %	Sigma Aldrich, UK
Sodium deoxycholate	0.25%	Sigma Aldrich, UK
Sodium Chloride	150mM	Sigma Aldrich, UK
EGTA	1mM	Sigma Aldrich, UK
PMSF	1mM	Sigma Aldrich, UK
Sodium Fluoride	1mM	Sigma Aldrich, UK
Protease inhibitor cocktail	2%	Calbiochem, UK

Table 1.5 – Western Blotting Gel Recipe

Resolving gel recipe	15%	Stacking gel recipe	5 %
Acrylamide	3.75 ml	Acrylamide	1 ml
Bis- Acrylamide	400 µl	Bis- Acrylamide	520 µl
1.5M Tris pH 8.8	3.73 ml	Tris pH 8.8	1 ml
10% SDS	100 µl	10% SDS	80 µl
10% APS	67 µl	10% APS	80 µl
TEMED	5 µl	TEMED	8 µl
Water	1.998ml	Water	5.312 ml

2.3 EnC treatments

Confluent EnC were treated in serum-free medium 199 (Invitrogen) with 0.05% BSA (Sigma Aldrich) with 1µg/ml of either PR3 (Athens Research & Technology Inc., USA, stock 100 µg/ml) or HNE (Calbiochem, UK, stock 100 µg/ml) for 2 hours or 24 hours or with 1 units/ml (0.01ng/ml) or 10 units/ml (0.025ng/ml) or 100units/ml (2.5ng/ml) TNF-α (NIBSC) for 4 hours. The enzyme activity of PR3 and HNE was tested against 100µl of the synthetic substrate (2mM) N-methoxy-succinyl-Ala-Ala-Pro-Val-4 nitroanilide (Sigma Aldrich) and incubated at room temperature protecting from light and the absorbance was read from 5 minutes to 4 hours at 405nm.

In some experiments, serine protease inhibitor, Alpha 1 anti trypsin (prolastin, a gift from Telecris Biotherapeutics, USA, Stock 100mg reconstituted in 40ml sterile water) was added to the cultures along with the proteases diluted in M199 with 0.05% BSA to a working concentration of 140nM.

2.4 Neutrophil Isolation from whole blood using percoll density gradient method

Blood from consented healthy volunteers, was collected into tubes with anticoagulant, 10% acid citrate dextrose (ACD), containing disodium hydrogen citrate (Sigma Aldrich) and D-glucose. Neutrophils were isolated using a two step density gradient. Whole blood was layered over percoll gradients (GE Health Care, UK) and centrifuged at 1500g for 30 minutes. The middle neutrophil fraction was harvested and washed twice in phosphate buffered saline (PBS) with calcium and magnesium (Gibco). The neutrophils were then re-suspended in 1 ml of PBS/BSA, count and cellular viability was assessed by trypan blue

exclusion using a hemocytometer and then re-suspended to a concentration of 1 x 10^6 cells per ml in PBS/BSA. The preparations contained >98% neutrophils.

2.5 Isolation of ANCA IgG and normal control IgG

ANCA-positive IgG (WG or MPA active patient's IgG) was isolated from plasma exchange fluid (PEX) collected from patients and normal control IgG (ANCA-negative IgG) was obtained from the serum of consented healthy volunteers. 50 ml aliquots of PEX samples were added with 1ml calcium chloride (1 molar) to remove clotting factors such as fibrin, heated in a water bath and then the precipitant was removed. The patient's serum and normal serum was diluted with PBS in equal volumes and filtered separately using 0.2μm and passed through a 5ml volume HiTrap Protein G column (Amersham Biosciences). IgG trapped in the column was eluted using 0.1 molar glycine, pH 2.6 and neutralised with 1 ml of 1 molar Tris base (Sigma Aldrich), pH 9.0. The samples were placed into pre-boiled dialysis tubing (10-14kDa cut off) and dialysed in PBS overnight at 4ºC. The samples were collected into vivaspin concentrators (Vivascience, Hannover, Germany) and spun to concentrate to approximately 1mL volume with a 10,000 Da cut off, and stored at 4˚C for up to one month.

The concentration of the eluted IgG was determined by spectroscopy. ANCA IgG samples were diluted 100X and the absorbance was measured at 280nm using PBS buffer as a blank in quartz cuvette. 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. Therefore, the resulting equation is:

Concentration (mg/ml) = A x dilution factor

= 1.43 x 1

2.5.1 Endotoxin removal from immunoglobulin preparations

Endotoxin removing gel (Detoxi – gel from Pieree) was used to remove endotoxin from ANCA IgG and normal IgG preparations. The gel beads were washed in PBS with 1% sodium deoxycholate (Sigma Aldrich) in an eppendorf and spun at 13000RPM for 30 seconds. The supernatant was discarded and the washing was repeated thrice. The IgG preparations were pipetted in 50% suspension with beads and left in rotation overnight at 4^{o}C. The samples were spun at 13000 RPM for 5 minutes and supernatants were collected. The beads were washed in PBS with 1% sodium deoxycholate as before and were stored in 25% ethanol at 4^{o}C for future use.

2.6 Adhesion assays

2.6.1 EnC static neutrophil recruitment assay

HUVEC and GEnC (primary and secondary) seeded at $2x10^{4}$/well in 96 well plates were grown to confluence. Wells were washed with medium 199 (GIBCO) with 0.15% BSA prior to treatment for 2 hrs with 0 & 1 µg/ml PR3 or HNE and for 4 hrs with TNFα 100 units/ml (NIBSC, a positive control). Neutrophils, that were isolated using the Percolll gradient method (as above), were added at concentration of $2.5x10^{5}$ cells/well and incubated at 37°C for 1 hour. A standard curve plate was prepared in parallel to the experiment with $2.5x10^{5}$ neutrophils and then serial diluted. At the end of the incubation period, non-adhered neutrophils in supernatants were very gently sucked off and EnC monolayer was

washed thrice with warm PBS. The cells were lysed with 0.1% triton X-100 and 100ul of MPO substrate (SIGMA ALDRICH*FAST*™ OPD) was added and absorbance read at 450nm after 1hr on a Thermo© Multiskan Ascent plate reader. The number of adherent neutrophils was determined by comparing the absorbance with a neutrophil standard curve (0-2.5x10^5 cells).

2.6.2 Adhesion assay on TNF-α activated HUVEC under flow model

2.2.6.2.1 Microslide preparation and testing using platelets

Glass capillaries or microslides (rectangular cross-section, 0.3x3mm; length, 50 mm, Camlab, Cambridge, UK) were cleaned with nitric acid, washed with water followed by acetone and coated with 4% 3-aminopropyltriethoxysilane (APS). The microslides were allowed to dry and autoclaved. To check the effectiveness of APES coating, platelets were layered into the microslide. For the experimens the slides were coated again with 1% gelatin (type B from bovine skin, Sigma Aldrich) or 2µg/cm^2 fibronectin (from human plasma, Sigma Aldrich).

2.6.2.2 Transfer of EnCs into microslides

The confluent cultures of HUVEC or primary GEnC grown in T25cm^2 were harvested with trypsin and EDTA 0.2%. The cells were spun at 1500rpm for 5 minutes and the pellet was re-suspended in 0.4mls of medium. Endothelial cells were seeded into gelatinized microslides at a concentration of 1.5x10^5 cells per slide and incubated for 2-3 hours at

37ºC allowing adherence and spreading of the endothelial cells. The slides were then placed in a modified glass petri dish filled with medium and the slides were connected via rubber tubing to a pump which exchange medium at a speed of 15rpm through the slides every hour. The endothelium was cultured in this system for 24 hours or until the experimental treatments allowing maintenance of a healthy monolayer.

2.6.2.3 The flow based adhesion assay

The microslides with healthy confluent EnC monolayers were individually treated with different treatments, 4h TNF-α (2, 10, 100 Units) treatment on HUVEC or 2h PR3/HNE (1 µg/ml) treatment on primary GEnCs and incubated at 37 ºC. After 4h incubation, flow adhesion experiments were performed in an enclosed Perspex chamber at 37ºC (Figure7). Each individual microslide was glued onto a glass microscope slide mounted on a phase-contrast microscope. One end of the microscope slide was attached via silicon rubber tubing to a syringe pump (set to 0.1Pa wall shear) allowing control of the rate of flow across the microslide surface. 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 (PBS with calcium and magnesium/0.1% BSA) and a reservoir containing neutrophil suspension isolated by percoll method ($1x10^6$/ml diluted in PBS with 0.1% BSA). Flow was switched between wash buffer and neutrophil suspension and was perfused for 4 minutes, followed by a washout of 1 minute. The number of neutrophils rolled over the endothelial monolayer, adhered or transmigrated were viewed and recorded both in real time and on video. Video microscope recordings were then made in 4-5 fields, 5 minutes after the initial perfusion of neutrophils to look at

rolling neutrophils and at 10minutes for transmigration along the centre of the microslide in the direction of flow. Video recordings were analysed using Image Pro-Plus software (MediaCybernetics).

Figure 7: Flow Model

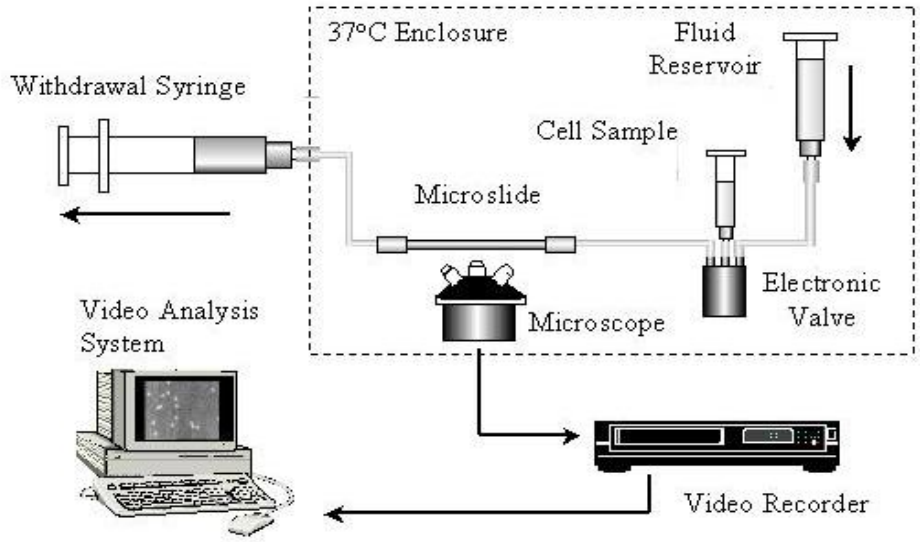


Figure 7: The flow model. All flow experiments were performed in a Perspex box at 37ºC. The microslide, mounted on a glass microscope slide was attached to a pump and to the sample and wash buffers. Events in the slide were viewed through the microscope, recorded on video and analyzed on the computer. (Image copied with permission from Dr Tanya Pankhurst)

2.7 Immunostaining of podocytes for differentiation marker, Podocin

The immunolabeling was done to identify the differentiation markers of Podocytes. Podocytes were grown in chamber slides and allowed to differentiate at 37^{o} C (with 5% CO_2) for 7 or 14 days. Briefly, slides were fixed with ice cold methanol for 10 min and then air dried at room temperature for 30 min. Nonspecific binding was blocked with 4% FBS with 0.1% Tween 20 (Sigma Aldrich) in PBS for 30 min. 1µg/ml of polyclonal rabbit anti

NPHS2 primary antibody (Abcam) was added onto the slide and incubated overnight at 4^{o} C. The slides were washed thrice with PBS with 2% FBS and 1% BSA and secondary antibody (Anti- Rabbit FITC, DAKO) was applied at the appropriate dilutions (1:10 to 1:40) and incubated for 1 hour protected from light. The slides were washed three times with PBS with gentle agitation. Cover slips were mounted on glass slides with slowfade (invitrogen). The pictures were taken using fluorescent imaging.

2.8 Co-culturing of podocyte cell line and EnCs

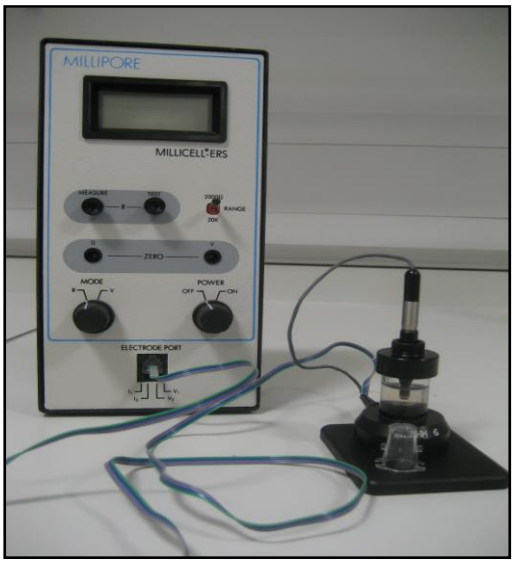
2.8.1 Seeding podocytes and EnCs on co-culture inserts

The confluent flask of podocytes was washed with 0.02% EDTA (Sigma Aldrich), trypsinised (Sigma Aldrich), centrifuged at 1500rpm for 5mins. The cells were counted using a CASY® Technology Cell Counter. The cells were resuspended at a seeding density of 6x10^{4} cells/ insert and seeded onto the external surface of the 3um porous polyethylene terepthalate transwell filter inserts (BD Pharmingen, UK) and allowed to settle for 2 hours adding medium after 1 hour. The inserts were placed in 24 well companion plates at 37^{o}C and the podocytes were left to differentiate for 7 or 14 days. Similarly, EnCs (6X10^{4} cells/ insert) were seeded on the inner surface of inserts in complete RPMI medium and left to settle overnight at 37^{o} C (adapted from (McGettrick et al., ; Rainger and Nash, 2001; Rainger et al., 2001)).

2.8.2 Electrical resistance (ER) measurement on inserts across cell cultures

The ER across the monolayers of HUVEC and/or podocyte cell line cultured on filters was measured using the Millicell-ERS resistance meter according to the manufacturer's instructions (Millipore, USA).

Figure 8: The millicell-ERS resistance meter



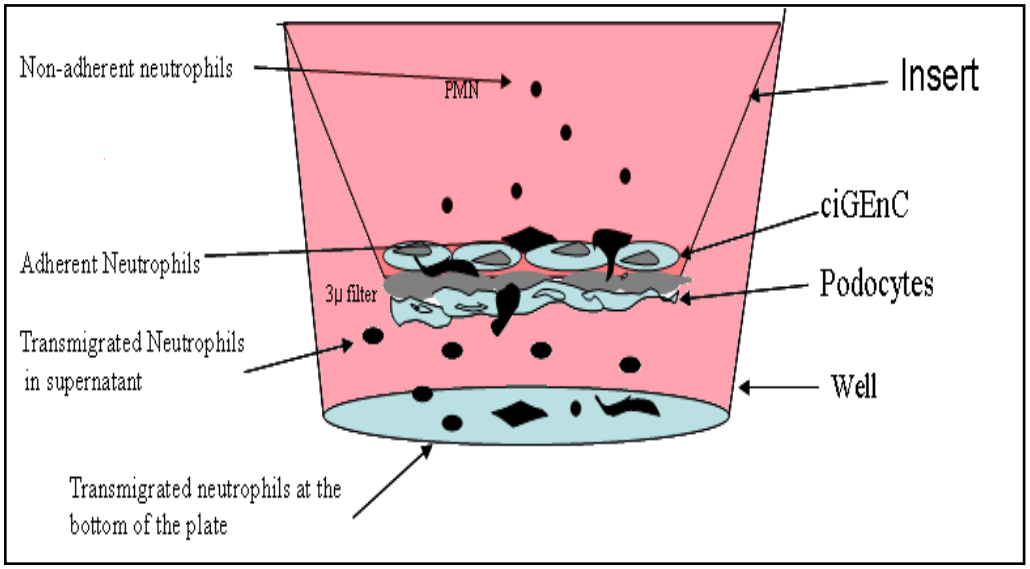
Initially the electrodes were immersed in fresh RPMI for 15 min followed by manually adjusting the resistance setting to zero. The filters were placed in a special electrode chamber, containing 700ul of culture medium, with centrally located electrodes to ensure reproducibility. The ER across the monolayer was measured and recorded. The electrodes were washed in fresh culture medium between resistance measurements. The resistance meter was stored according to manufacturer's instructions. Cell monolayer resistance was

obtained by subtracting the resistance of a blank filter from the resistance of the sample filter. This was obtained by subtracting the area of the filter, to calculate resistance in ohms cm2.

2.8.3 Transwell assay with endothelial and epithelial co-culture to assess neutrophil recruitment

Podocytes were seeded onto the 3um porous transwell filter inserts (BD Pharmingen, UK) as described in section 2.7. EnCs were seeded on the inner surface of inserts in RPMI complete medium 24hrs prior to an experiment and left to settle overnight at 37^{o} C. For comparison, parallel cultures of EnCs alone and podocytes alone were seeded inside inserts as controls in RPMI complete medium. This assay is illustrated in figure 9 below.

Figure 9: Illustration of co-culture



The viable confluent layers of cells on the inserts were stimulated with TNF-α (NIBSC) diluted in RPMI medium in a dose dependent manner (0 -100Units/ml) and added to both upper and lower chambers for 4 hours and incubated at 37^{o}C. Following incubation, supernatants were collected from the upper insert and from the lower wells and stored at 80^{o}C for multiplex bead assay using LUMINEX (Invitrogen, UK).

After stimulation with cytokines, the inserts were washed with fresh medium and were transferred into a fresh plate. Fresh medium (RPMI+0.1% Bovine serum albumin (BSA)) was added in the upper and lower chambers. The neutrophils isolated by percoll density gradient method (as described earlier) were added into the upper insert (2X10^{5} cells/well in RPMI+BSA). Neutrophils were added either directly without priming or activating by TNF-α (2 and 10 units/ml) and IgG [normal or ANCA (200ug/ml)] before perfusion. Neutrophils were allowed to settle, adhere and migrate at 37^{o} C for 1 hour. The experiment was stopped by transferring the filter into a fresh well. The neutrophils in the upper chamber were collected and the filter was washed twice. The cells from the washes were pooled with the upper sample and were taken to represent non-adherent neutrophils. The neutrophils in the lower chamber were collected and washed twice with fresh medium. The washes were pooled with the sample from the lower chamber; these pooled samples contained transmigrated neutrophils. The neutrophil counts from the samples collected (counted using Casy counter) represent the number of non-adherent and the transmigrated cells. From this data, the percentages of non-adherent, adherent and migrated neutrophils were determined.

The results were represented as total recruited and transmigrated neutrophils. Total recruited neutrophils represent those that were adherent and those subsequently

transmigrated to the bottom chamber (% recruited = adherent + total transmigrated/total added neutrophil* 100) and the % transmigrated represent the total transmigrated neutrophils of total recruited neutrophils (% transmigrated = total transmigrated / total recruited * 100).

The wells were examined microscopically to ensure that all cells are removed and washed further if necessary. The inserts were fixed in order to analyze the location of neutrophils on the insert by using 2% isotonic glutaraldehyde (Sigma Aldrich) containing 1ug/ml bisbenzimide (Sigma Aldrich) for 15 minutes in the dark followed by 4 washes in PBS to get rid of excess Fluorochrome from the pores. They were then cut out directly onto microscope slides, and cover slips were mounted using the mountant, Vectashield H1000 (in the dark).

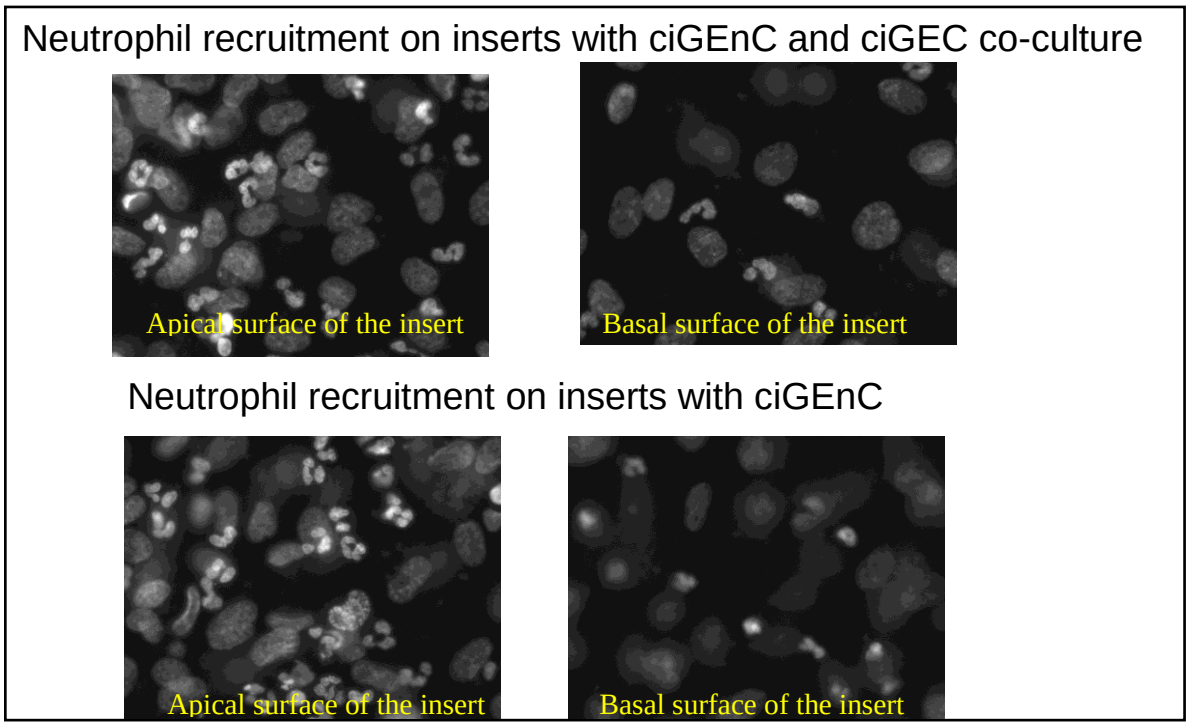
Observations were made using UV fluorescent microscopy and images were recorded for analysis using Image pro-plus software package (Data Cell Ltd, UK). The total number of neutrophils were counted and converted to cells / mm2 using the known field dimensions, multiplied by the area of the filter, divided by the total number of neutrophils added, and then multiplied by 100 to express percentage neutrophil recruitment of total added.

$$\text{Number of Pictures in well area (X)} = \frac{\text{area of filter or insert}}{\text{Area counted}}$$

$$\text{Neutrophil count/mm}^2 = \frac{\text{average counts}}{X}$$

Area of a 24 well is 1.767cm^2 (given by BD falcon), area of insert filter is 1.3cm^2 (given by BD falcon).

Figure 10: Video images of cells on apical and basal surfaces of the filter



2.9 Neutrophil recruitment assay to HUVEC and GEnC following conditioning with podocyte medium

HUVEC and GEnC (primary and secondary) seeded at $6x10^4$/well in 24 well plates were grown to confluence in EBM-2 (Lonza, UK). Cells were treated with conditioned medium from 14 day differentiated podocytes and incubated at 37^o C for 24 hours. Endothelial monolayers were stimulated with 0–100 Units/ml of TNF-α (NIBSC) in podocyte conditioned medium for 4 hours, at 37^o C. Neutrophils were isolated using the percoll gradient method (as previously described) and resuspended in RPMI+0.1% BSA, added to the wells at concentration of approximately $2x10^5$cells/well and incubated for 1 hour. After

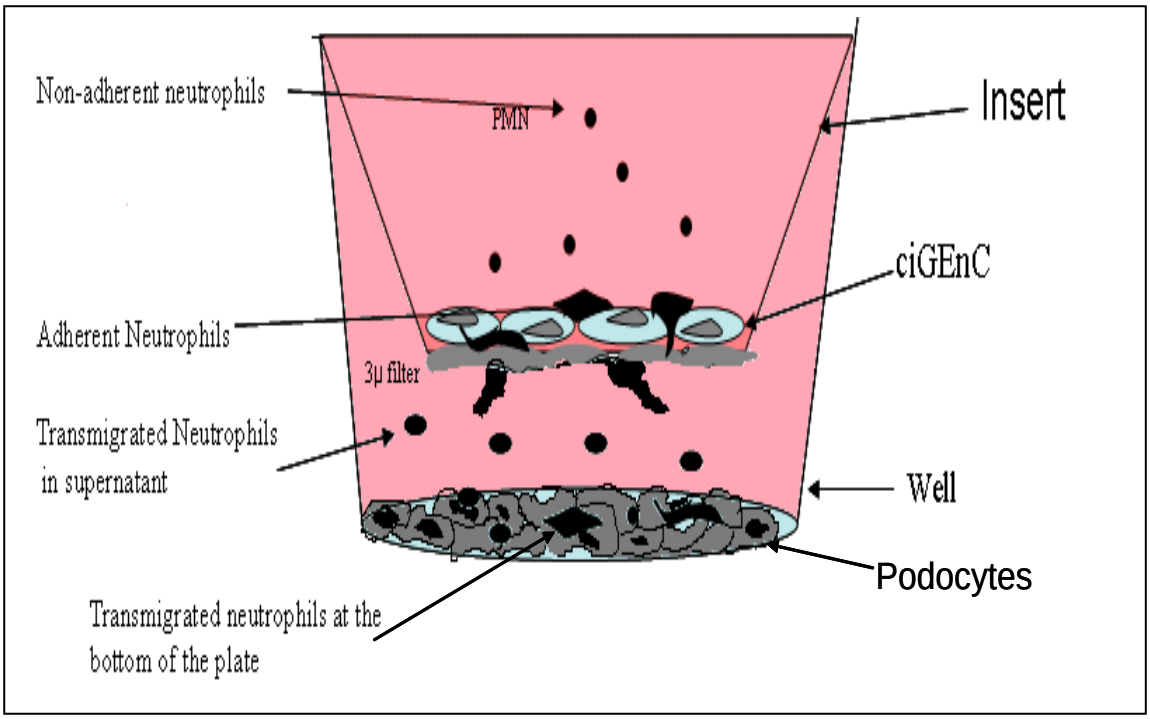
incubation, wells were washed with RPMI containing BSA thrice and non-adhered neutrophils were washed off. Cells were stained with 1ug/ml bisbenzimide (Sigma Aldrich) for 15 minutes in the dark followed by 4 washes in PBS to get rid of excess fluoro-chrome from the pores (in the dark).

Observations were made using UV fluorescent microscopy and images were recorded for analysis using Image pro-plus software package (Data Cell Ltd, UK). The total number of neutrophils were counted and converted to cells/mm^2 using the known field dimensions, multiplied by the area of the filter, divided by the total number of neutrophils added, and then multiplied by 100 to express percentage neutrophil recruitment of total added.

2.10 Transwell assay with endothelial cells in the presence, but not in contact with epithelial cells to assess neutrophil migration by soluble mediators

Podocytes were seeded onto the 24 well insert companion plates (BD Pharmingen, UK) at a seeding density of 60000/well and left to differentiate for 7 or 14 days in a 37^o C incubator (with 5% CO_2) (Figure 11). EnCs were seeded on the inner surface of inserts in RPMI complete medium 24 hours prior to use in an experiment and left to settle overnight at 37^o C (as described in section 2.2.8.1). For comparison, parallel cultures of EnCs alone as well as Podocytes alone were seeded inside inserts as controls in RPMI complete medium. The confluent layers of cells in the inserts were stimulated with TNF-α (NIBSC) in a dose dependent manner (0 -100Units/ml) for 4 hours in RPMI medium. The rest of the procedure was identical to Section 2.2.8.3.

Figure 11: Structure of co-culture with EnCs and podocytes with no direct contact



2.11 Neutrophil recruitment assay following treatment with IL-6 on GEnC mono-cultures

GEnC were seeded at 6 x 10^4 cells / well in 24 well plate insert. Cells were treated with 10ng/ml of IL-6 incubated at 37^o C for 24 hours. The concentration was chosen as this was the approximate amount of IL-6 that was detected in co-cultures by LUMINEX. Endothelial monolayers were stimulated with 0–100 Units/ml of TNF-α (NIBSC) in RPMI medium for 4 hours, at 37^o C. Neutrophils were isolated using the percolll gradient method (as previously described) and resuspended in RPMI+0.1% BSA, added to the wells at concentration of

approximately $2x10^5$cells/well and incubated for 1h. After incubation, wells were washed with RPMI containing BSA thrice and non-adhered neutrophils were washed off. Cells were stained with 1ug/ml bisbenzimide (Sigma Aldrich) for 15 minutes in the dark followed by 4 washes in PBS to get rid of excess fluoro-chrome from the pores (in the dark).

Observations were made using UV fluorescent microscopy and images were recorded for analysis using Image pro-plus software package (Data Cell Ltd, UK). The total number of neutrophils were counted and converted to cells / mm^2 using the known field dimensions, multiplied by the area of the filter, divided by the total number of neutrophils added, and then multiplied by 100 to express percentage neutrophil recruitment of total added.

2.12 Multiplex cytokine immunoassay and ELISA to detect soluble mediators

Invitrogen's multiplex bead immunoassay was used to detect cytokines and chemokines in the supernatants collected from co-cultures. Cytokines measured included IL-1β, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 , IL-13, IL-15, IL-17, interferon-γ (IFN-γ), IFN- α, TNF-α, GM-CSF, MCP-1, CCL3 (MIP-1α), CCL4 (MIP-1β), GM-CSF, CCL11 (eotaxin), CCL5 [regulated upon activation, normal T cell expressed and secreted (RANTES)], TNFRI and TNF-RII. A Quantikine ELISA kit for ENA-78 (R&D systems) was used as per manufactures instructions.

2.13 Blocking Studies

2.13.1 CXC – chemokine receptor blocking in co-cultures and mono-cultures

To investigate the role of neutrophil chemokines receptors CXCR1 and CXCR2 in neutrophil recruitment and migration, the receptors were blocked. Neutrophils were treated with function-blocking antibodies against CXCR1 and CXCR2 (IgG1, Biosource, Belgium, clones 501 and 19, respectively) and the antibodies used at 4 ug/ml (1ul of stock 0.1g/0.1ml) in 100ul of neutrophils (up to $3x10^6$ cells/ml) and incubated for 30 min prior to addition to the cultures. 4 ug/ml antibodies against CXCR1 & 2 were used after optimising the concentration, as preliminary studies with 2 ug/ml (concentration adapted from (Calderwood et al., 2005)) did not work in co-cultures. The function neutralizing antibodies against CXC chemokines were incorporated into the co-culture system to see the effect of anti CXCR1 & 2 antibodies on neutrophil recruitment to EnCs co-cultured with podocytes.

2.13.2 Neutralization of IL-6 activity in culture supernatants

IL-6 was measured in the supernatants from GEnC mono-cultures and GEnC Podocyte co-cultures using LUMINEX assay (Invitrogen, UK). To neutralize IL-6 activity, monoclonal anti IL-6 antibody (5ug/ml, R&D systems, clone 6708) was added to the medium with the establishment of co-cultures and maintained throughout the experiment. The concentration was chosen after optimising it with different concentrations ranging from 1-10µg/ml. In some experiments, antibody was removed with the addition of neutrophils, while other experiments were carried with the presence of neutralising IL-6 antibody along with

neutrophils as well. Static adhesion assay is carried as explained earlier with co and mono-cultures of ciGEnCs.

2.13.3 Blocking of IL-8, ENA 78 and GRO-α in co-culture studies

GRO-α and IL-8 were measured in the supernatants from GEnC mono-cultures and GEnC/Podocyte co-cultures using LUMINEX assay (Invitrogen, UK). To block GRO- α and IL-8 monoclonal anti GRO antibody (2ug/ml, R&D systems, clone 31716) and IL-8, monoclonal anti IL-8 antibody (10ug/ml, R&D systems, clone 6217) were added to the cultures after cytokine stimulation along with the addition of neutrophils. Static adhesion assay is carried as explained earlier with co and mono-cultures of ciGEnCs.

ENA-78 was measured in the supernatants from GEnC mono-cultures and GEnC/Podocyte co-cultures using human ENA-78 immunoassay (R&D systems). ENA-78 was blocked in cultures as described above using monoclonal anti ENA-78 antibody (5ug/ml, R&D systems, clone 33160).

The concentration were used following optimisation with range of concentrations and picked the appropriate one to carry on further.

2.14 Statistics

All experiments were performed at least three times and presented as the mean±SEM. Variation between multiple treatments was evaluated using ANOVA followed by bonferroni multiple comparison post-test or Dunnetts post-test and student T tests. $P<0.05$ were considered statistically significant.

Chapter 3: Investigating the role of SP on EnC activation and viability in VGN

3.1 Introduction

Neutrophilic SP have been identified as the targets of ANCA associated vasculitic diseases. Raised plasma levels of PR3 or HNE, either complexed with their inhibitors or alone, have been detected during active disease (Haubitz et al., 1997; Henshaw et al., 1994). The SP mediated EnC damage is thought to play a very important role in the vasculitic process but the importance of SP in GEnC activation that occur during the development of VGN, has not been fully defined. Under normal conditions these SP assist in clearance of pathogens within phagolysosomes and in neutrophil migration across the endothelium as part of the immune response (Janoff, 1985a). The process of diapedesis does not generally result in EnC damage (Huber and Weiss, 1989), however, during disease states, ANCA against SP present in the sera of patients, are shown to be capable of activating cytokine primed neutrophils, resulting in degranulation and an oxidative burst resulting in endothelial damage (Charles et al., 1991; Ewert et al., 1991; Keogan et al., 1992). Several mechanisms of EnC damage have been identified involving EnC detachment induced by SP that degrade a variety of matrix proteins including fibronectin, laminin, vitronectin, and collagen type IV (Ballieux et al., 1994; Varani et al., 1989; Westlin and Gimbrone, 1993). Under serum free conditions, increasing concentrations of PR3 or HNE can induce cleavage of thrombomodulin and detachment of EnC *in vitro* (Boehme et al., 2002). This proteolytic activity of SP is shown to be inhibitable by the presence of α1 antitrypsin (α1-AT) (Ballieux BE, 1993; Rao et al., 1993), but in the circulation, the concentration of the inhibitors is generally low (approx. 60ng/ml) and are easily inactivated

by ROS produced during an oxidative burst, thereby enhancing damage mediated by SP (Janoff, 1985b; Kramps JA, 1984, 1988). The pathogenic significance of PR3 or HNE certainly needs further investigation.

We hypothesized that PR3 or HNE provoke the endothelium by initial activation and thereby alter neutrophil endothelial interactions leading to EnC detachment and cytolysis during VGN. In this chapter the pathogenic role of SP on EnC is explored - by studying the activation, functionality and viability. My study also determined if the effects of SP are completely enzymatic and inhibitable by the role of protease inhibitor α1-AT. EnC cultures were incubated with an increasing range of concentrations of these inflammatory mediators in order to determine the time and concentration dependent effects on EnC stimulation or activation leading to the neutrophil recruitment or on EnC injury and whether the effects were independent of proteolytic effects.

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3.2 Methods

Endothelial damage was tested following increasing concentrations of PR3 or HNE:

- Following the treatments with SP, the cells were were tested for their mitochondrial activity using MTT assay.
- EnC detachment was tested using crystal violet staining
- PI staining was done to check the membrane integrity of the EnC
- EnC apoptosis was measured using DAPI staining, Caspase -3 cleavage and Annexin V and PI binding.

EnC stimulation or activation following SP treatment was measured by static neutrophil recruitment assay and physiological flow based neutrophil recruitment assay.

3.3 Results

3.3.1 Effect of 2 hours treatment of PR3 or HNE on EnC survival:

3.3.1.1 EnC viability was determined by measuring mitochondrial activity:

Initially, we exposed EnCs (HUVEC, ciGEnC & Primary GEnC) to a range of concentrations of PR3 or HNE (0.5 - 15 µg/ml) for 2 hrs to identify the point where viability slips away under serum-free conditions testing the mitochondrial activity using the MTT assay as described previously (Fig.12A). The results revealed that the treatments with PR3 (0.5-15 µg/ml) for 2 hours on all the cell types did not show any significant reduction in viability compared to untreated controls. Treatment with HNE (0.5-15 µg/ml) did not affect viability of ciGEnCs, but on HUVEC there was significant decrease in cell viability with increasing concentrations over 5µg/ml (Fig.12A). The viability of HUVEC was reduced significantly to 38.9%±15.15 of control values (**$p<0.01$ by one-way ANOVA with Dennett's Post test with 11 data sets). This infers that at the 2 hours time point, concentrations of upto 5 µg/ml of PR3 or HNE do not reduce cell viability. The significant damage to HUVEC on treatment with concentrations over 5µg/ml of HNE may reflect the sensitivity of primary cultures. ciGEnC were not affected even with the higher concentrations. Primary GEnCs were treated only with lower concentrations of PR3 or HNE (0.5 - 2 µg/ml) and no loss in mitochondrial activity was observed following the 2 hours treatment (Fig. 12B). The effect shown by HNE at higher concentrations (10µg/ml) on EnC viability was completely inhibited by α1-AT.

Figure 12: Mitochondrial activity measuresment following 2 hours SP treatment

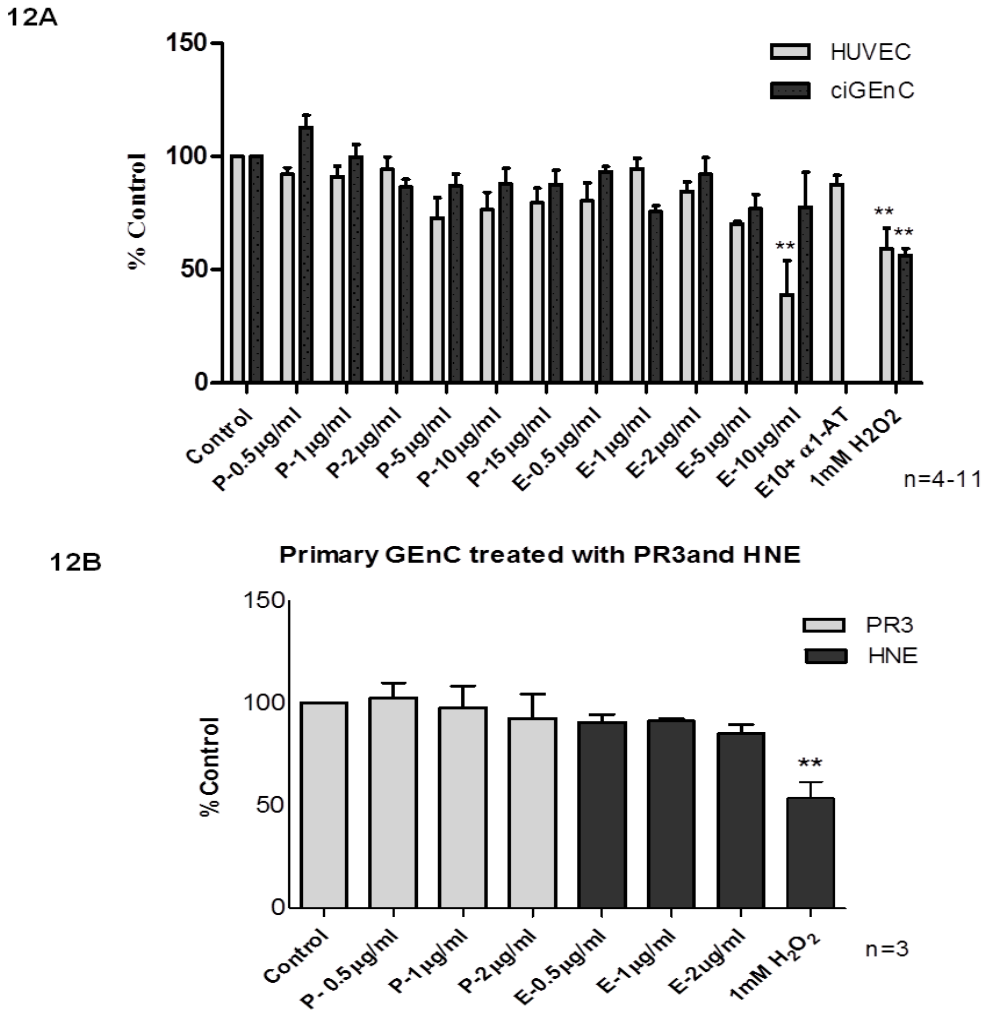


Fig 12: Effect of 2h treatment with PR3 or HNE on mitochondrial activity. Figure 12A ciGEnC and HUVEC were treated with 0.5-10µg/ml of PR3 (P) or HNE (E) for 2hours and cell viability was assessed using the MTT assay. α1-AT was used to determine any alterations in EnC viability were due to proteolysis. Figure 12B shows the effect of treatments (0.5 – 2 µg/ml PR3 or HNE) on primary GEnC. 1mM H_2O_2 treatment was used as positive control. Statistics: One way ANOVA with Dunnett Post Test, protease treated vs controls **p<0.01 and One way ANOVA with Bonferroni post test. **P<0.01, ***P<0.0001.

3.3.1.2 EnC monolayer detachment measured by crystal violet Assay

Crystal violet assay was used to enumerate the percentage of adherent viable cells following SP treatments. HUVEC and ciGEnCs were treated with concentrations (0.5, 1, 2 and 10 µg/ml) of PR3 or HNE and a crystal violet assay was performed. The results obtained confirmed results from the MTT assay, where PR3 did not affect cell attachment even at higher concentration of 10 µg/ml on both cell types, whereas HNE (10 µg/ml) treatment showed increased cell detachment significantly in HUVEC to 55.98%±2.27 of control values (**p<0.01 by one-way ANOVA with Bonferroni's post test with 3 data sets), but in ciGEnCs it did not reach significance when compared to control (Figure 13).

Figure 13: EnC attachment measured by crystal violet assay

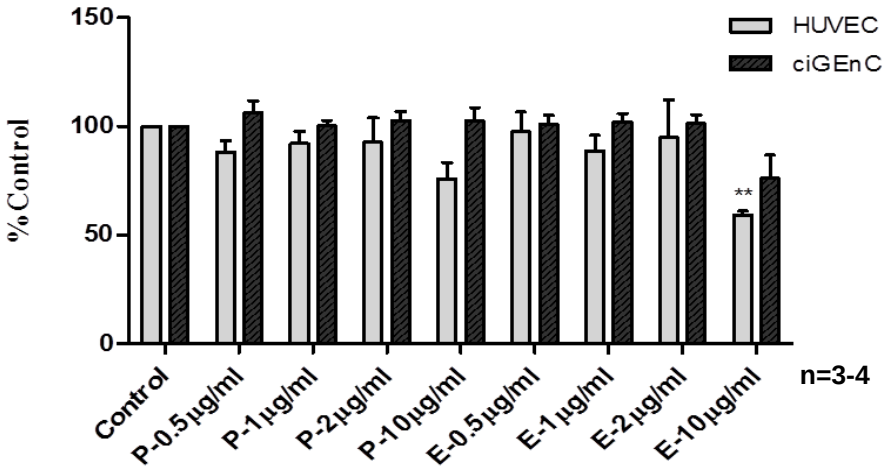


Figure 13: Effect of 2h SP treatment on EnC attachment measured by crystal violet assay. GEnC and HUVEC were treated with 0.5-10µg/ml PR3 (P) or HNE (E) for 24 hours and cell attachment was assessed using the crystal violet assay. Statistics: One way ANOVA with Bonferroni post test, control vs SP.

3.3.1.3 Membrane integrity assessed by PI staining following the treatment with PR3 or HNE

The effect of SP on the membrane integrity of a healthy cell was determined by PI staining. Confluent monolayers of HUVEC, primary and secondary GEnCs were treated with different concentrations (0.5, 1, 2 µg/ml) of PR3 or HNE for 2 hours and tested for the loss in membrane integrity (Fig. 14). This assay showed no decrease in cell membrane integrity following these treatments and thus confirmed the MTT and crystal violet assay data.

Figure 14: Membrane integrity tested by propidium iodide staining

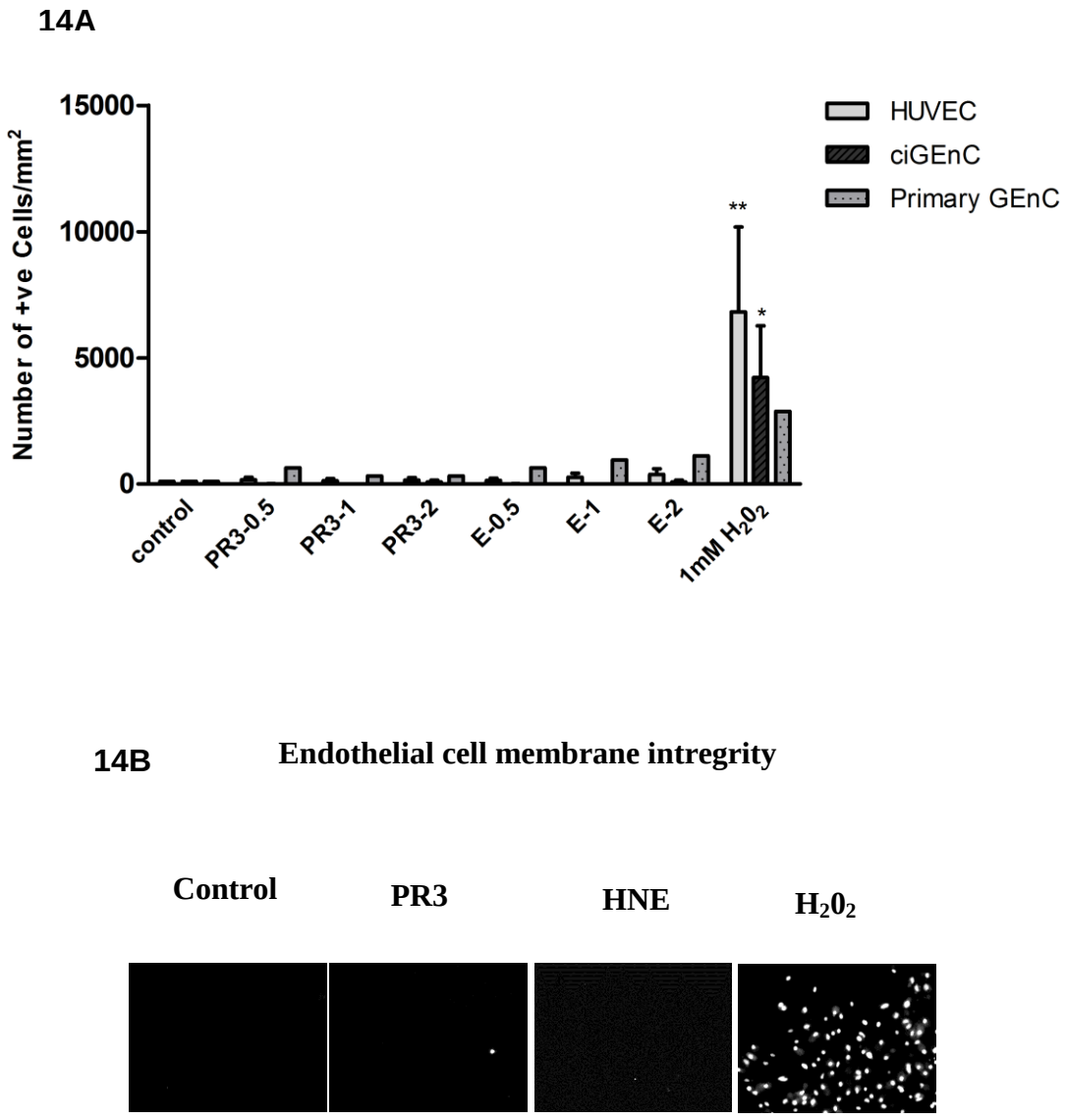


Figure 14: Membrane integrity of ciGEnC, HUVEC and primary GEnC detected by PI staining following treatment with PR3 or HNE (0.5 to 2 µg/ml) for 2 hours, 1mM H_2O_2 treatment for 1hr was used as a positive control. Figure 14A shows the counts of the PI positive cells and figure 14B shows the phase contrast images representing all cell types. Statistics: One way ANOVA with Bonferroni post test.

3.3.1.4 Monolayer integrity assessed by phase contrast microscopy following treatment with PR3 or HNE

Confluent monolayers of HUVEC, primary and secondary GEnC's were treated with different concentrations (0.5, 1, 2 µg/ml) of PR3 or HNE (Fig. 15) and cell layers were observed for loss of monolayer integrity by using phase contrast microscopy. There was no loss of monolayer integrity with PR3 treatment, but HNE 2 µg/ml showed retraction of the monolayer following 2 hours treatment. A similar effect was observed with all cell types.

Figure 15: The effect of PR3 or HNE on EnC monolayers following 2 hours treatment

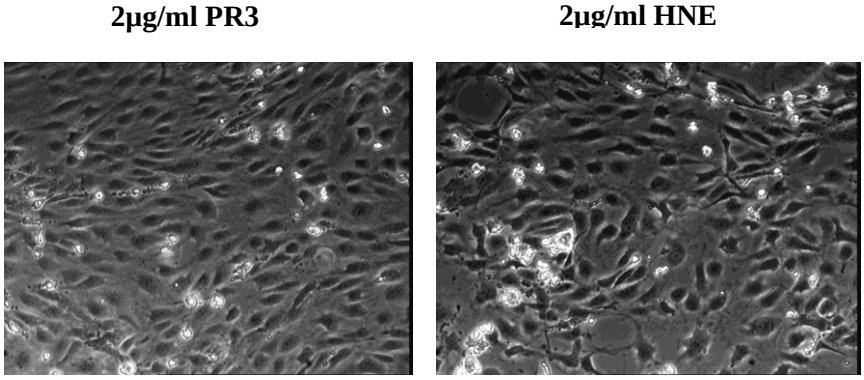


Figure 15 represents the damage caused to monolayers of EnCs following 2 hours treatment with 2µg/ml PR3 or HNE. The phase contrast images (Fig 15) are representative for all cell types.

3.3.1.5 Apoptosis detection following the treatment with PR3 or HNE

Endothelial monolayers treated with PR3 or HNE at concentrations of 1 or 10 µg/ml for 2 hours, were tested for apoptosis using different techniques

(i)UV light microscopy using DNA specific DAPI staining to visualize intact nuclei (Fig 16)

(ii) Caspase 3 dependent apoptosis by western blotting (Fig 17)

(iii) FACS using annexin V and PI staining (Fig 18) to detect early and late apoptotic stages.

The results have shown no detectable apoptosis at the concentration of 1 µg/ml with both the enzymes on HUVEC and ciGEnC. 10 µg/ml of PR3 or HNE treatment for 2 hours on both cell types resulted in reduction of monolayer integrity but no detectable apoptosis as visualized by DAPI staining. These changes were inhibited by treatment with α1-AT (Prolastin) at molar excess concentration (Figures 16 B,D,F,H).

Figure 16: DAPI staining to visualize nuclear fragmentation induced by SP on EnC in the presence and absence of alpha1- AT

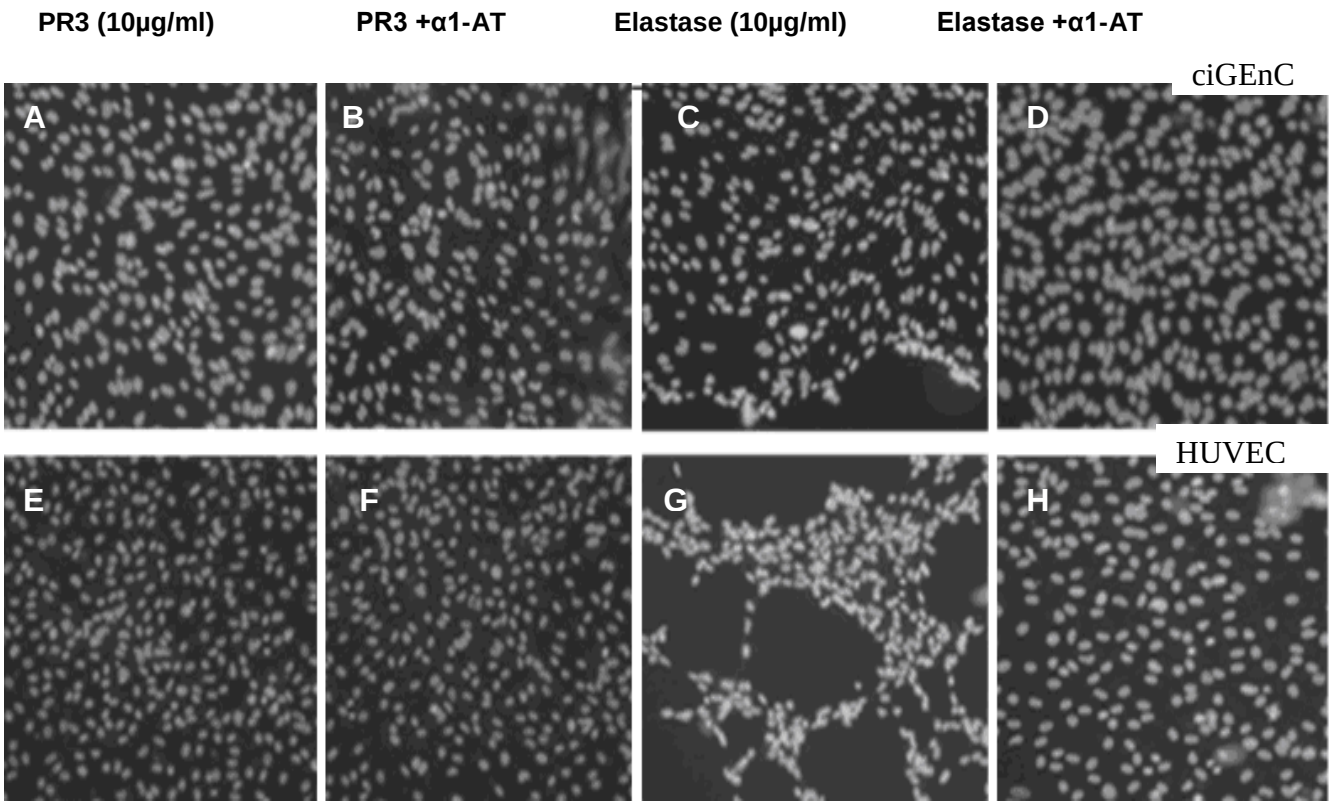


Figure 16: UV fluorescence photomicrographs of PR3 (P) or HNE (E) treated EnCs stained by DAPI. ciGEnC (Images A-D) and HUVEC (Images E-H) were incubated with 10µg/ml PR3 or HNE for 2 hours and stained with DAPI to detect apoptotic morphology in the presence or absence of α1-AT.

Figure 17: SP induced caspase-3 activation in EnC

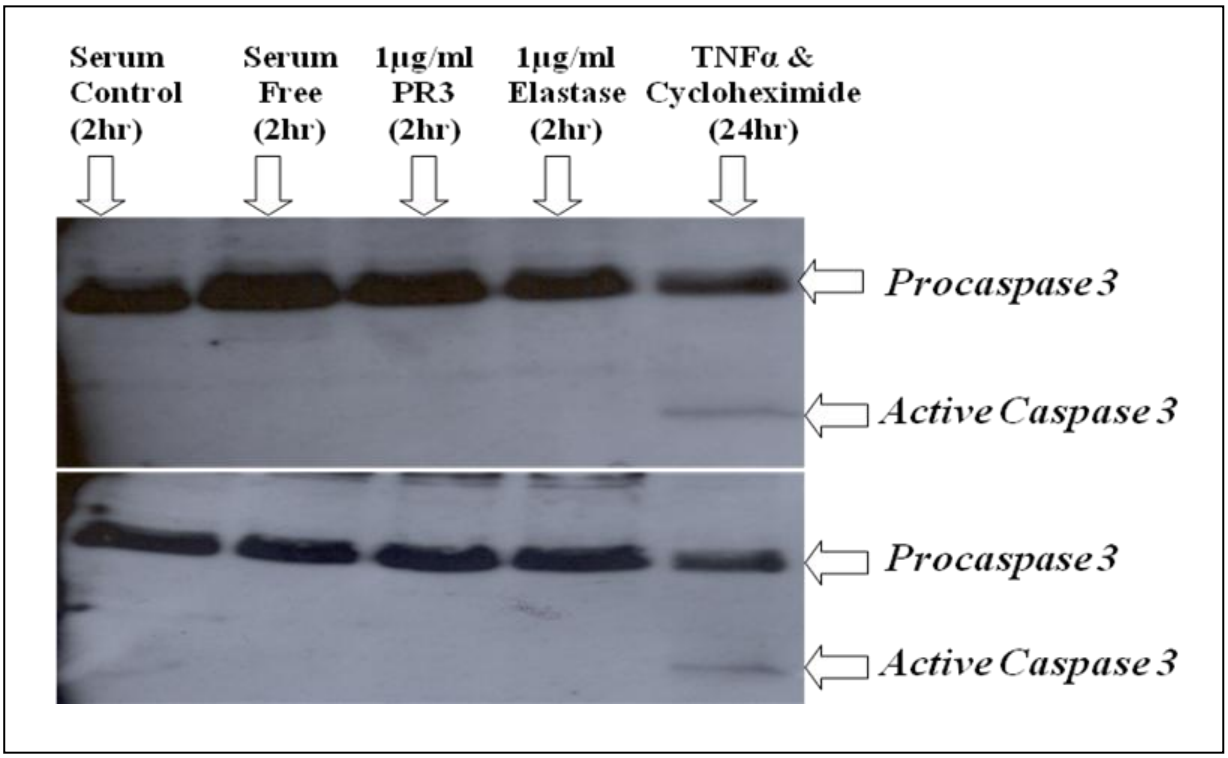


Figure 17 represent western blots showing the effect of 1µg/ml PR3 or HNE on EnC apoptosis by cleaving active caspase 3. Activation of caspase 3 is determined as marker of apoptosis and the activation was absent from these protease-treated EnC. Cells were treated with 3µg/ml CHX+10ng/ml TNF for 24 hours as a positive control. Representative images of 3 individual experiments.

Figure 18: Apoptosis detection on EnC stained with annexin V and PI by FACS

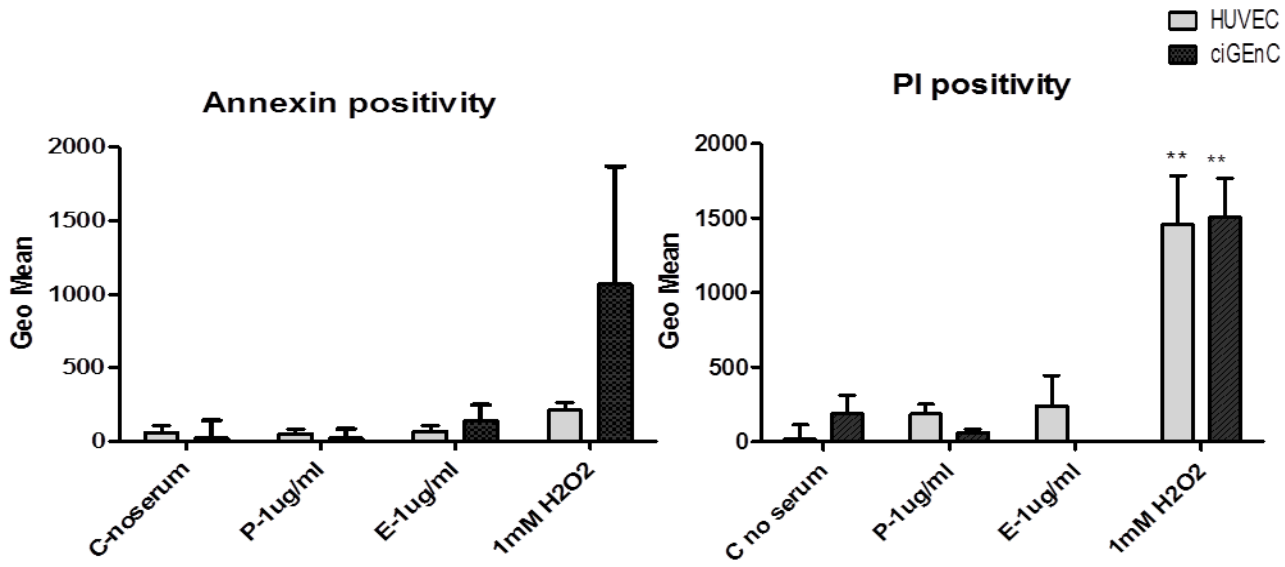


Figure 18 represents the effect of 1µg/ml of PR3 or HNE on EnC in inducing apoptosis by staining with Annexin V and PI. ciGEnC and HUVEC were treated with 1µg/ml of SP for 2 hours and then stained to detect apoptosis. The cells were treated with 1mM H_2O_2 as a positive control. Statistics: One way ANOVA with Bonferroni post test.

3.3.2 Effect of 2 hours treatment of PR3 or HNE on EnC activation:

3.3.2.1 Time dependent effect of SP on GEnCs in inducing neutrophil recruitment under static conditions

Having found the concentration of SP that did not disturb endothelium using viability experiments, we proceeded to determine the time point where the EnC favors neutrophil recruitment in the presence of PR3 or HNE at 1µg/ml. Time course was performed until 4 hours on GEnC treated with SP. Greater level of neutrophil recruitment on GEnC was observed with 2 hours treatment and later decreased rapidly (fig 19, +$p<0.05$ for PR3 compared to control by one-way ANOVA with Dunnett's Post test with 4-8 data sets).

Figure 19: Neutrophil recruitment to GEnC following PR3 or HNE treatment

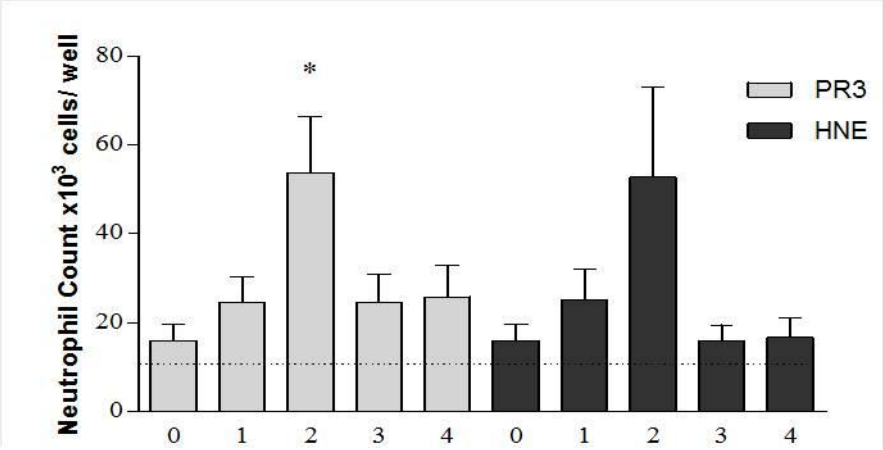


Figure 19 - Effect of PR3 or HNE (1µg/ml, 0-4 hr) on GEnC neutrophil recruitments under static conditions. EnC were incubated with PR3 or HNE and the neutrophils were allowed to adhere for 1 hour at 37ºC. Number of adherent neutrophils was determined from a neutrophil standard curve. Statistics: One- way ANOVA with Dunnetts Post Test, *protease vs. control.

3.3.2.2 Effects of SP pre-treatment on EnC-neutrophil recruitment under static conditions

The effect of SP in the activation of HUVEC and GEnC (primary and secondary) and in promoting endothelial-neutrophil interactions was assessed following treatment with 1µg/ml PR3 or HNE for 2 hours under static conditions. The results revealed that pre-treatment with PR3 or HNE significantly increased neutrophil recruitment on HUVEC and ciGEnC (figure 20A ***$p<0.001$ for PR3 or HNE treatment compared to control by one-way ANOVA with Dunnett Post test with 23 data sets) with 1µg/ml of either protease that was partly inhibitable with α1-AT under static conditions (figure 20B, +$p<0.05$ for either PR3 or HNE only compared to SP with α1-AT by one-way ANOVA with Bonferroni Post test with 6 data sets). In the case of primary GEnC (Fig. 20A), there was no significant increase in the adhesion in contrast to other cell types.

Figure 20: Neutrophil recruitment by EnC pre-treated with SP for 2 hours under static conditions and inhibition in the presence of alpha 1- AT

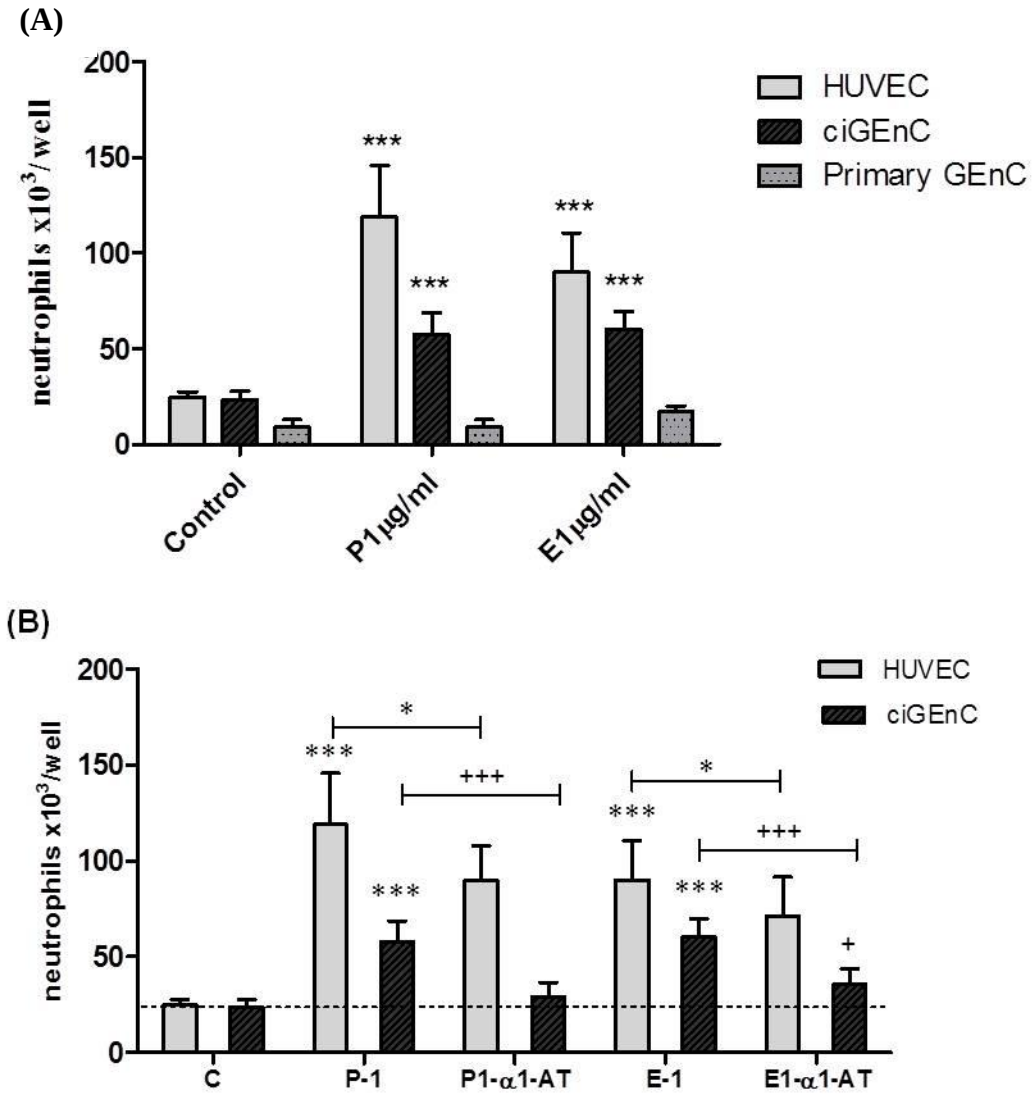


Figure 20 – Adhesion of unprimed neutrophils under static conditions was observed by treating EnCs with PR3 or HNE (1µg/ml, 2 hours) and also in the presence or absence of α1-AT. EnC were incubated with PR3 or HNE and the neutrophils were allowed to adhere for 1 hour at 37ºC. Number of adherent neutrophils was determined from a neutrophil standard curve and inhibition by α1-AT was carried out at molar excess concentration. Statistics: One- way ANOVA with Dunnett Post Test, protease vs. control (n=3).

3.3.2.2 Effects of SP pre-treatment on EnC-neutrophil recruitment under physiological flow conditions

Under physiological flow conditions, the PR3 or HNE induced neutrophil recruitment was observed on EnC's pre-treated with SP (1µg/ml) for 2 hours or TNF-α (100 Units) for 4 hours. Increased neutrophil interactions were observed on HUVEC more with PR3 and to a much lesser extent with HNE (figure 21, **p<0.001 for PR3-treated cells compared to controls by one-way ANOVA with Bonferroni Post test with 5-6 data sets). However, GEnCs did not present a similar pattern of neutrophil recruitment and showed greater variation than HUVEC (data from 1- 4 data sets).

Figure 21: Neutrophil recruitment by EnC pre-treated for 2 hours with SP under physiological flow conditions

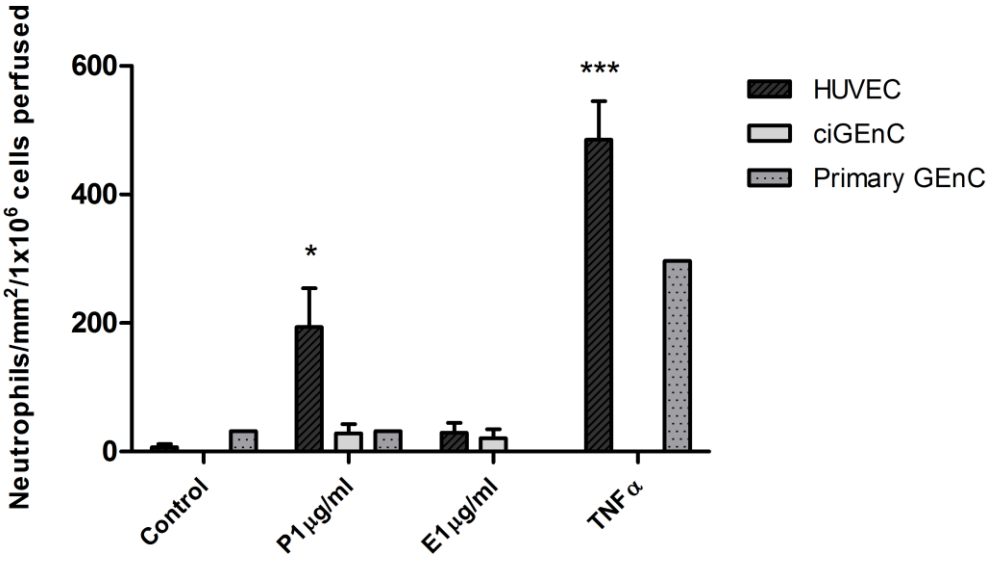


Figure 21 – Adhesion of unprimed neutrophils under flow conditions was observed by treating EnCs with PR3 or HNE (1µg/ml, 2 hours) or TNF-α (100 Units) for 4 hours. EnC were incubated with PR3 or HNE and a bolus of neutrophils was perfused over the surface of EnCs under wall shear rate of 0.1Pa and the effects were observed by phase contrast microscopy. Number of adherent neutrophils was determined by counting using Image Pro Plus software (Media Cybernetics, USA).

3.3.3 Effect of post treatment incubation (2 hours treatment followed by 22 hour incubation) of PR3 or HNE treated EnCs:

3.3.3.1 Mitochondrial activity tested with MTT Assay

EnCs (HUVEC, ciGEnC) were tested for their mitochondrial activity following treatment with different concentrations (1 - 15 µg/ml) of PR3 or HNE for 2 hours and left for 22 hours incubation at 37^{o}C and observed post treatment for any chronic effects of the enzymes (Fig.22). The results revealed that the treatments showed significant reduction in mitochondrial activity especially with HNE treatments. HUVEC demonstrated significantly reduced mitochondrial activity with HNE treatment even with lower concentration treatments. GEnCs also lost their mitochondrial activity significantly with higher concentrations of HNE. However, PR3 also showed reduction in activity on HUVEC and ciGEnC with higher concentrations, which did not reach statistical significance in all instances.

Figure 22: Long term effects of PR3 or HNE treatment on mitochondrial activity measured by MTT assay following 2 hours treatment

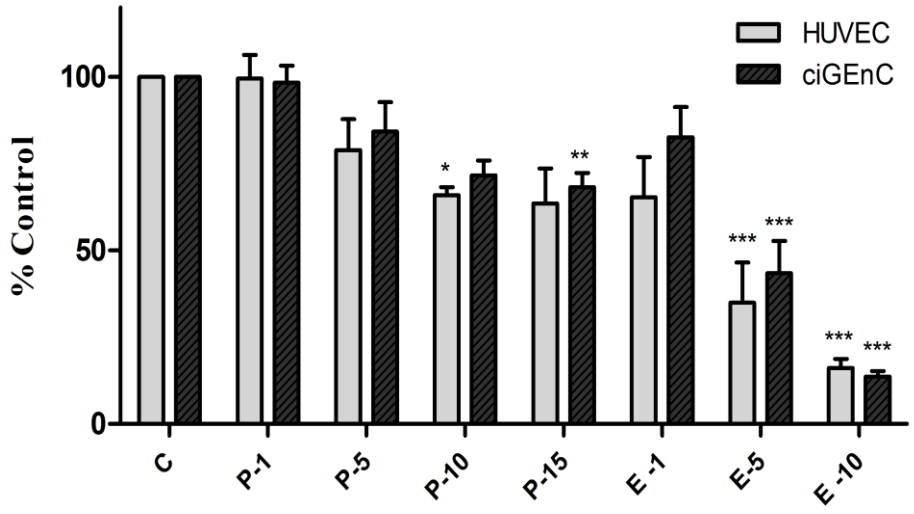


Figure 22 represent the ciGEnC and HUVEC treated for 2 hours with PR3 (1-15 µg/ml) or HNE (1-10 µg/ml) and incubated for another 22 hours at 37^{o}C to see the chronic effects of the enzymes. Cell viability was assessed using the MTT assay. Statistics: *p<0.05, **p<0.01, ***p<0.0001 by one-way ANOVA with bonferroni post test, treatment compared to control.

3.3.3.2 Apoptosis detection with 2 hours treatment of PR3 or HNE followed by 22 hours post treatment incubation:

EnC monolayers treated with PR3 or HNE at lower and higher concentrations for 2 hours and then incubated for 22 hours in serum (20%) containing medium, were tested for apoptosis using different techniques (i) UV light microscopy using DNA specific DAPI staining to visualize intact nuclei (Fig 23), (ii) caspase 3 dependent apoptosis by western blotting (Fig 24).

UV light microscopy images of DAPI staining shows the signs of apoptosis on cultures of ciGEnC and HUVEC following the treatment with 10µg/ml of PR3 or HNE. The demonstrated apoptosis was clearly inhibitable in the presence of α1-AT at molar excess concentration.

Western blots of active caspase 3 dependent apoptosis detection have shown that HUVEC and ciGEnC treated with lower concentrations (1µg/ml) of PR3 or HNE for 2 hours and following incubation for 22 hours in normal medium treatment did not undergo apoptosis when compared to the positive control (CHX (3ug/ml) +TNF (10ng/ml) treatment for 24hours). The serum starved cells were used as positive control and demonstrated apoptosis.

Figure 23: Detection of nuclear fragmentation of EnC induced by longer incubations following 2 hours SP treatment

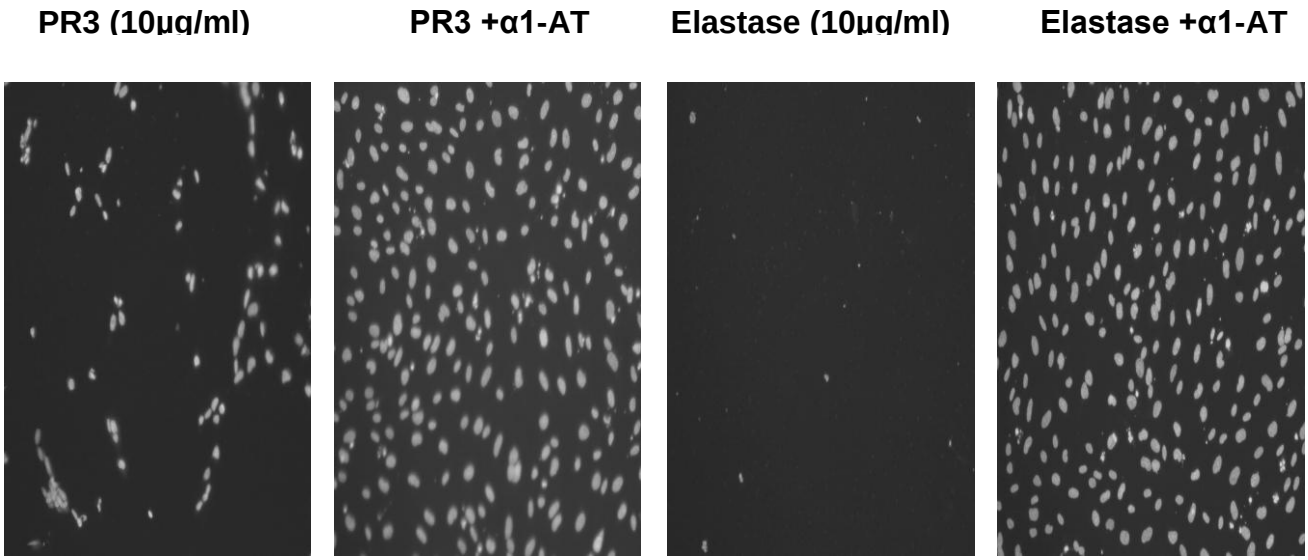


Figure 23 UV fluorescence photomicrographs of PR3 or HNE treated EnCs stained by DAPI in the presence or absence of α1-AT. Apoptotic cells depict nuclear fragmentation and homogeneous DNA staining in granules of different sizes. The images are representation of 3 individual experiments and were demonstrated by HUVEC as well as ciGEnC.

Figure 24: Caspase 3 cleavage in EnC induced by longer incubations following 2 hours SP treatment at 37°C

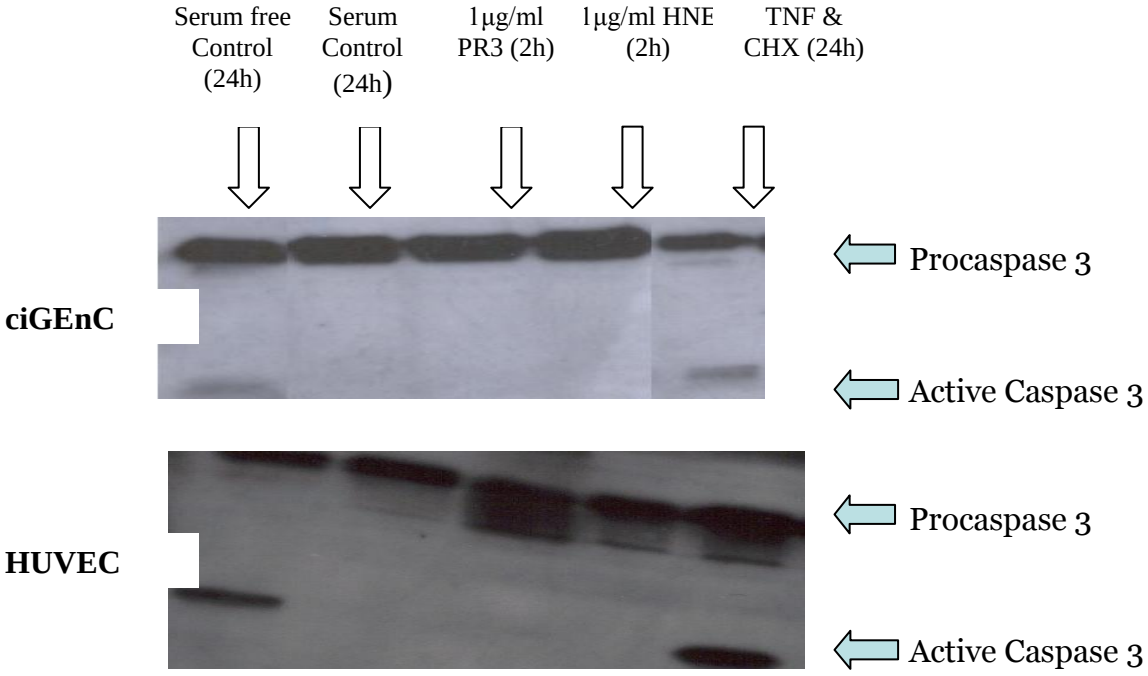


Figure 24 represent western blots testing for caspase 3 dependent apoptosis following the treatment of EnCs with PR3 or HNE (1µg/ml) for 2 hours and post incubation in the presence of 20% serum medium for 22 hours at 37°C. The blots show no signs of caspase 3 cleavage following the treatments compared to positive control (3µg/ml CHX+10ng/ml TNF for 24 hours) (lane 5). Lack of serum did cause detectable apoptosis (lane 1) in both cell types.

3.3.4 Effect of 24 hours treatment of PR3 or HNE on EnCs:

3.3.4.1 Mitochondrial activity tested with MTT Assay

Following the treatment for 24 hours, there was loss of mitochondrial activity of GEnC following treatment with 10µg/ml of PR3 and in HUVEC with concentrations as low as 2µg/ml, compared to untreated controls (Fig 25A). Treatment with 2µg/ml of HNE impaired viability in both GEnC and HUVEC compared to untreated controls (Fig 25A). The effect of higher concentration was significantly nullified by the presence of α1-AT (Fig 25B).

Figure 25: Measurement of mitochondrial activity following 24 hours SP treatment in the presence and absence of α1- AT

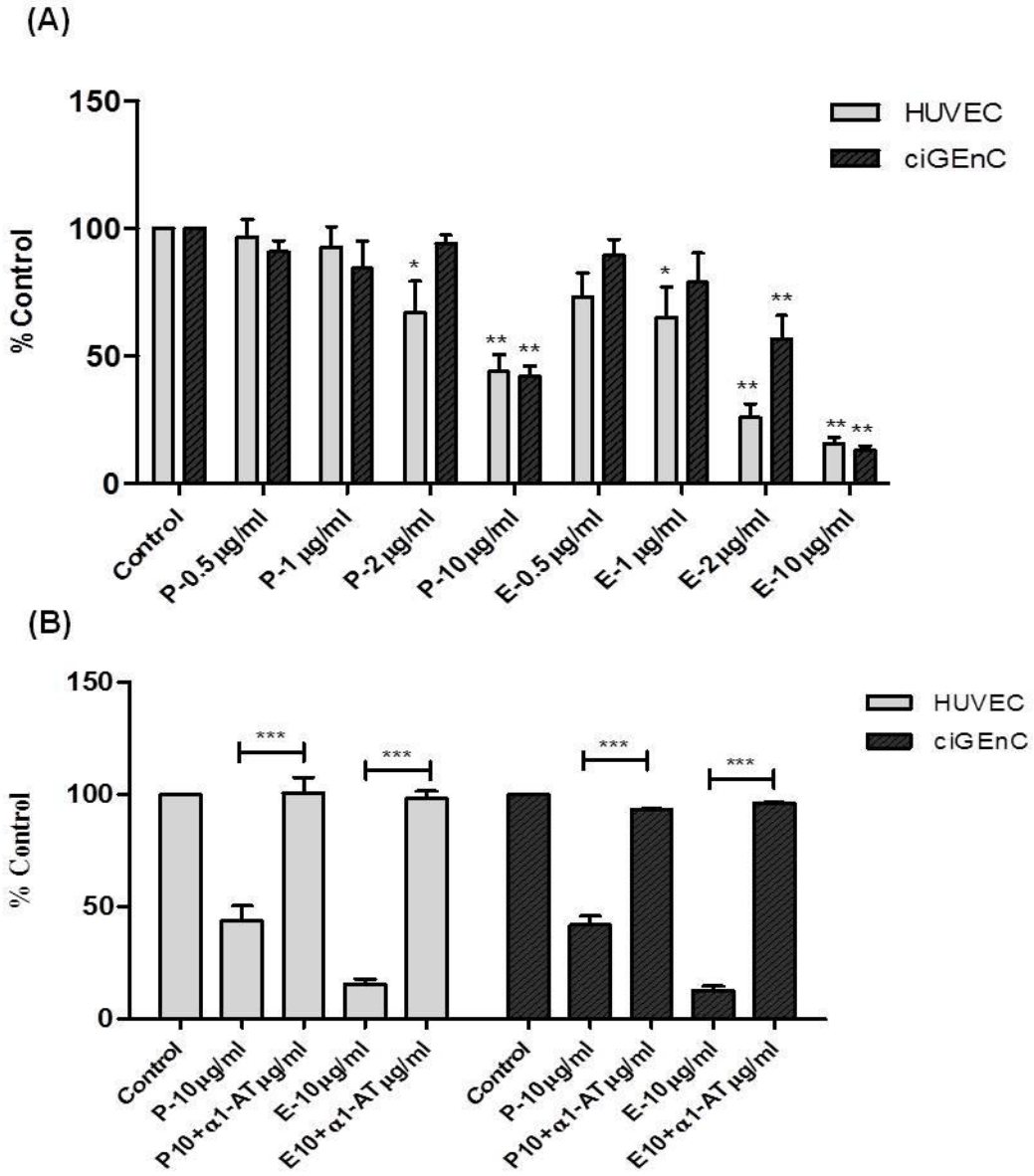


Figure 25 A,B -Effect of 24 hours treatment with SP and in the presence of α1–AT on EnC mitochondrial activity. GEnC or HUVEC were treated with 0.5-10µg/ml PR3 (P) or HNE (E) for 24 hours and mitochondrial activity was assessed in the presence and absence of protease inhibitor using the MTT assay.

Statistics: One way ANOVA with Dunnetts Post Test from 6 data sets, control vs SP.

3.3.4.2 Apoptosis detected using DAPI staining by UV light microscopy

EnC monolayers treated with PR3 or HNE at lower and higher concentrations for 24 hours were tested for apoptosis using UV light microscopy using DNA specific DAPI staining to visualize intact nuclei (Fig 26). DAPI staining on 24 hours SP treated cells revealed that PR3 or HNE at 10 µg/ml caused maximum chromatin condensation of EnC after 24 hours and these changes were inhibitable in the presence of α1-AT at molar excess concentration.

Figure 26: Nuclear fragmentation in EnC induced by 24 hours SP treatment

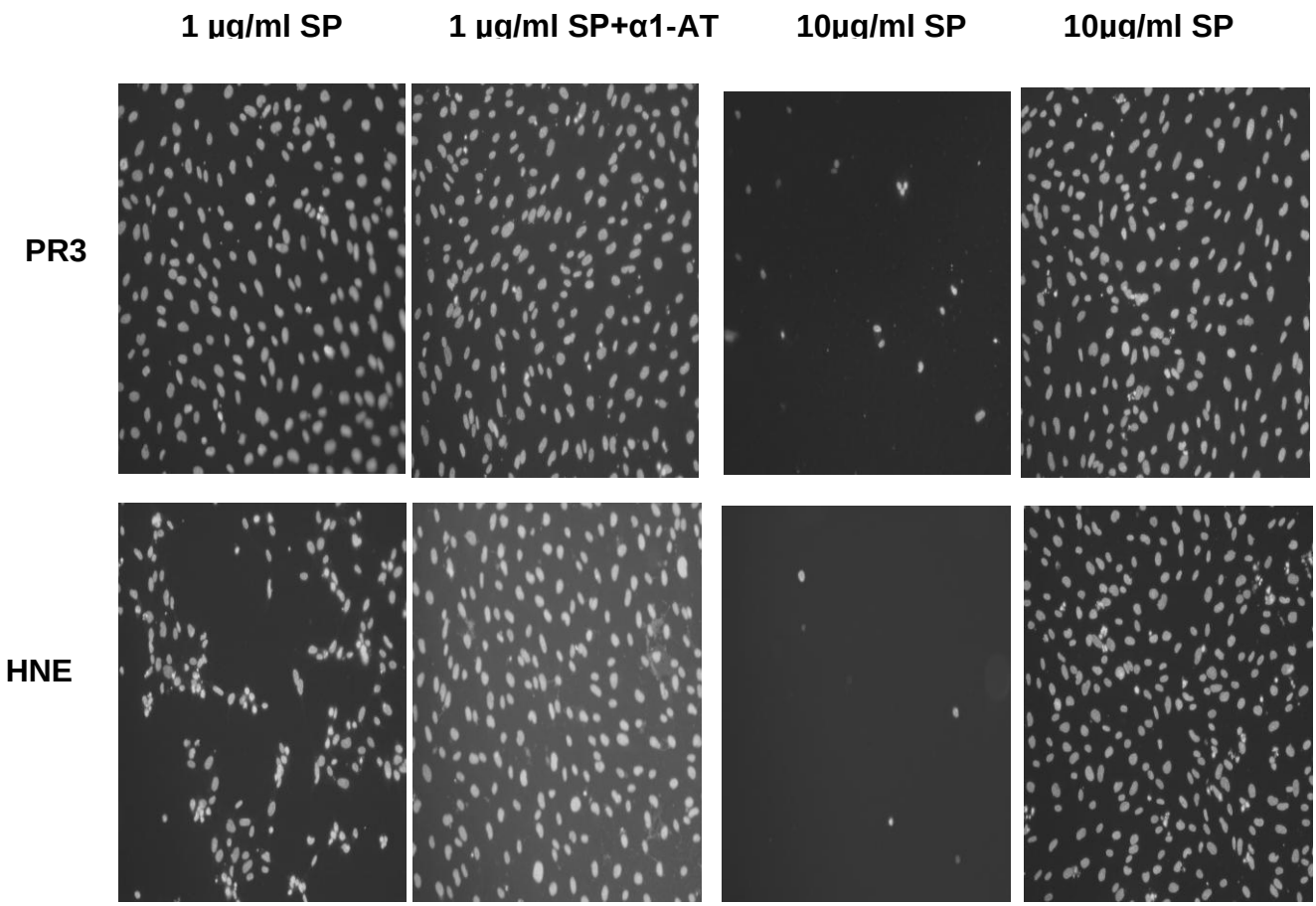


Figure 26 UV fluorescence photomicrographs of PR3 or HNE treated EnCs stained by DAPI in the presence or absence of α1-AT. Apoptotic cells depict nuclear fragmentation and homogeneous DNA staining in granules of different sizes. The images are representation of 3 individual experiments.

3.4 Discussion

ANCA induced neutrophil-derived inflammation pathophysiologically leads to EnC injury caused by proteolytic SPs as well as oxygen radicals and by neutralisation protective antiproteinase shield at inflammatory sites (de Bono and Yang, 1995; Varani et al., 1989; Weiss, 1989). The initiation of vascular damage starts with activation of endothelium upregulating adhesion molecules carried on to leukocyte infiltration further proceeding towards damage (Pall and Savage, 1994; Yamada et al., 1981; Zeck-Kapp G, 1990). SPs are the second source of cell injury and our study investigated the ability of SP to induce damage EnC in dose dependent manner and have shown the transition from health to proadhesive phenotype enhancing neutrophil adhesion and eventually leading towards damage. It is conclusive that the lower concentrations of SP and shorter incubation periods do not cause any significant damage to the endothelium; instead they do induce the endothelium to express a weak adhesive phenotype which supported neutrophil capture. This may be due to a limited degree of endothelial activation since parallel studies from my colleague have demonstrated increased expression of P-selectin (Bevins unpublished). Our work demonstrated that neutrophil adhesiveness to endothelium was enhanced by mobilising endothelial P-selectin expression and stabilising this adhesion by inducing IL-8 signalling. This could be due to initial EnC stimulation which does not require de novo protein synthesis and occurs rapidly as described by Hunt and Jurd (Hunt and Jurd, 1998). This would be compatible with the likely mobilization of Weibel-Palade bodies to the endothelial cell surface resulting in the release of P-selectin and von Willebrand factor (vWF) by the action of SP. Our data also suggests that PR3-induced neutrophil capture under flow conditions may require a threshold endothelial P-selectin level, as

neutrophil recruitment only occurred under conditions which significantly increased P-selectin expression. Alternatively, the adhesion observed might be charge dependent. Under static conditions, SP being cationic proteins, bind covalently to the anionic endothelium and might be damaging the vascular endothelium by digesting the matrix proteins and junctional proteins, thus facilitating the adhesion of neutrophils (Campbell et al., 2000). However, only partial inhibition of adhesion by α1-AT, suggests that the effect is only partly dependent on the enzymatic actions of SP.

We have also documented the stages of EnC damage in a concentration and time dependent manner and have confirmed the previous observations that high levels of both proteases can induce cell retraction and detachment (Bart, 1994). All the cell types (HUVEC, Primary and secondary GEnC) had showed similar effect with no significant difference. Lower concentrations and shorter durations of exposures showed minimal or no evidence of EnC apoptosis, loss of membrane integrity or cell viability. At 2 hour time point, PR3 and HNE (1 µg/ml) induced neutrophil recruitment by both HUVEC and ciGEnC but not primary GEnC under static conditions. Under flow conditions, PR3 but not HNE induce neutrophil recruitment. EnCs were susceptible to damage with HNE even at lower concentrations disturbing monolayer integrity with no detectable apoptosis. Indeed, HNE has been linked to endothelial cell injury more so than PR3 (Carden et al., 1998; Nakatani et al., 2001; Smedly et al., 1985). In addition, we also found protease treatment was not directly correlated with immediate cell destruction and thus observed activation of EnCs. However, exposure of EnC to higher concentrations (10 and 15 µg/ml) of SP demonstrated a significant decrease in mitochondrial activity.

Further, our studies proceeded to looking at the effects of long term exposure to proteases on endothelial monolayers. At 24 hours treatment with lower concentrations up to 1µg/ml,

PR3 did not cause any considerable damage to the cells. However, HNE 1µg/ml caused considerable damage to monolayer integrity but no significant reduction in mitochondrial activity was observed. These findings indicate that 24 hours time point is long enough for 1µg/ml HNE in starting to digest the matrix proteins and reduce the monolayer integrity, but did not decrease the cell mitochondrial activity that further leads to death. Higher concentrations of 10µg/ml did cause detachment, significant reduction in viability and apoptosis. Yang *et al*., 1996 have shown the induction of apoptosis by PR3 treatment on endothelial cells following 24 hours treatment (Yang et al., 1996). Other *in vitro* studies have demonstrated the direct detachment and cytolysis of EnCs by the effect of SP (Ballieux et al., 1994);(Westlin and Gimbrone, 1993) and inducing emphysematous lesions upon intratracheal instillation *in vivo* (Janoff et al., 1977). It has been demonstrated that treatment with PR3 and HNE resulted in the degradation of several constituents of the glomerular basement membrane, including fibronectin, laminin and collagen type IV and heparan sulfate leading to detachment of GEnCs which further effects cell metabolism and activate the process of cell death and thus glomerular injury (Klebanoff et al., 1993; Re et al., 1994). Yang *et al*., (Yang et al., 2001), resolved the confusion whether detachment triggers apoptosis or vice versa, by showing the percentage of apoptotic cells proportional to the degree of cell detachment indicating that detachment of EnCs from the matrix correlates with the apoptosis induced by PR3/HNE and our results corroborate the above findings. Our data also demonstrates that HNE when compared to PR3 was more effective inducer of endothelial cell damage. Thus, the analysis of studies using concentrations from 0.5 - 15 µg/ml of PR3/HNE, suggest that exposure of EnCs to low levels of neutrophil-derived proteases activated the EnCs without causing any damage to the monolayer and

higher concentrations for 2 hours might be the initiative to the damage of endothelial monolayer by digesting the matrix proteins.

Accumulation of neutrophils at focal sites is involved with acute inflammation which was observed with the presence of SP along with the damage that they cause to endothelium. Later our interest was directed to observe the chronic effects of SP where we looked at post treatment effects as these proteases were shown to have equal contributions to chronic inflammation, as monocytes are also a source of PR3 and HNE. The results have shown that there are chronic effects of treatments at higher concentrations of PR3 where as HNE decreased mitochondrial activity following even 1 µg/ml. Western blotting did not show any caspase-3 cleavage with treatment of 1ug/ml of either PR3/HNE. However treatment with higher concentrations demonstrated detachment of cells and apoptotic bodies with DAPI staining. The actions of α 1 antitrypsin, at a molar excess concentration, outlasted the 2 hour incubation period and nullified the effect of SP on EnCs, thereby acting to protect the cells from post-treatment effects. Both HUVEC and ciGEnC showed similar effects. These results indicate that the damage to the endothelial surface occurs not only in the presence of the enzyme but also post effects leads to increased damage.

Prolastin - Therapeutic target of Vasculitis:

Our work also demonstrated that all the effects were inhibitable by the presence of prolastin suggesting the balance between SP and their inhibitors and protease-independent mechanisms. The presence of protease inhibitors in plasma and tissue fluid offer some defense against elevated protease concentrations, however, in some occasions this balance can be disturbed (Bank et al., 1999). It has been reported that the deficiency of protease inhibitors in some individuals due to heterozygosity for alpha 1 anti trypsin is associated in systemic vasculitis (Segelmark et al., 1995). Under experimental conditions, Prolastin seems to diminish the damage caused to EnCs by PR3/HNE, and thus confirms that the proteolytic activity of the enzymes is causing the damage and partial inhibition of neutrophil recruitment to the endothelial monolayers represents the mechanisms independent to the enzyme activity. Prolastin is currently in use for chronic replacement therapy of individuals with congenital alpha-1 antitrypsin deficiency. The series of experiments suggests that SP may represent a viable target for the inhibition of inflammation. This work contributes to our understanding of the mechanism of endothelial injury in vasculitis. Learning more about the functional capabilities of these SP might result in the development of manipulative methods of the inflammatory process.

Chapter 4: Podocytes modulates neutrophil recruitment by cytokine activated GEnCs

4.1 Introduction:

Being major detoxicating and osmo-regulative units, glomeruli are exposed to many disturbing stimuli that can trigger inflammatory episodes. Recruitment of neutrophils by glomerular endothelium mediates the first stage of defence during health and disease. Circulating neutrophils marginate towards glomerular endothelial cells (GEnC) under the influence of cytokines and inflammatory agonists, which stimulate the cell surface expression of pro-adhesive molecules and activate adhesive interactions (Brady et al., 1992);(Mulligan et al., 1993). The role of several adhesion molecules and their ligands in the recruitment process has been elucidated (Bishop and Hall, 1989; Brady et al., 1992; Kawasaki et al., 1993; Mulligan et al., 1993; Seron et al., 1991; Tang et al., 1997; Tipping et al., 1994). The involvement of neighbouring cells in the regulation of physiological and immunological processes by vascular endothelium is of great interest. Previous in vitro studies have shown that when EnCs are co-cultured with other stromal cells, such as fibroblasts or smooth muscle cells, their ability to recruit leukocytes following cytokine stimulation is altered (Gerhardt H, 2000; Helen et al., 2009; Hsiai, 2008; Lally et al., 2005; McGettrick, 2010; McGettrick et al., ; Rainger and Nash, 2001). Similarly, in glomerular capillaries, the communication between neighbouring cells might be crucial for regulating different physiological functions. Glomerular podocytes, despite having limited regenerative capacity, influence the growth, proliferation and regeneration of GEnCs. The symbiosis between these cells is well explained by the exclusive expression of VEGF and

angiopoietin-1 by podocytes, while GEnCs express high levels of the cognate receptors VEGFR2 and Tie 2, thereby ensuring the normal maintenance functions and angiogenesis (Fan L, 2002; Hirschberg et al., 2008; Kitamoto et al., 1997; Liang et al., 2006; Satchell et al., 2002; Simon et al., 1998). Both the cells have been shown to play a major role in the progression and regression of many disease states (Liang et al., 2006). The cross talk between GEnCs and podocytes mediated by soluble factors has been studied and reviewed (Camici, ; Hirschberg et al., 2008; Satchell et al., 2002; Vicki and David, 2005). There is evidence for a possible role of podocytes in regulating glomerular inflammation and in the initiation and propagation of glomerular damage (Eremina et al., 2003) although the underlying cell biological mechanisms are not completely understood

In order to demonstrate the importance of the physical relationship between the glomerular cells and their ability to communicate during inflammatory episodes, we recreated glomerular micro-environment *in vitro* and studied neutrophil recruitment by GEnCs in the presence of podocytes and inflammatory stimuli, and defined the molecular mechanisms involved.

4.2. Methods:

1. Characterisation of podocytes: Immunostaining of podocytes for the differentiation marker, podocin.

2. Co-culturing of podocytes and EnCs:

- Electrical resistance (ER) measurement on inserts across cell cultures.
- Transwell assay to assess neutrophil recruitment in endothelial and epithelial cell co-culture.
- Neutrophil recruitment assay to HUVEC and GEnC following conditioning with podocyte derived medium
- Transwell assay with endothelial cells co-cultured, but not in contact with epithelial cells to assess neutrophil migration by soluble mediators

3. Detection of Soluble mediators: Multiplex cytokine immunoassay & ELISA

4. Neutrophil recruitment assay following IL-6 treatment of GEnC mono-cultures.

5. Blocking Studies:

- CXC – chemokine receptor blocking in co-cultures and mono-cultures.
- Neutralization of IL-6 activity in culture supernatants.
- Blocking of IL-8, ENA 78 and Gro-α in co-culture studies.

4.3 Results:

4.3.1 Expression of the podocyte specific marker, podocin, by differentiated podocytes

One of the specific markers for differentiated podocytes is podocin which is localized on podocyte foot process membranes at the insertion site of the slit diaphragm. Based on the previous work by the Saleem group (Saleem et al., 2002), Podocin was used as a specific differentiation marker to compare the phenotype of the cells under permissive (33^{o}C) and non-permissive temperatures (37^{o}C). Podocin was not expressed by undifferentiated podocytes and was expressed at comparable levels in all cells at non-permissive temperatures following 7 days and 14 days differentiation at 37^{o}C. These findings also provide evidence that the differentiated cell lines are of primary podocyte origin.

Figure 27: Immunofluorescence images of podocytes expressing the differentiation marker, podocin

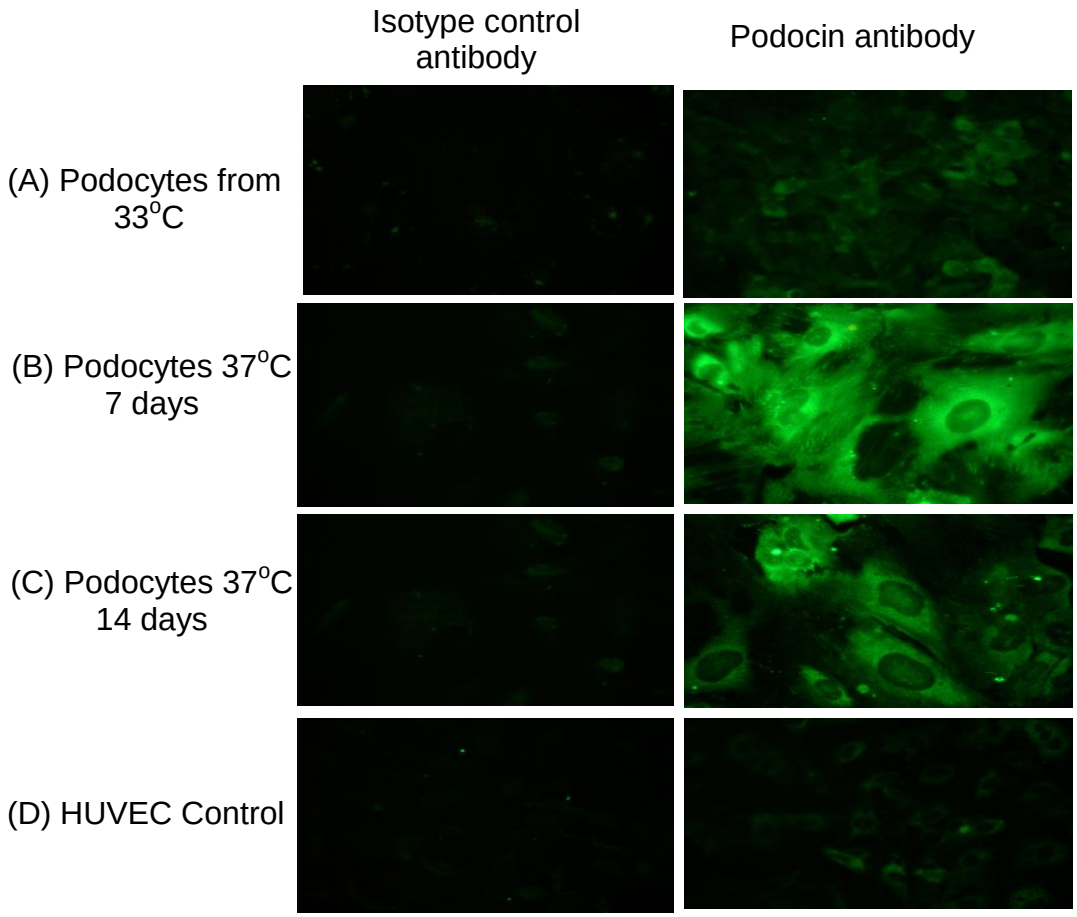


Figure 27: Immunofluorescence microscopy of the podocyte differentiation marker, podocin: Little or no expression in (A) undifferentiated podocytes from 33°C and (D) control cells, HUVEC. Podocytes differentiated at 37°C for 7 and 14 days expressed podocin on intracellular filaments and on the cell surface (B, C). Left panel are isotype controls showing no non-specific staining for podocin antibody. Magnification, X40

4.3.2 Trans-culture electrical resistance (ER) testing of co-cultures and monocultures

Electrical resistance across the podocyte monolayer grown on the basal side of a 3µm insert was tested, following the seeding of podocytes from differentiation up to 14 days. The podocyte monolayer on the insert following 1 day differentiation showed ER values of 7.61 Ωcm^2. Following the differentiation process up to 14 days, the ER was checked on days 3, 6, 10 and 14, and the ER decreased to a minimum of 1.109 Ωcm^2 by day 14 (Figure 28A). The ER of EnC mono-cultures (HUVEC or GEnC) cultured on the apical side of the transwell insert was also measured along with co-cultures. The EnC & podocyte co-cultures showed no difference when compared to the inserts with single endothelial cultures. The ER of HUVEC and podocyte co-cultures was 4.95 Ωcm^2 and HUVEC alone showed an ER of 4.29 Ωcm^2; similarly the ER of ciGEnC and podocyte co-cultures was 11.22 Ωcm^2 and ciGEnCs alone showed an ER of 10.23 Ωcm^2 (Figure 28B). HUVEC cultures showed a lower ER than ciGEnC cultures.

Figure 28: Electrical resistance tested across EnC and podocyte monolayers and co-cultures on the inserts

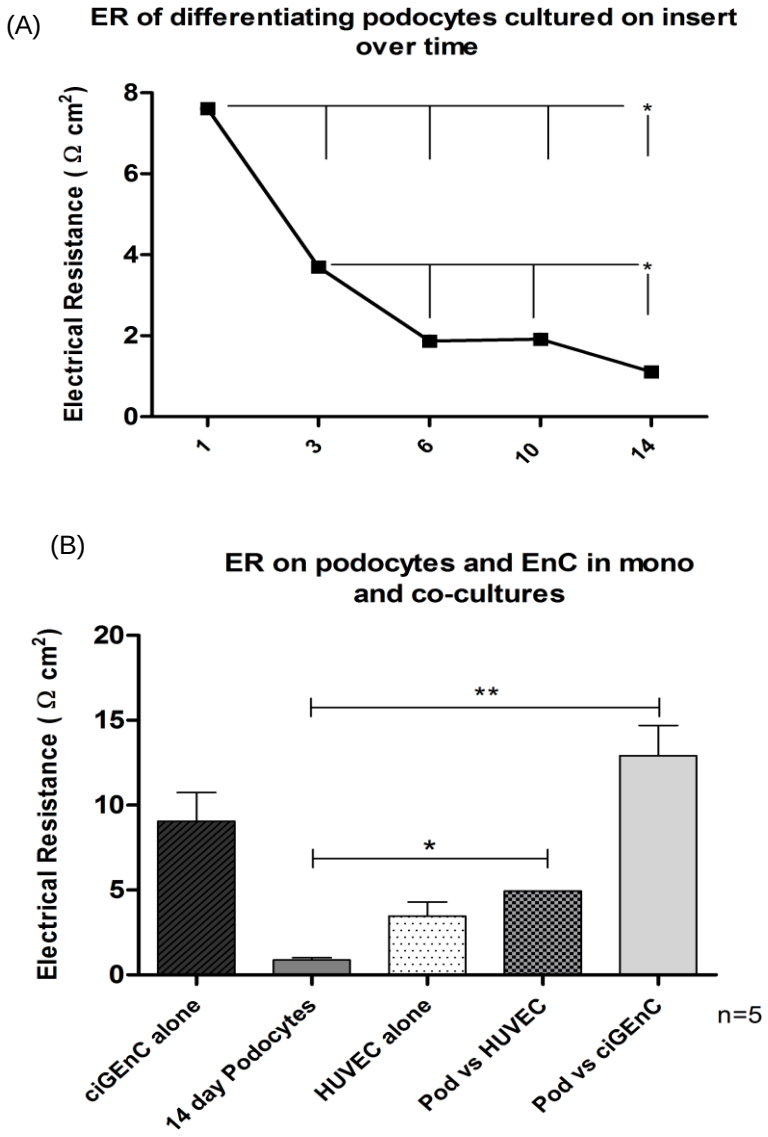


Figure 28: ER measured on mono-cultures and co-cultures. (A) Podocytes cultured on the insert over period of differentiation (14 days) after initial seeding of the monolayer and was tested on all inserts used for experiments. (B) Represent the ER on filters with co-cultures and single cultures of EnCs and podocytes.Stats: the monocultures were compared to co-cultures by Mann-Whitney test and *P<0.05 was considered significant.

4.3.3 Culture medium effect on neutrophil recruitment by HUVEC

Neutrophils were suspended in different types of medium (M199+0.1 % BSA, RPMI+0.1% BSA, RPMI complete medium (described in previous section)) and the neutrophil recruitment pattern by HUVEC monolayers was examined. Neutrophils that were suspended in RPMI with 0.1% BSA showed an increase in adhesion by 5-10% on HUVEC monolayers treated with higher concentrations of TNF-α (10 and 100 Units/ml), when compared to the neutrophils suspended in other types of medium. Hence, future experiments were carried out by resuspending the neutrophils in RPMI with 0.1% BSA.

4.3.4 Co-culture of EnCs and podocytes allowing direct contact

The effect of podocytes in regulating EnC neutrophil interactions was studied in a co-culture system using 3µm pore filters enabling direct cell-cell contact between the cell types. We analysed whether direct intercellular contact in the co-cultures generated different neutrophil recruitment patterns compared to EnCs grown alone inside the filters. Both the cultures were subjected to cytokine (TNF-α) treatments for 4 hours in a dose dependent manner (0 – 100Units/ml).

4.3.4.1 Optimisation of co-culture technique:

4.3.4.1.1 Effects of seven- day differentiated podocytes on neutrophil recruitment by ciGEnC and HUVEC

EnC that had been co-cultured with podocytes that in turn had been differentiated for 7 days (podocytes$_{7\ day}$) were subjected to TNF-α treatments in a dose dependent manner (0-100 Units/ml). In this experiment TNF-α was only added to the upper chamber of cultures. EnC mono-cultures supported greater neutrophil recruitment compared to co-cultures of EnCs and podocytes.

Following 100Units/ml TNF-α treatment, ciGEnC mono-cultures showed a 2-fold increase in neutrophil recruitment compared to ciGEnC and podocytes$_{7\ day}$ co-cultures (figure 29A).

On the other hand, HUVEC co-cultures did not show a significant increase compared to monocultures presenting neutrophil recruitment of 31762 cells/mm^2/5x10^5 neutrophils added when compared to HUVEC co-cultured with podocytes$_{7day}$ 18794cells/mm^2/5x10^5 neutrophils added (figure 29B). There was no significant difference in transmigration of neutrophils following increasing concentrations of TNF treatments except at TNF 100 U/ml on ciGEnCs as shown in figure 29 C&D.

Figure 29: Effect of 7-day differentiated podocytes on neutrophil recruitment by EnCs

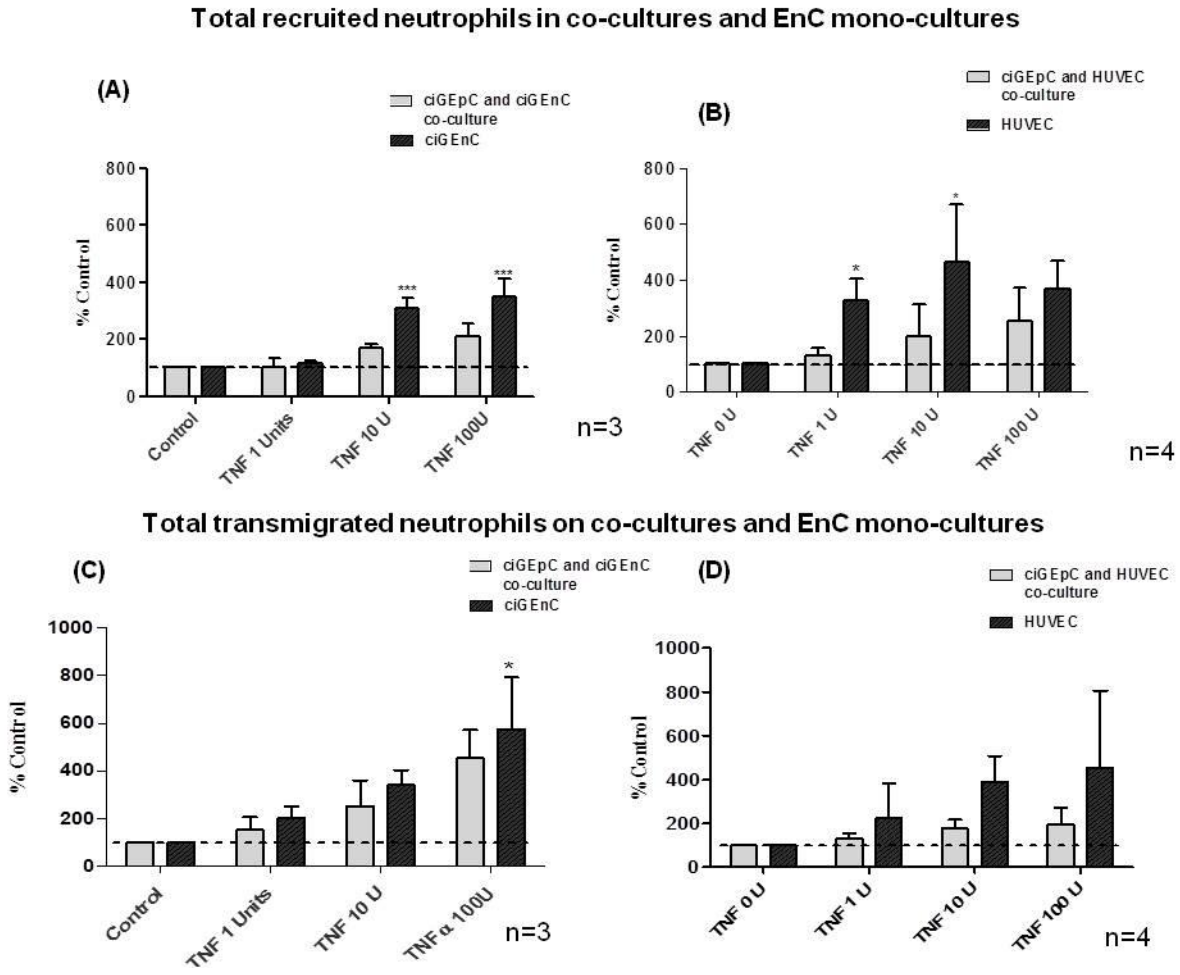


Figure 29; Neutrophil recruitment and migration to EnCs (ciGEnCs and HUVEC) cultured alone or with 7-day differentiated podocytes stimulated with TNF-α at 0-100U/ml. (A) Total recruited neutrophils by ciGEnC co-cultured with podocytes or cultured alone. (B) Total recruited neutrophils by HUVEC co-cultured with podocytes or cultured alone. (C) Total transmigrated neutrophils in co-cultures or mono-cultures. (D) Total transmigration by HUVEC co-culture with podocytes or cultured alone. Data shown is mean of n=7 independent experiments. In all experiments, co-cultures were compared with mono-cultures. Data expressed is normalized to control. Statistics: ***P<0.001 or *P<0.05 represents significance of the treatment compared to the control by one way ANOVA.

These findings suggest that EnCs and podocytes have the ability to influence each other's behavior and that the presence of podocytes$_{7\ day}$ suppressed neutrophil recruitment.

4.3.4.1.2 Drawbacks with the seven-day culture experiments and representation of the data:

- In these experiments, the upper and lower chambers of the co-culture system had equal volumes of medium which would possibly increase the hydrostatic pressure in the system.
- The cultures were treated in a dose dependent manner with TNF-α only in the upper chamber facing EnCs and the lower chamber facing podocytes were left untreated. This diluted the treatments due to the exchange of medium between the chambers.
- The number of neutrophils added to the system during static adhesion assay was approximately 500,000/well, and they were not re-counted after adding into the well, so it was assumed that the counts were exactly 500,000/well. This did not provide perfect counts to calculate the percentages of neutrophil recruitment and transmigration. So, the counts were normalized to controls.

The later experiments were corrected and counted exactly after collecting all the washes containing neutrophils from the wells.

4.3.5 Effects of 14-day differentiated podocytes on neutrophil recruitment by ciGEnC and HUVEC

Covering the previous issues, EnCs co-cultured with podocytes that have been differentiated for 14 days ($podocytes_{14day}$) were subjected to treatment with TNF-α in a dose dependent manner (0-100 Units/ml). The cultures demonstrated dose dependent increase in neutrophil recruitment with TNF-α treatments, when compared to their individual controls in both co-cultures and mono-cultures (Figure 30 & 31).

Interesting finding was that, similar to 7-day differentiated podocytes and ciGEnC co-cultures, the ciGEnCs co-cultured with 14-day differentiated podocytes also demonstrated reduced neutrophil recruitment when compared to ciGEnC mono-cultures. In these cultures, the reduced neutrophil recruitment by co-cultures was observed with and without TNF-α treatment. The reduction was significant at higher concentrations of TNF-α (10 & 100 Units/ml).

There was 12% reduction in neutrophil recruitment in unstimulated co-cultures compared to mono-cultures. Following 10U/ml TNF-α treatment, ciGEnC co-cultured with $podocytes_{14\ day}$ showed a reduction in neutrophil recruitment by upto 33%. Neutrophil recruitment (% of total neutrophils added) in co-cultures vs mono-cultures was 20.36 $\pm$ 2.9% and 34.27 $\pm$ 4.8%, n=14. At 100U/ml TNF-α treatment, co-cultures of GEnC in direct contact with podocytes demonstrated significant reduction in neutrophil recruitment by up to 50% when compared to GEnC mono-cultures. Neutrophil recruitment (% of total neutrophils added) in immortalised co-cultures vs mono-cultures was 23.93 $\pm$ 1.8% and 49.7 $\pm$ 4.3%, n=38 (Figure 30A).

Figure 30: Effect of 14-day differentiated podocytes on neutrophil recruitment by ciGEnCs

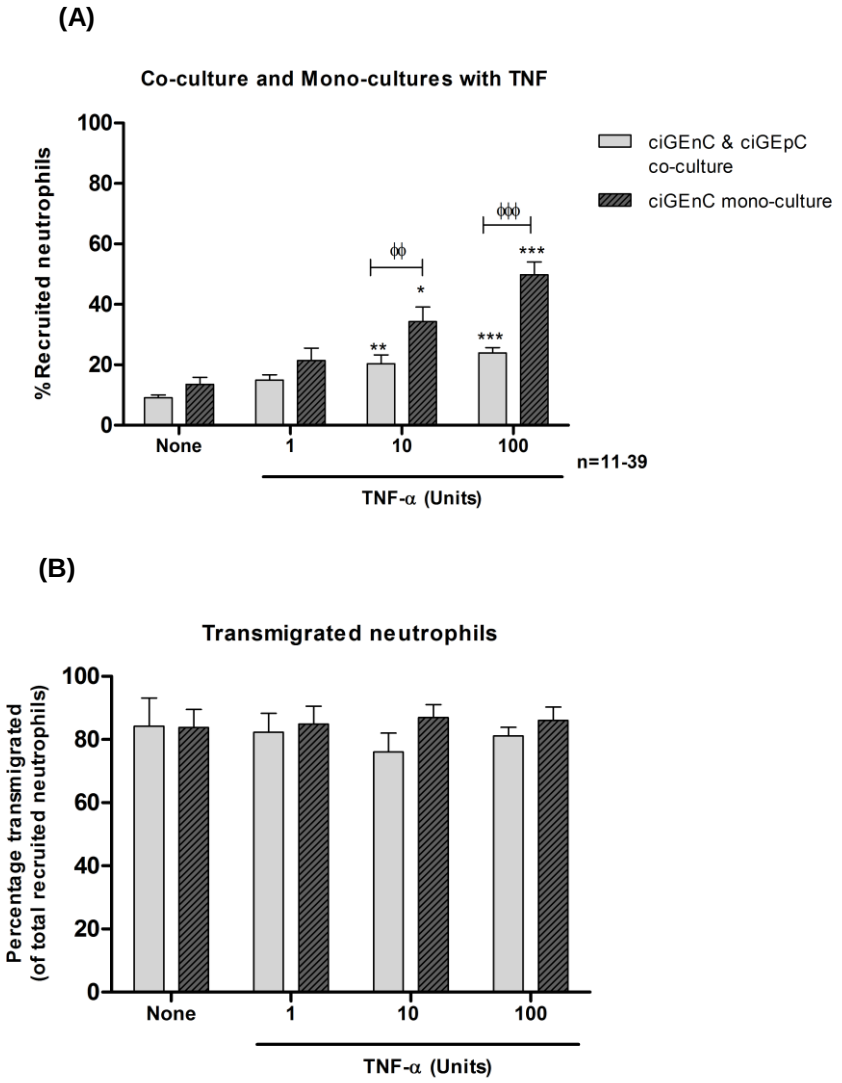


Figure 30: Neutrophil recruitment by ciGEnC mono-cultures or ciGEnC co-cultured with podocytes stimulated with TNF-α at 0 (none) to 100 Units/ml. (A) Percentage of the total recruited neutrophils to unstimulated and TNF stimulated co-cultures of ciGEnC and podocytes or ciGEnC cultured alone. (B) Percentage of neutrophils that demonstrated transmigration from the total recruited neutrophils across ciGEnC co-cultured with podocytes or cultured alone. In all experiments, co-cultures were compared with mono-cultures, and were run in parallel.

Statistics: The symbols (* P<0.05, ** P<0.01 & *** P<0.0001 by one way ANOVA) represents significance of TNF treatments compared to the control by one way ANOVA in cultures. The symbols ($^{\phi\phi}$P<0.01 & $^{\phi\phi\phi}$P<0.0001 by two way ANOVA) represents significance of TNF treatments between cultures by Dunnet's and Bonferroni's multiple comparison test.

In contrast, HUVECs when co-cultured with podocytes$_{14day}$ demonstrated no significant reduction in neutrophil recruitment when compared to HUVEC mono-cultures (Figure 31A) despite increasing TNF-α concentrations.

With both the cell types, the majority ($\geq$80%) of total recruited neutrophils were observed to transmigrate through the cell layers and only a small sub-population were left firmly adherent at the EnC surface at the time the assay was terminated (3.61–5.19% of adherent cells on EnC co-cultured with podocytes$_{14\ day}$) with both ciGEnC (Figures 30 B) and HUVEC cultures (figures 31 B).

All the above data suggests that ciGEnCs and podocytes are capable of cross talk resulting in suppression of neutrophil recruitment. Comparable crosstalk between HUVEC and podocytes was not observed.

Figure 31: Effect of 14-day differentiated podocytes on neutrophil recruitment by HUVEC

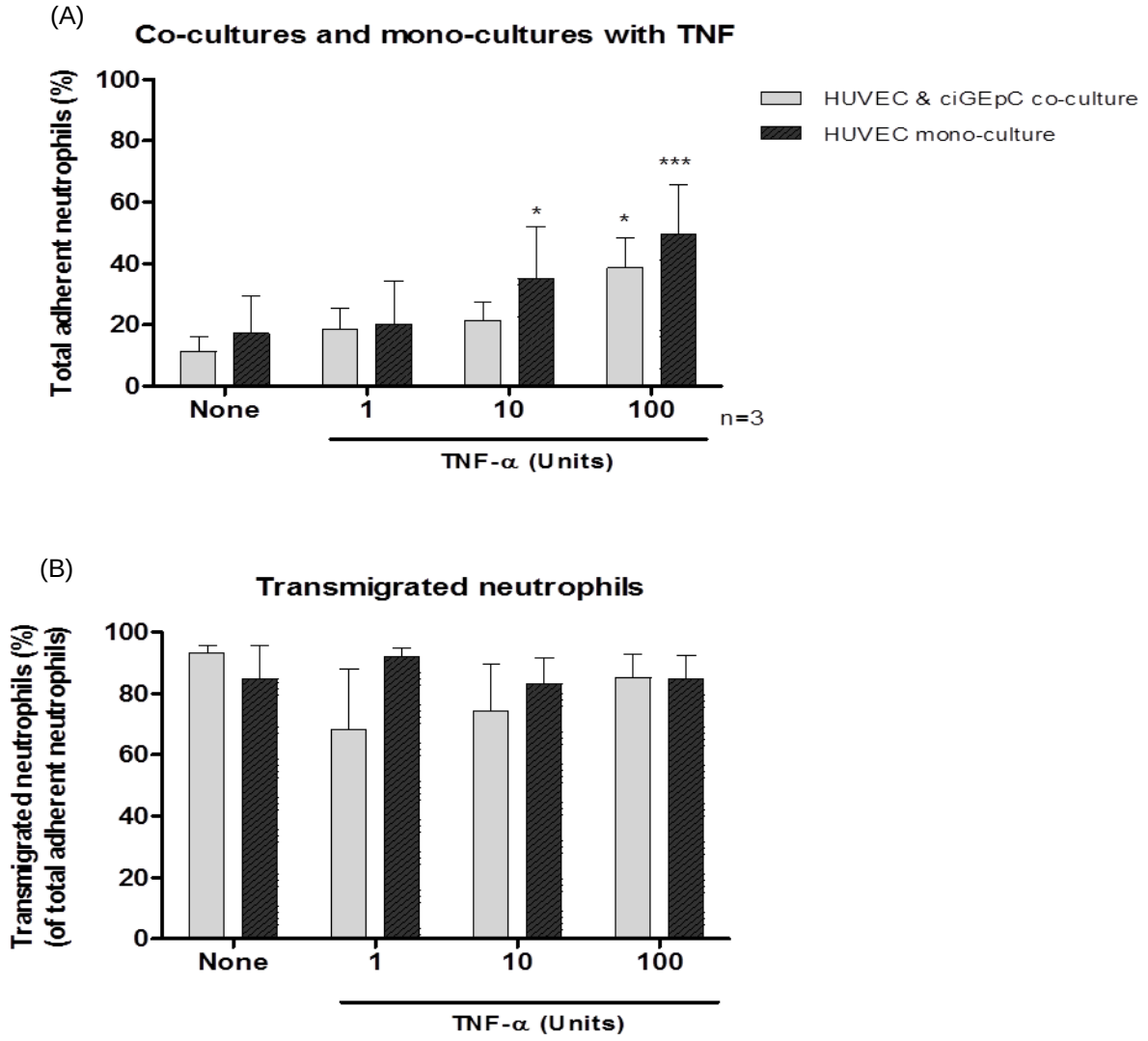


Figure 31: Neutrophil recruitment by HUVEC mono-cultures or HUVEC co-cultured with podocytes stimulated with TNF-α at 0 (none) to 100 Units/ml. (A) Percentage of total recruited neutrophils to unstimulated and TNF stimulated HUVEC co-cultured with podocytes or cultured alone. (B) Percentage of neutrophils that demonstrated transmigration from the total recruited neutrophils across HUVEC co-cultured with podocytes or cultured alone. In all experiments, co-cultures were compared with mono-cultures, and were run in parallel.

Statistics: *The symbols (* P<0.05 & *** P<0.0001 by one way ANOVA) represents significance of TNF treatments compared to the control by one way ANOVA.*

4.3.6 Effects of primary podocytes on neutrophil recruitment by primary GEnC

Primary GEnCs were co-cultured with primary podocytes and were subjected to TNF-α treatment in a dose dependent manner (0-100 Units/ml). The cultures demonstrated a dose dependent increase in neutrophil recruitment when compared to their individual controls in both co-cultures and mono-cultures. Primary GEnCs co-cultured with primary podocytes also demonstrated reduced neutrophil recruitment as was observed with cell line cultures. In these cultures, the reduced neutrophil recruitment by co-cultures was observed with and without TNF-α treatments and the reduction was significant at higher concentration of TNF-α (100 Units/ml).

There was 5% reduction in neutrophil recruitment in unstimulated co-cultures compared to mono-cultures. Following 10U/ml TNF-α treatment, primary GEnC co-cultured with primary podocytes showed a reduction in neutrophil recruitment by up to 14%. Neutrophil recruitment (% of total neutrophils added) in co-cultures vs mono-cultures was 8.6 $\pm$ 1.3% and 15.07 $\pm$ 5.4%, n=4. At 100U/ml TNF-α treatment, co-cultures of GEnC in direct contact with podocytes demonstrated significant reduction in neutrophil recruitment by up to 30% when compared to GEnC mono-cultures. Neutrophil recruitment in primay co-cultures vs mono-cultures was 18.8 $\pm$ 4.8% and 31.3 $\pm$ 5.5%, n=4 (Figure 32A). Similar to the cell line cultures, the majority of total recruited neutrophils were observed to be transmigrated through the cell layers (figures 32B). The above data also suggests that primary GEnCs and podocytes have the ability to cross talk and play a collaborative role in regulating neutrophil recruitment.

Figure 32: Neutrophil recruitment by primary cultures of GEnC and podocytes

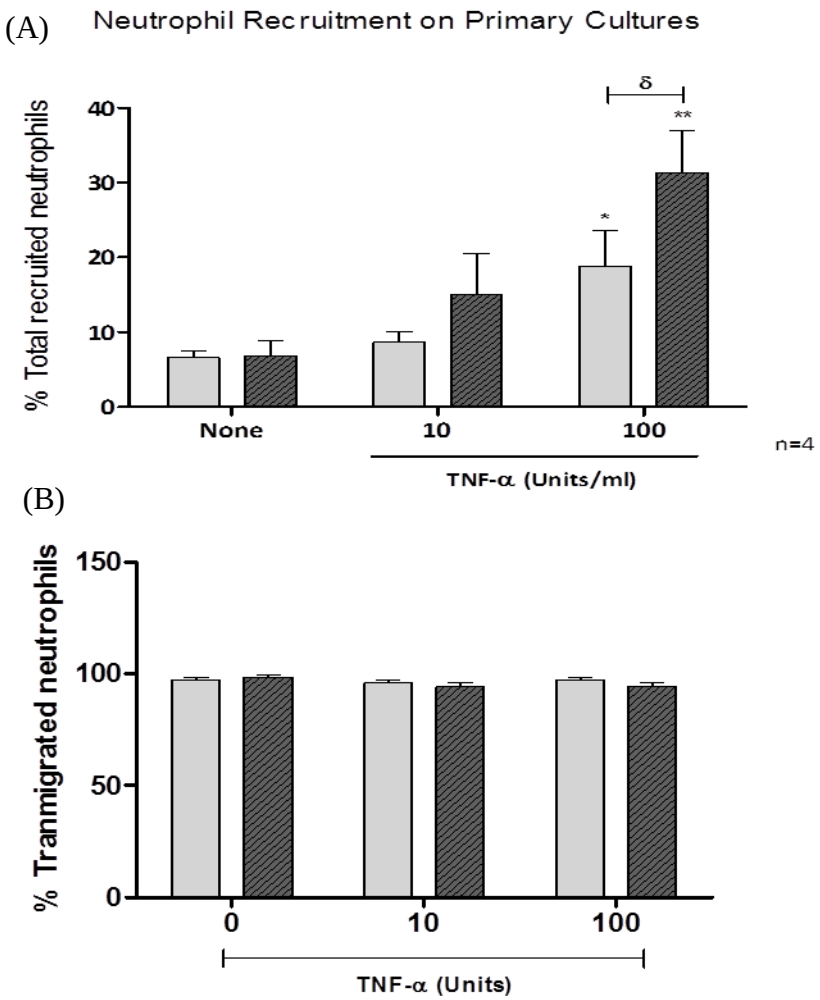


Figure 32: Neutrophil recruitment by primary GEnC mono-cultures or co-cultured with primary podocytes stimulated with TNF-α at 0 (none) to 100 Units/ml. (A) Percentage of total recruited neutrophils to unstimulated and TNF stimulated GEnCs co-cultured with podocytes or cultured alone. (B) Percentage of neutrophils that demonstrated transmigration from the total recruited neutrophils across GEnCs co-cultured with podocytes or cultured alone.

Statistics: *The symbols (* P<0.05 & *** P<0.0001 by one way ANOVA) represents significance of TNF treatments compared to the control by one way ANOVA. The symbol ($^{\delta}$P<0.05 by two way ANOVA) represents significance between cultures by Dunnet's multiple comparison test.*

4.3.7 Co-culture of EnCs and podocytes in the absence of direct contact

Previous experiments showed that podocytes in direct contact could reduce endothelial dependent neutrophil recruitment. In order to examine whether the effects observed were contact based or due to soluble mediators released by the cells, we cultured podocytes and EnCs such that no cellular contact was possible as in figure 10, ensuring that the cross talk could only occur via mediators.

The results observed showed a similar pattern to that seen previously with the contact based co-cultures of ciGEnC with podocytes$_{14\ day.}$ ciGEnC that had been in the presence of podocytes bound neutrophils less efficiently in the system when compared to ciGEnC mono-cultures, although this did not reach significance except at 100 U/ml TNF-α treatment. The effect of TNF-α treatment was significant in individual cultures; there was dose dependent increase in neutrophil recruitment with increasing TNF-α concentration (0-100Unitsml).

Following 100U/ml TNF-α treatment, there was 40% reduction in neutrophil recruitment in co-cultures compared to mono-cultures which reached significance. Neutrophil recruitment (% of total neutrophils added) in co-cultures vs mono-cultures was 28.5 $\pm$ 7% and 47.3 $\pm$ 15.2%, n=3. At lower concentrations of TNF-α treatment and in unstimulated cultures there was no reduction in neutrophil recruitment (Figure 33A).

In the case of HUVEC co-cultured with podocytes$_{14day}$ without direct contact, the neutrophil recruitment was similar in both co-culture and HUVEC mono-cultures. The effect of TNF-α treatment was significant in individual cultures; there was dose dependent increase in neutrophil recruitment with increasing TNF-α concentration (0-100Unitsml). This suggests

that HUVEC are not affected by the soluble mediators released by podocytes (Figure 33B).

The presence of podocytes that had no direct contact with ciGEnCs had significant effect on neutrophil recruitment. In contrast, podocytes only reduced neutrophil recruitment to HUVEC by 0.5%, when compared to control mono-cultures of HUVEC. However, the magnitude of the responses induced in the absence of direct contact tended to be 10% less than those observed where cells were only separated by a porous filter. Thus, close juxtaposition of podocytes with EnC may potentiate the activity of putative soluble mediators.

Figure 33: Neutrophil recruitment by ciGEnCs and HUVEC co-cultured with podocytes with no contact, compared to mono-cultures

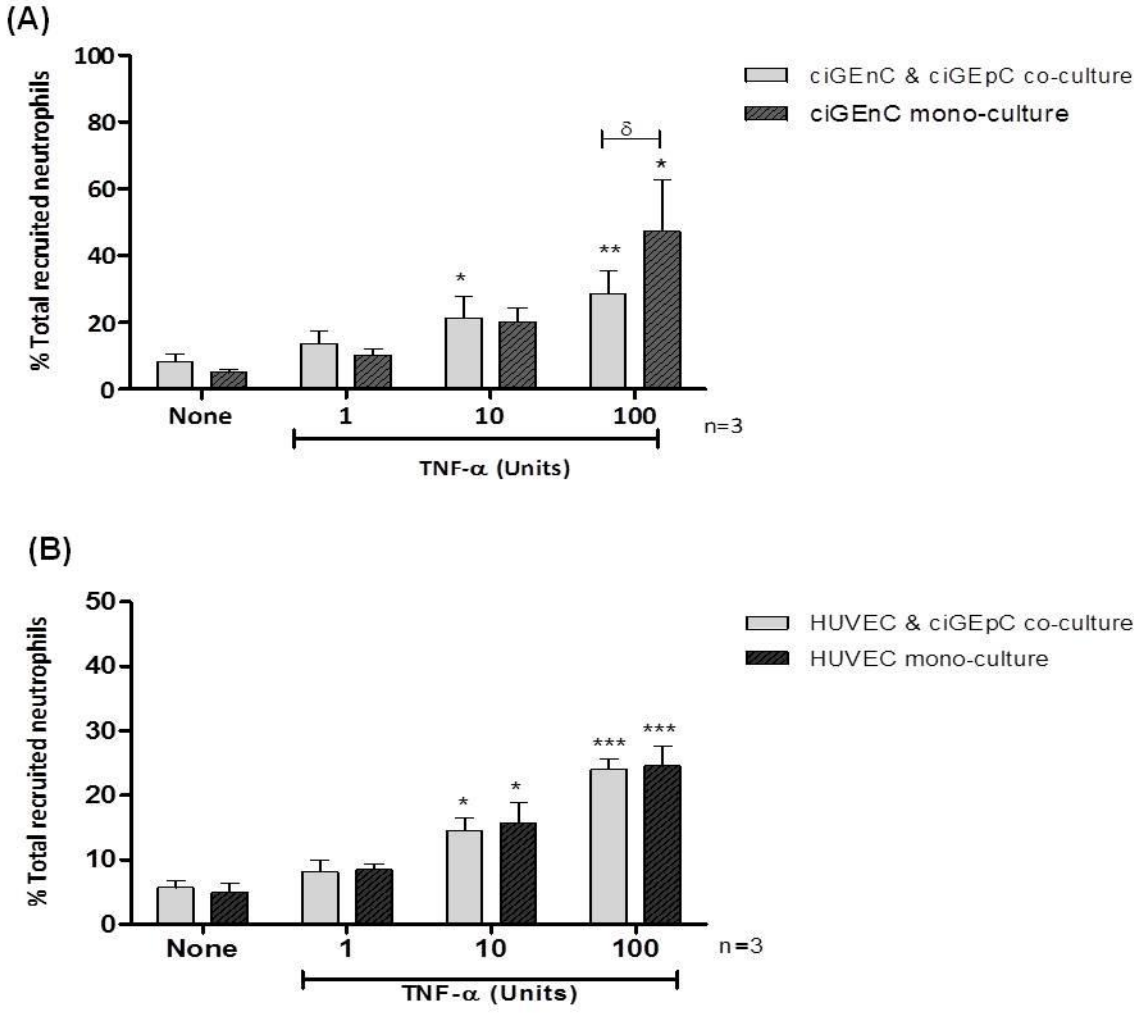


Figure 33: Neutrophil recruitment to ciGEnCs & HUVECs cultured alone or with podocytes, assuring no direct contact between the cell types and the cultures were stimulated with TNF-α at 0-100U/ml. (A) Percentage of the total recruited neutrophils to ciGEnC co-cultured with podocytes or cultured alone. (B) Percentage of the total recruited neutrophils to HUVEC co-cultured with podocytes or cultured alone. Statistics: *The symbols (* P<0.05, ** P<0.01 & *** P<0.0001 by one way ANOVA) represents significance of dose dependent TNF treatments compared to the control by one way ANOVA. The symbol ($^{\delta}$P<0.05 by two way ANOVA) represents significance between cultures by Dunnet's multiple comparison test.*

4.3.8 Effects of soluble mediators released into podocyte medium on EnC-neutrophil recruitment

The effect of conditioned media from 14-day differentiated podocytes on neutrophil recruitment following treatment with different concentrations of TNF-α was examined. The physical presence of podocytes in close contact with EnCs significantly reduced neutrophil recruitment by EnCs particularly at higher concentrations of TNF-α. Interestingly, the supernatants from podocytes treated for 24 hours had a suppressing activity on neutrophil recruitment by ciGEnCs reaching significance especially with TNF 1Unit/ml treatment (Figure 34A). Similarly, HUVEC treated with podocyte conditioned medium for 24hrs also showed a decrease in endothelial responses with TNF 100Units/ml treatment although there was a lot of variation between the experiments and so this effect did not reach statistical significance (Figure 34B). The above data complements the results from previous experiments which suggested that mediators produced in the presence of podocytes do influence recruitment of neutrophils by ciGEnCs but not HUVEC cultures and may be suggesting organ specific cross talk between the cells.

Figure 34: Effect of soluble mediators released into podocyte conditioned medium on neutrophil recruitment by EnCs

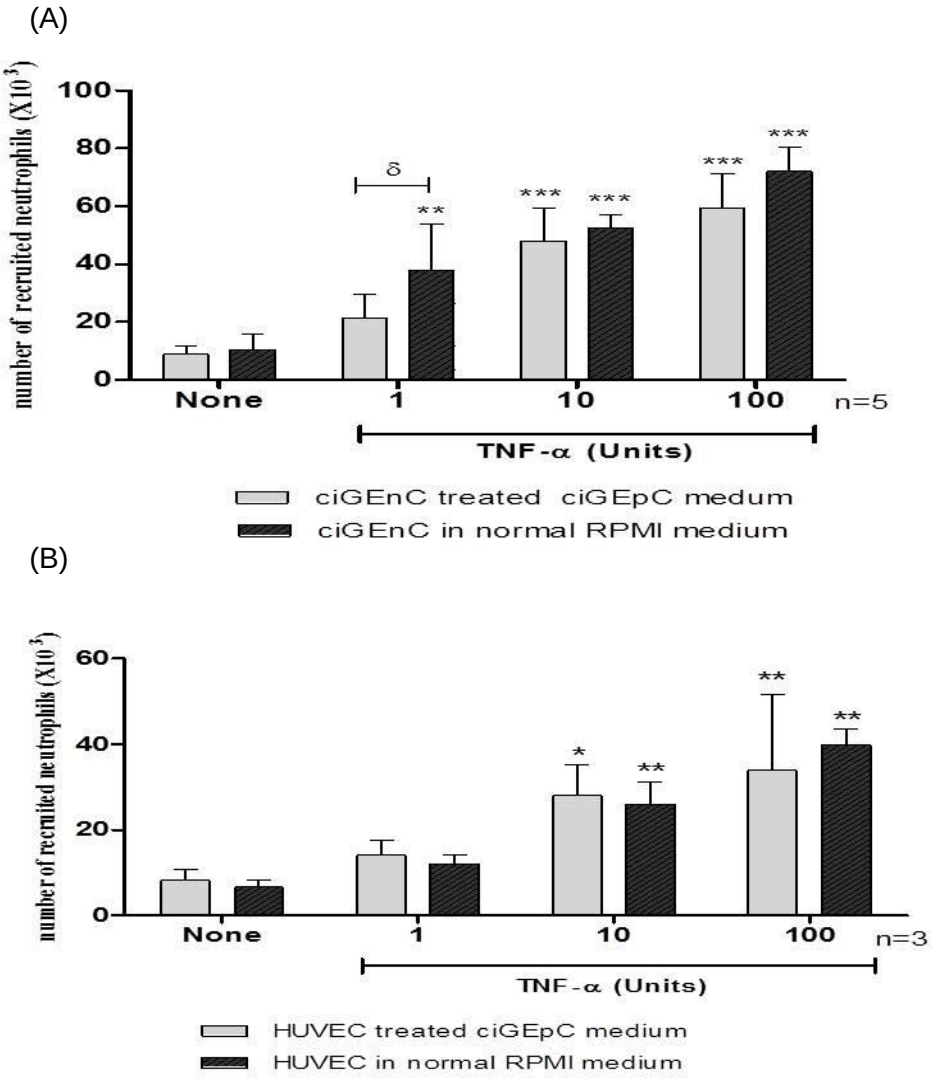
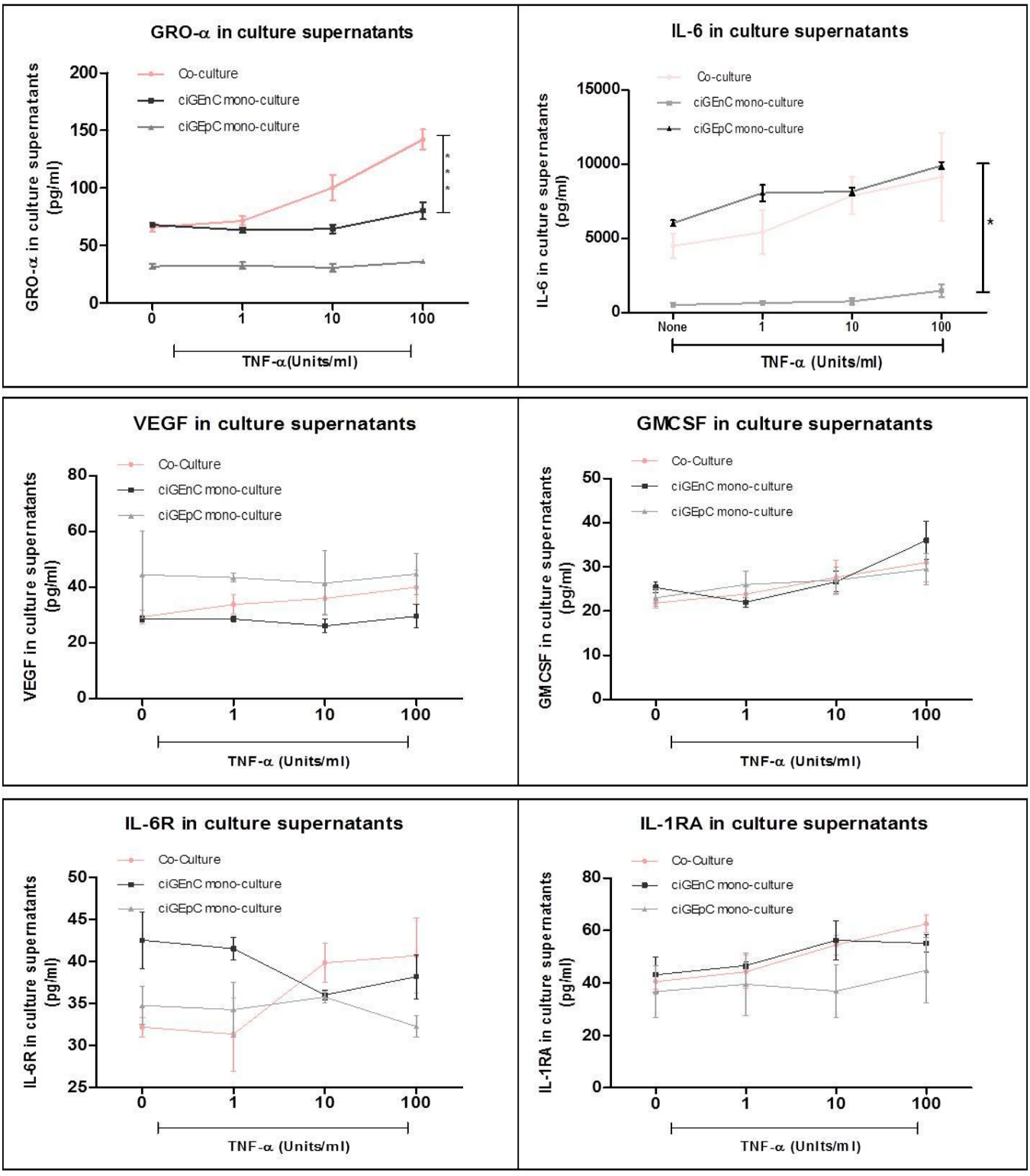


Figure 34: Neutrophil recruitment by ciGEnCs and HUVEC treated with and without podocyte conditioned medium for 24h and stimulated with TNF-α at 0-100U/ml. (A) Total number of neutrophils recruited by ciGEnC cultured with and with out podocyte conditioned medium where conditioned medium is the medium collected from 14-differentiated podocytes cultures. (B) Total number of neutrophils recruited by HUVEC cultured with and without podocytes conditioned medium. HUVEC and ciGEnC experiments were run in parallel. Statistics: *The symbols (* P<0.05, ** P<0.01 & *** P<0.0001 by one way ANOVA) represents significance of dose dependent TNF treatments compared to the control by one way ANOVA and the symbol ($^{\delta}$ P<0.05 by two way ANOVA) represents significance of TNF 1unit/ml treatment between cultures by Bonferroni's multiple comparison test.*

4.3.9 Detection of cytokine and chemokine profiles from the supernatants collected from EnC and podocytes co-cultures and single cultures following TNF-α treatment and neutrophil recruitment

Samples were subjected to multiplex bead assay by LUMINEX to detect levels of a panel of cytokines and chemokines that had a potential mechanistic relation to neutrophil recruitment by ciGEnC and HUVEC. The results showed detectable amounts of neutrophil specific ELR positive CXC chemokines, including IL-8 and GRO-α in ciGEnC co-cultured with podocytes. There was significant release of IL-6 release in co-cultures of ciGEnC and podocytes compared to single cultures, suggesting its possible involvement.

Figure 35: Soluble mediators released into culture supernatants by ciGEnC and podocytes in mono-cultures and co-cultures by Luminex



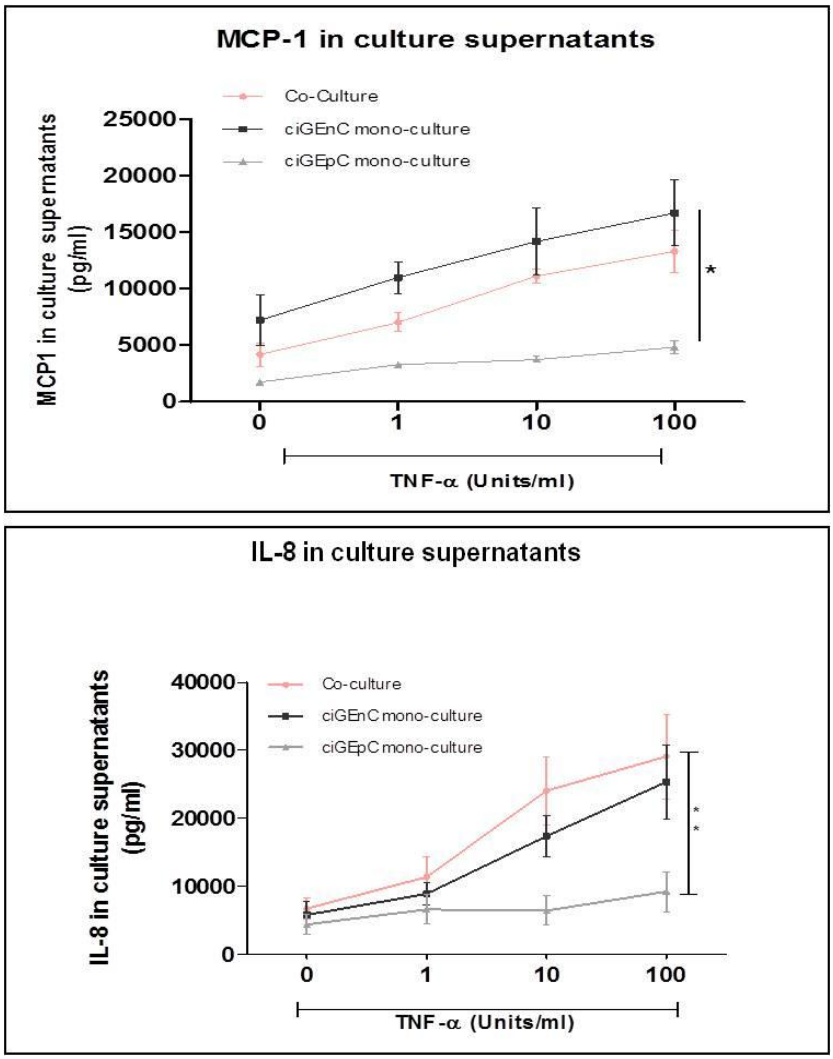


Figure 35: Cytokine and chemokine levels obtained by subjecting the supernatants from mono-cultures of ciGEnC or podocytes or from ciGEnC co-cultured with podocytes by LUMINEX.

4.3.10 Detection of Angiopoeitin-2 in supernatants from co-culture and mono-cultures

In the process of detecting the molecules involved in neutrophil recruitment by GEnC in co-culture with podocytes and in mono-cultures, the supernatants collected from the unstimulated and stimulated cultures were tested for the presence of angiopoietin-2 using quantikine ELISA kit from R & D systems. The results revealed no significant difference in release from the cultures of GEnC in co-culture with podocytes and in mono-cultures.

Figure 36: Detection of angiopoeitin-2 in supernatants from co-culture and mono-cultures

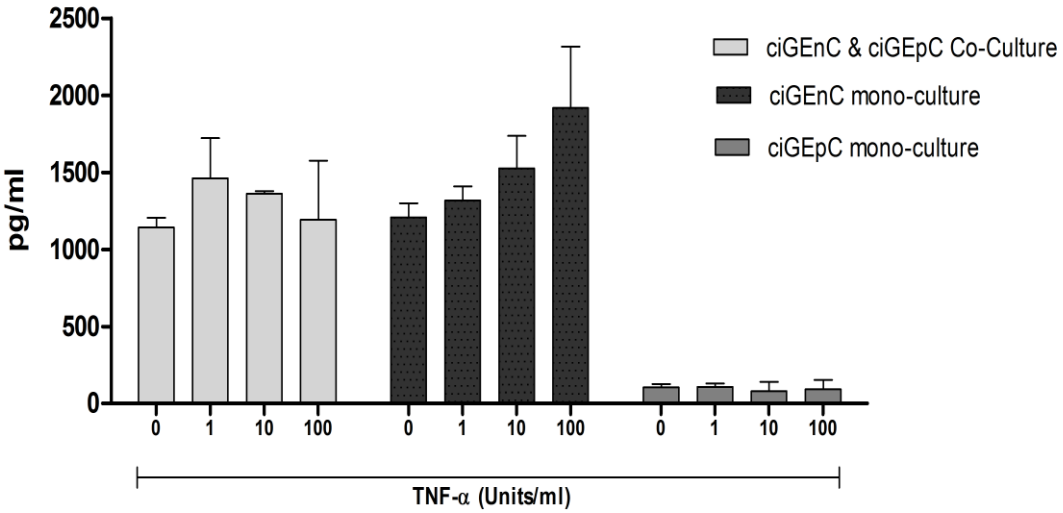


Figure 36: Angiopoeitin-2 levels in culture supernatants from mono-cultures of ciGEnC or podocytes or from ciGEnC co-cultured with podocytes by ELISA.

4.3.11 Detection of tumor growth factor (TGF)-β in supernatants from co-culture and mono-cultures

In the process of detecting the molecules involved in the neutrophil recruitment by GEnC in co-culture with podocytes and in mono-cultures, the supernatants collected from the unstimulated and stimulated cultures were tested for the presence of TGF-β using quantikine ELISA kit from R & D systems. The results revealed no significant difference in release from the cultures of GEnC in co-culture with podocytes and in mono-cultures.

Figure 37: Detection of active TGF- β in supernatants from co-culture and mono-cultures

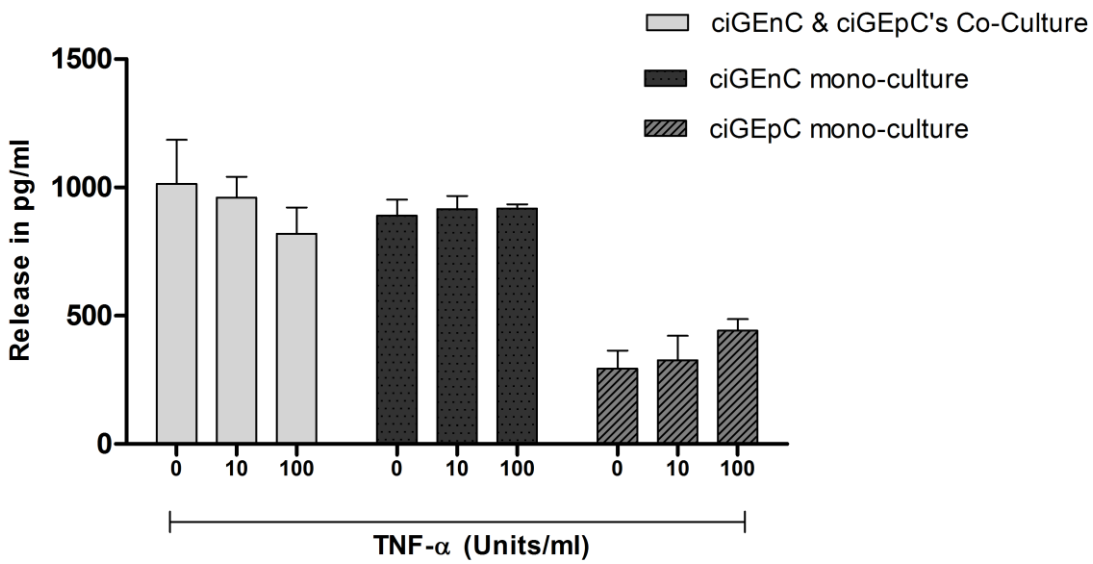


Figure 37: TGF-β levels in culture supernatants from mono-cultures of ciGEnC or podocytes or from ciGEnC co-cultured with podocytes by ELISA.

4.3.12 Involvement of CXC receptors in neutrophil recruitment by ciGEnC in mono-culture and in co-culture with podocytes

We dissected the involvement of CXC chemokine receptors in neutrophil recruitment by testing antibodies against the CXC receptors 1 & 2 (CXCR1 & CXCR2). CXCR1 & 2 are the receptors that are expressed on the surface of neutrophils and recognise CXC chemokines with an ELR motif. CXCR1 displays greater ligand specificity than CXCR2 and binds with high affinity to IL-8 and granulocyte chemotactic protein. CXCR2 binds multiple CXC chemokines in addition to IL-8, including epithelial neutrophil-activating peptide 78 (ENA-78), neutrophil-activating peptide-2, growth-related oncogene (GRO)-α, -ß, -γ, and LPS-induced CXC chemokines.

In co-cultures, the neutrophil recruitment was significantly reduced with CXCR2 blockade but not with CXCR1 blockade. This trend was evident at all the TNF-α concentrations, but reached significance in cultures treated with TNF-α 100U/ml (Figure 38). As IL-8 is specific to CXCR1, this shows that the transmigration is not mediated by IL-8 alone, indicating the involvement of molecules other than (or in addition to) IL-8 that signal through the CXCR2 receptor (Figure 38).

Figure 38: Role of CXC chemokine receptors in neutrophil recruitment by EnCs in co-cultures and mono-cultures

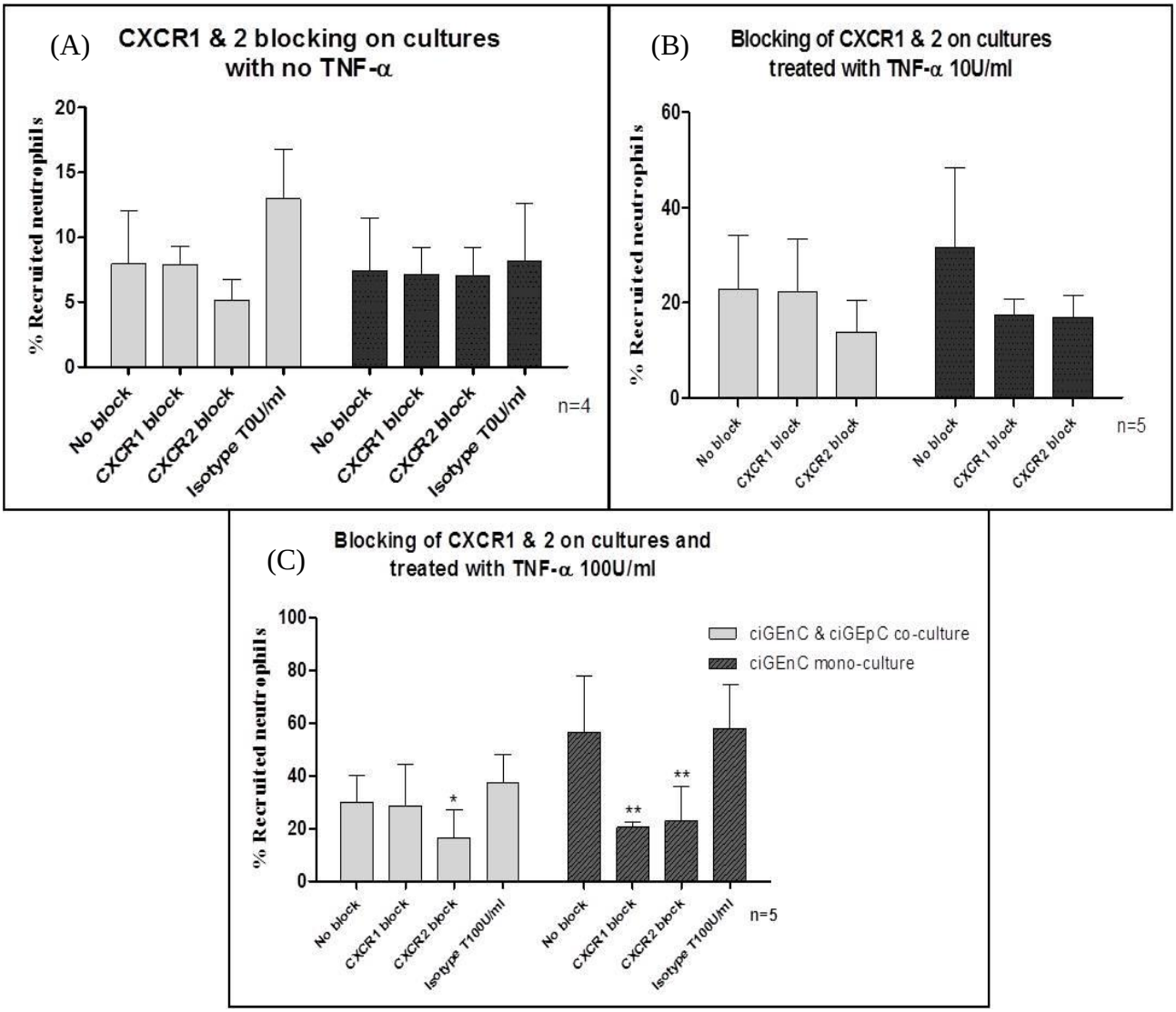


Figure 38: Effect on neutrophil recruitment on blocking CXC receptors 1 & 2 in both co-cultures and mono-cultures treated without (A) and with TNF-α 10U/ml (B), TNF-α 100U/ml (C). Mouse IgG used as isotope control. Statistics: ANOVA followed by bonferroni post test showed significant effect of blocking on neutrophil recruitment in TNF-α stimulated (*<0.05) co-cultures and mono-cultures (**P<0.001).

4.3.13 Mechanism of neutrophil recruitment in EnC - podocyte co-cultures; the role of CXC chemokines

Following the data obtained by LUMINEX and ELISA, the mechanism by which the neutrophils are recruited by the ciGEnCs in both co-cultures and mono-cultures has been evaluated.

The levels of certain CXC chemokines including IL-8, GRO-α and ENA-78 were measured in the supernatants of ciGEnC and podocyte co-cultures and mono-cultures by multiplexing and ELISA. Co-culture supernatants showed significantly increased levels of IL-8, GRO-α and ENA-78 (Figure 39A to C respectively). The amount of IL-8 released in co-cultures was higher (29087 pg/ml) compare to ciGEnC mono-cultures (25,363 pg/ml) and significantly more compared to podocyte mono-cultures (9236pg/ml). This data suggested a role of IL-8 in enhancing recruitment of neutrophils in cultures (Figure 39A).

There was also a prominent and significant increase in soluble GRO-α in the co-culture supernatants (142pg/ml) compared to mono-cultures of either ciGEnC (80.3pg/ml) or podocytes (36pg/ml) (Figure 39B). Detection of soluble ENA-78 also showed similar findings. There was a significant increase in soluble ENA-78 in the co-culture supernatants (281pg/ml) compared to mono-cultures of either ciGEnC (128.3 pg/ml) or podocytes (18.5 pg/ml) (Figure 39C).

Figure39: Detection of IL-8, GRO-α and ENA-78 in culture supernatants

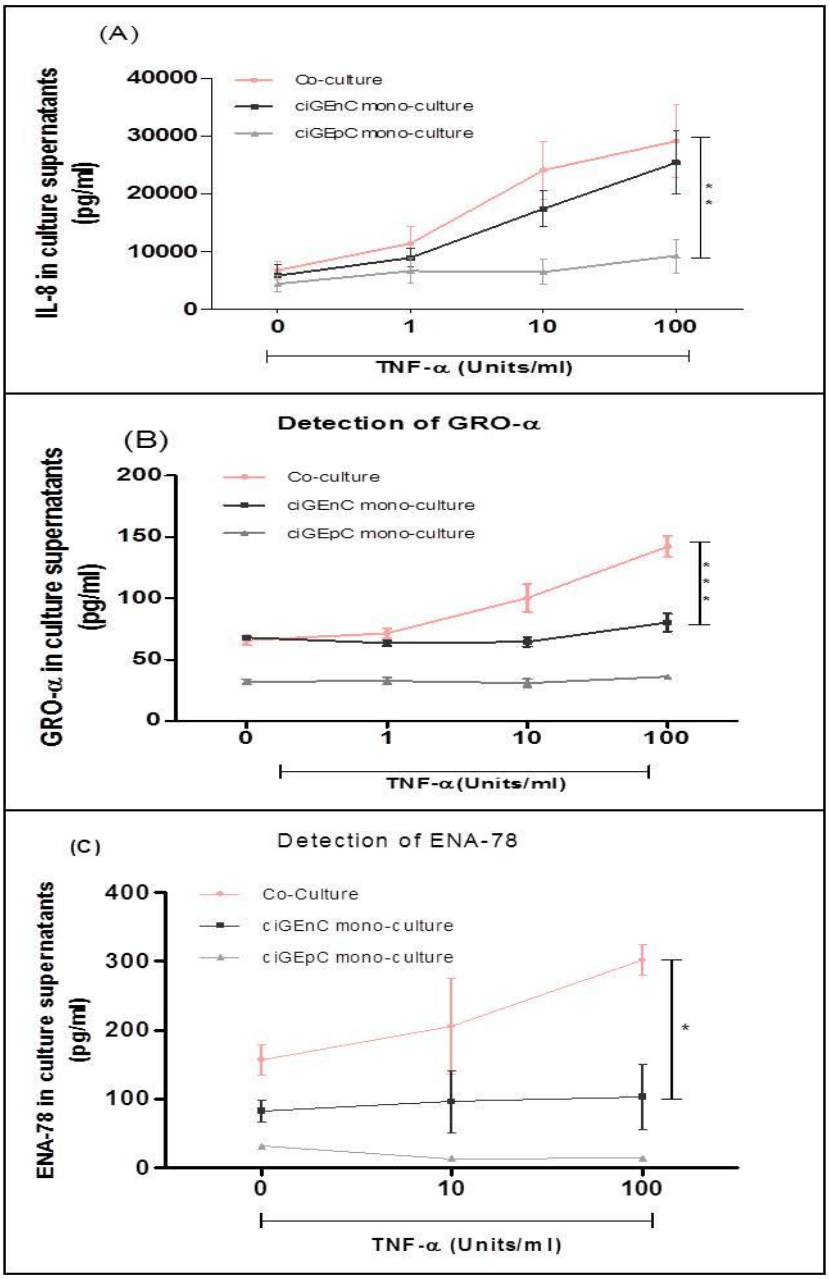


Figure 39: Detection of CXC chemokines (IL-8, GRO-α, ENA-78). (A) Detection of soluble IL-8 in supernatants collected from co-cultures and mono-cultures of ciGEnC and podocytes. (B) Detection of soluble GRO-α in supernatants collected from co-cultures and mono-cultures of ciGEnC and podocytes. (c) Detection of soluble ENA-78 in culture supernatants collected from co-cultures and mono-cultures of ciGEnC and podocytes. Data shown is the mean from 3 independent experiments. Statistics: *The significance * P<0.05, ** P<0.01 & *** P<0.0001 between the cultures is by two way ANOVA.*

Blocking the CXC chemokines:

Blocking antibodies against IL-8, GRO-α and ENA-78 were incorporated in the co-culture system to determine the chemokines mediating the neutrophil recruitment in co-cultures. Blocking of IL-8 in both mono-cultures and co-cultures revealed that neutrophil recruitment in ciGEnC mono-cultures was significantly mediated by IL-8, whereas in co-cultures the effect did not reach significance (Figure 40A). Interestingly, despite detecting significantly increased levels of GRO- α in co-cultures, blocking did not show any effect on neutrophil recruitment (Figure 40B). However, blocking of ENA-78 in cultures revealed that ENA-78 operates through CXCR2 and is playing a significant role in recruiting neutrophils in co-cultures (Figure 40C).

Figure 40: Blocking of chemokines to detect their involvement in neutrophil recruitment

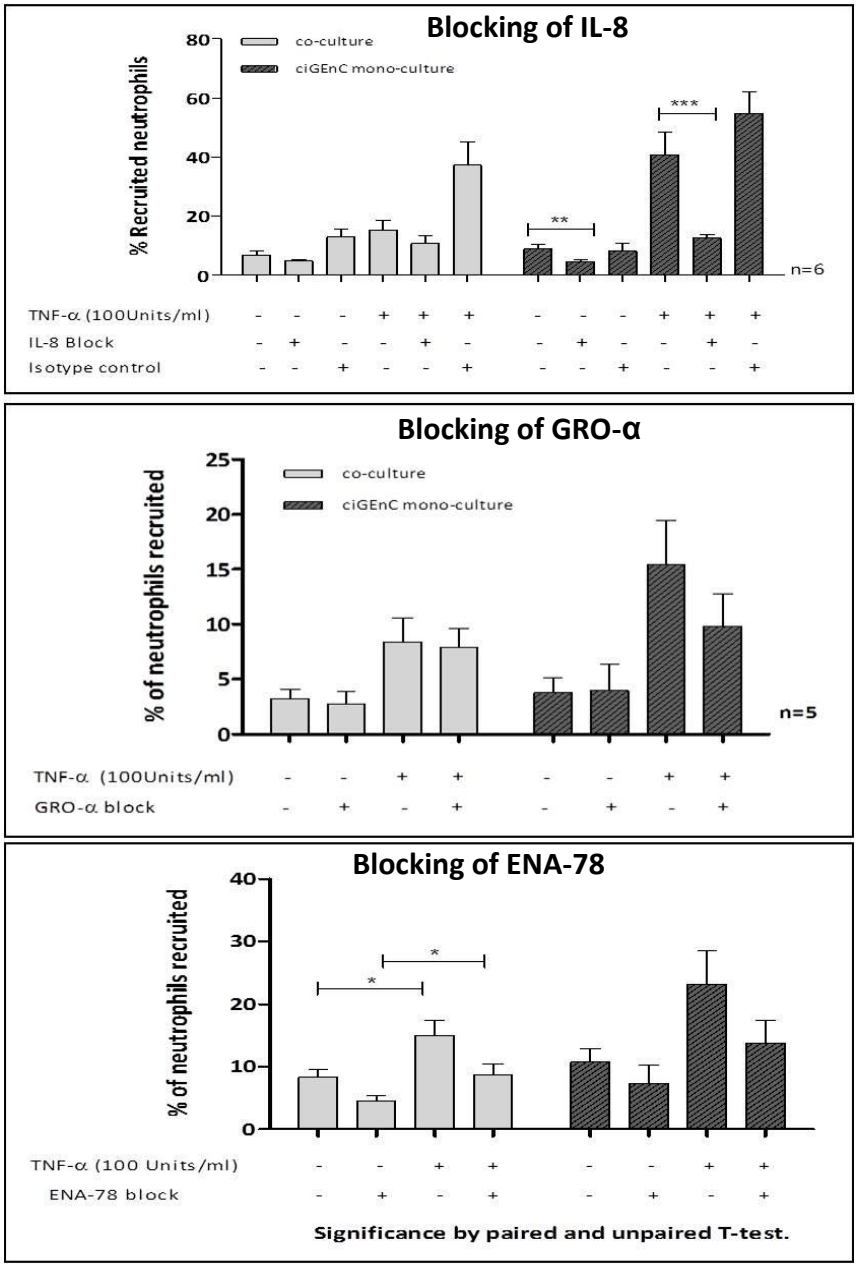


Figure 40: Blocking of CXC chemokines (GRO-α, ENA-78 & IL-8) to detect their role in neutrophil recruitment. (A) Blocking of IL-8 in co-cultures and mono-cultures. (B) Blocking of GRO-α in mono-cultures and co-cultures. (C) Blocking of ENA-78 in both co-cultures as well as mono-culture. Data shown is the mean from 5-6 independent experiments, using different blood donors for each experiment. Mouse IgG used as isotope control and all the three antibodies were raised in mouse. Statistics: The significance * P<0.05, ** P<0.01 & *** P<0.0001 between the treatments by one and two way ANOVA.

4.3.14 Effect of IL-6 neutralisation on neutrophil recruitment in co-cultures

IL-6 was detected by multiplex assay as a principle soluble mediator since it was present at significant levels in co-culture supernatants (up to 10,000pg/ml), and it appeared to be released by podocytes in the cultures (Figure 41A). To determine the involvement of IL-6 in neutrophil recruitment, a function neutralising antibody was added at the commencement of co-cultures and maintained all the way through the experiment making sure that there was no IL-6 exposure to ciGEnCs. The results showed significant recovery of neutrophil recruitment in both unstimulated and cytokine stimulated GEnC co-cultured with podocytes (Figure 41B & C). Hence, suggesting the possible role of IL-6 in the reduction of neutrophil recruitment in co-cultures of ciGEnC and podocytes.

Figure 41: Detection and neutalisation of IL-6 in cultures

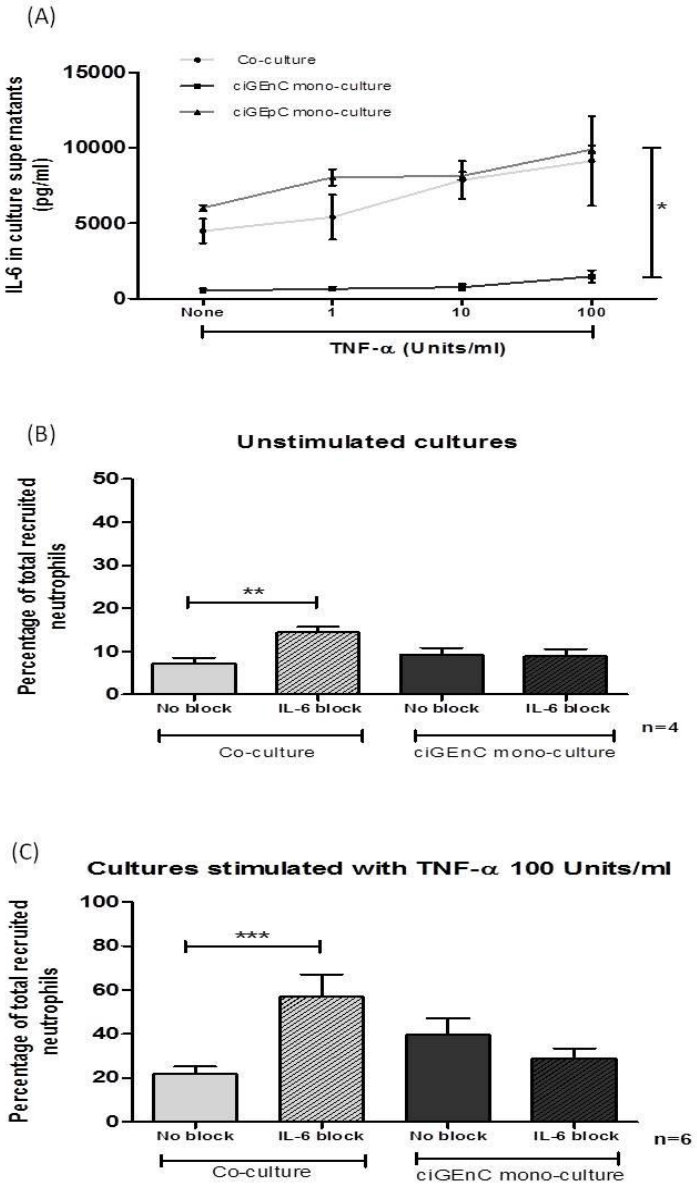


Figure 41: Detection and neutralising IL-6 on neutrophil recruitment on co-cultures and mono-cultures: (A) Detection of IL-6 in supernatants collected from co-cultures and mono-cultures. (B) Neutralisation in unstimulated ciGEnC and podocytes co-cultures, and ciGEnC mono-cultures; (C) neutralisation in TNF–α stimulated cultures. Data shown is the mean from 4-8 independent experiments, using different blood donors for each experiment. Statistics: One way and two way ANOVA followed by bonferroni post test showed significant effect of IL-6 blocking on neutrophil recruitment in both unstimulated (** $P<0.01$) and stimulated (***$P<0.001$) co-cultures when compared to mono-cultures.

4.3.15 Recapitulation of the co-culture response in response to TNF with exogenous IL-6 (10ng/ml) in glomerular endothelial cell cultures

The role of IL-6 was further elucidated by adding exogenous IL-6 into mono-cultures of ciGEnC. IL-6 (10ng/ml) was added exogenously into the mono-cultures, using a concentration similar to that observed in co-culture supernatants and maintained throughout the experiment in order to recreate the co-culture response. Reduction in neutrophil recruitment was observed in both the cultures of unstimulated and cytokine stimulated cultures. Significant reduction in neutrophil recruitment was observed in the cultures stimulated with 100Units/ml TNF-α when compared to the ciGEnC mono-cultures with no exogenous IL-6 (Figure 42).

Figure 42: Recapitulation of co-culture effect by exogenous IL-6 in ciGEnC mono-cultures

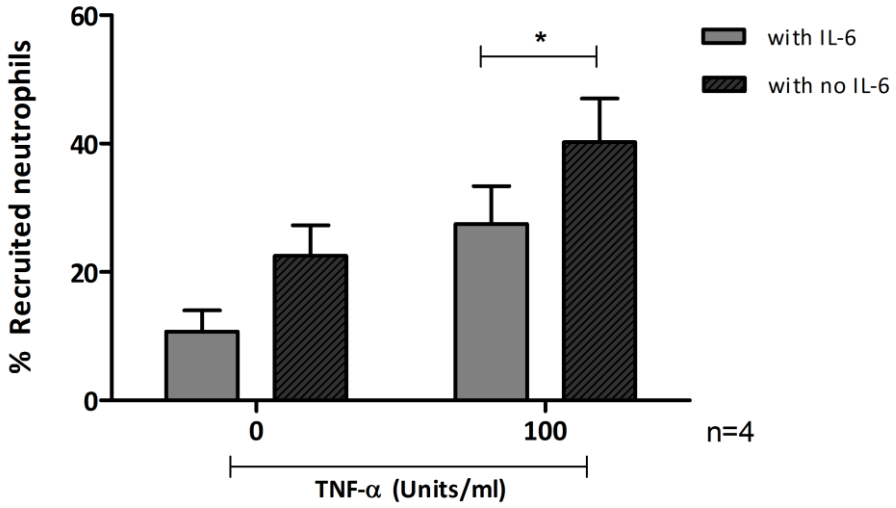


Figure 42: ciGEnC mono-cultures with and without exogenous IL-6 maintained for 24hrs prior and throughout the experiment. Data is represented as percentage of the neutrophils added in each experiment.
Statistics: Two way ANOVA followed by bonferroni post test showed significant effect in cultures with and without IL-6.

4.3.16 Co-culture of ciGEnC with different epithelial cells of other origins

Podocytes in co-cultures were replaced with other epithelial cells like renal tubular epithelial cells (HKC), Epithelial like liver cell line (AKN) and human retinal pigment epithelial cell line (ARPE-19), in order to test whether the replacement of podocytes with other epithelial cells mediate the similar response that was observed in co-cultures of podocytes and GEnCs. When compared to podocyte co-culture, HKC cell line presented similar reduction in neutrophil recruitment (Figure 43A). The other cell lines from liver and retina showed dramatic decrease in neutrophil recruitment. Testing the ER of these co-cultures showed that co-cultures of ciGEnC & podocytes as well as ciGEnC & HKC presented similar ER value of 20 Ωcm^2, where as other co-cultures showed increased ER values up to 60 Ωcm^2 (Figure 43B).

Figure 43: Effect of different epithelial cells in co-culture with ciGEnC - Podocytes replaced by HKC (renal tubular epithelial cells), AKN (epithelial-like liver cell line) and ARPE-19 (human retinal pigment epithelial cell line)

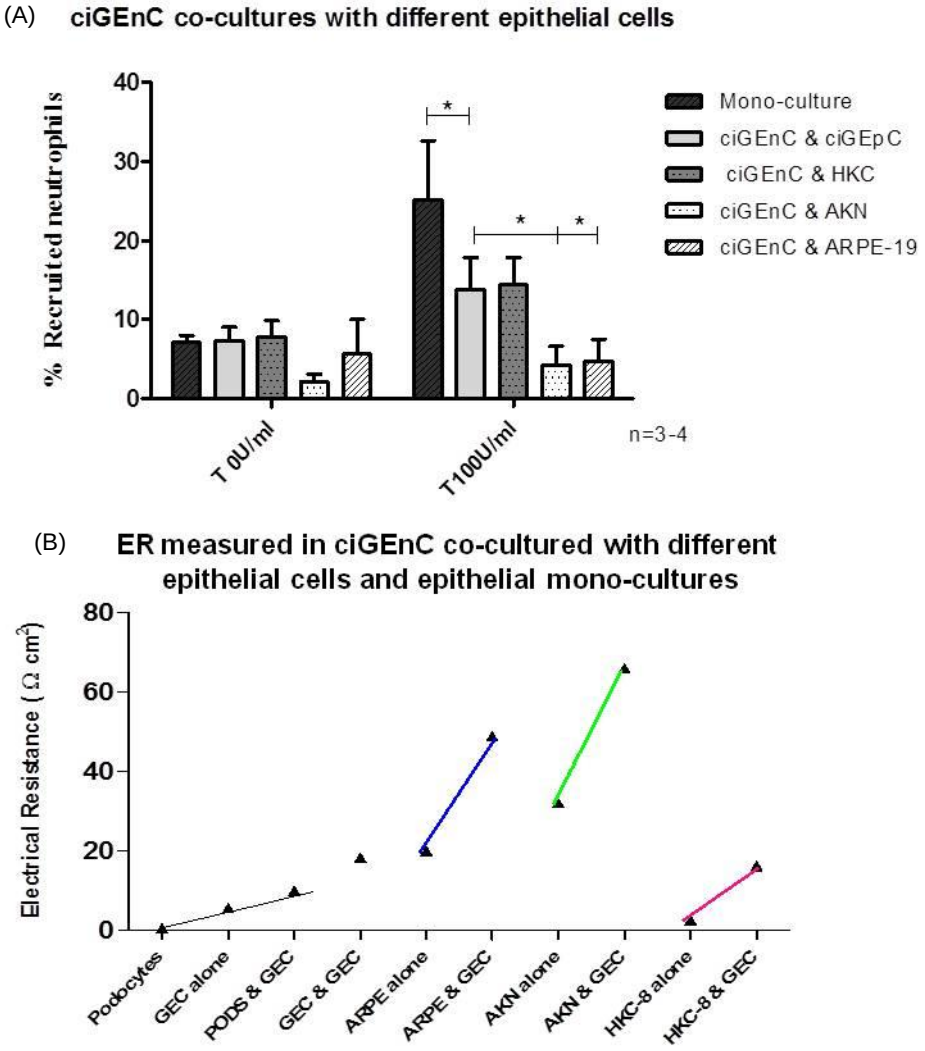


Figure 43: (A) ciGEnC were co-cultured with epithelial cells of different origins and maintained for 24hrs prior to the experiment and a static neutrophil recruitment assay was performed. (B) Electrical resistance measured in ciGEnC co-cultured with different epithelial cells and epithelial mono-cultures Statistics: Two way ANOVA followed by bonferroni post test showed significant effect between cultures.

4.3.17 Does Endothelial/endothelial co-cultures and epithelial/epithelial co-cultures reduce the neutrophil recruitment compared to GEnC mono-cultures?

Podocytes in co-cultures were replaced with ciGEnC or GEnC were replaced by podocytes having endothelial co-culture and epithelial co-culture, in order to test whether the double layer of cells can reproduce the reduced effect of neutrophil recruitment as was seen in GEnC co-cultured with podocytes. When compared to podocyte co-culture with ciGEnC, endothelial co-culture increased neutrophil recruitment similar to ciGEnC mono-cultures. The epithelial co-culture showed increased neutrophil recruitment compared to podocytes in mono-culture.

Figure 44: Co-culture of ciGEnC with ciGEnC and podocytes with podocytes

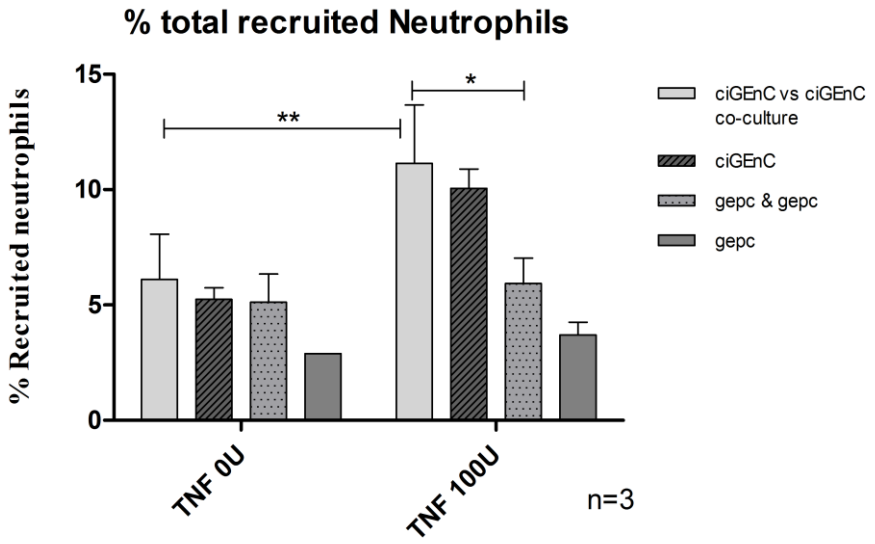


Figure 44: ciGEnC were co-cultured with ciGEnCs forming endothelial co-culture and similarly podocytes were cultured with podocytes forming podocyte co-culture and maintained for 24hrs prior to the experiment and a static neutrophil recruitment assay was performed.

4.3.18 Detection of IL-6 in HUVEC supernatants

IL-6 was detected by multiplex assay in podocyte and HUVEC co-culture supernatants (Figure 41A). HUVEC culture supernatants from HUVEC mono-cultures had increased levels of IL-6, in response to TNF-α along with increased levels in co-cultures (upto1700 pg/ml). The levels in HUVEC cultures were lower to that GEnC co-culture supernatants (upto10,000 pg/ml). The effect of IL-6 released in these cultures had no effect in regulation of neutrophil recruitment when HUVEC were co-cultured with podocytes.

Figure 445: Detection of IL-6 in HUVEC co-culture supernatants

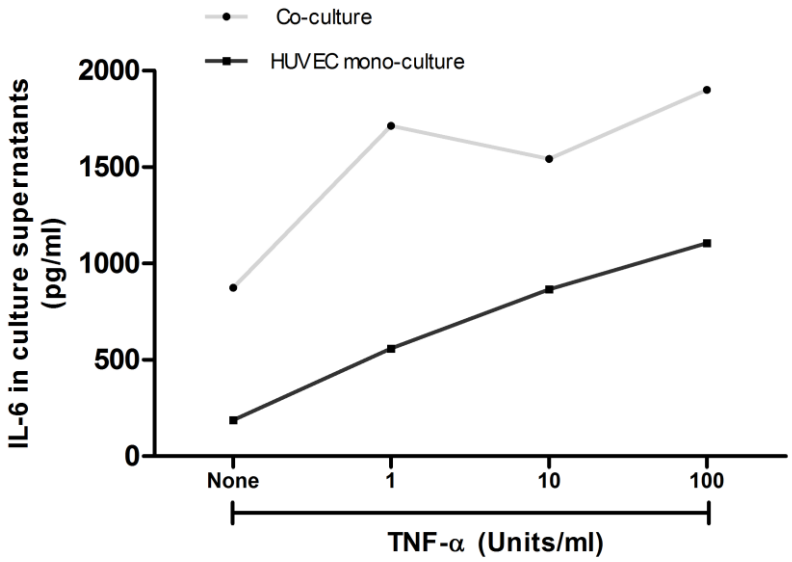


Figure 45: IL-6 levels in culture supernatants from mono-cultures of HUVEC or from HUVEC co-cultured with podocytes by LUMINEX.

4.4 Discussion

In glomeruli, the maintenance of vascular endothelial functions depends on a symbiosis between the podocytes and GEnCs. However, little is known about the involvement of podocytes in immune regulation. Thus, it was of interest to elucidate the role of podocytes in inflammation in particular to determine whether they could help protect GEnCs from various forms of glomerular injury.

Previous *in vitro* studies on leukocyte recruitment have been largely limited to endothelial monolayer models that have provided key information regarding the involvement of different adhesion molecules, cytokines and chemo attractants (reviewed by (Adams and Shaw, 1994; Alon et al., 1996; Baggiolini, 1998; Springer, 1995)). However, the *in vivo* situation is more complex since infiltrating leukocytes need to pass through the basement membrane and will likely contact stromal cells. Thus we developed an *in vitro* co-culture experimental model to mimic the complex glomerular micro-environment and to investigate neutrophil infiltration in the context of glomerular inflammation. Monolayers of glomerular endothelial and epithelial cells, both primary cells and cell lines that were separated by a semi permeable membrane allowing cell-cell contact enabled us to study the interactions between these glomerular cells. A key observation was significantly reduced level of neutrophil binding to TNF-α activated GEnCs in the presence of podocytes particularly at higher concentrations of TNF-α treatment that was indicative of a more activated inflammatory environment. Interestingly, these findings may be specific to renal glomerular cells as similar findings were not observed in the co-cultures of HUVEC with podocytes.

The reduction in neutrophil recruitment was apparent whether the podocytes and GEnC were in juxtaposition or separated within the same well. However, effects were more marked when cells were in juxtaposition such that cell to cell contact was possible.

Observations of transepithelial ER over time might suggest loss of compact monolayer integrity in podocyte cultures, but phase contrast images provide evidence of presence of podocytes on the insert and nuclear staining confirmed this. Similar findings have been observed by other investigators (Coers et al., 1996; Van Den Berg et al., 2000). Observations from our cultures also suggest that they do not show monolayer integrity as they become differentiated, as shown in the picture of differentiating podocyte monolayer (Figure 5). These observations support the results of the co-cultures with GEnCs, in that the reduced neutrophil recruitment is not due to the presence of two monolayers across the filters but instead, it is the presence of podocytes and their soluble mediators that are playing a role in the reduction. These observations point to an important role for soluble mediators, as well as a mechanistic role for direct cell to cell contact.

Usually within the glomerulus the inflammatory processes are switched off by the release of anti-inflammatory mediators that oppose the actions of proinflammatory mediators. Niemir et al., showed concomitant expression of IL-1 receptor type I and II (IL-1 RI) as well as IL-1 RA in podocytes which suggests a protective mechanism within glomeruli to counteract the effects of intrinsic glomerular production of IL-1 against continuing injury (Lovett and Sterzel, 1986; Niemir et al., 1997). IL- 10, also known as human cytokine synthesis inhibitory factor (CSIF), has also been shown to be greatly involved in suppressing glomerular inflammation due to its strong anti-inflammatory effects. Podocytes were shown to have an activated phenotype in two-dimensional cultures releasing

cytokines which affect the function of surrounding cell types [2, 29]. Podocytes have previously been shown to be a potential source of IL-6 that could play role in the regulation of processes critical to the initiation of progressive glomerular disease (Moutabarrik et al., 1994b; Osawa et al., 1994). Given a potential role for soluble mediators in our cultures, we were interested to observe that higher levels of IL-6 was detected both in co-cultures, as well as in supernatants derived from podocytes alone. Addition of exogenous IL-6 in GEnC mono-cultures led to reduced neutrophil recruitment, when compared to cultures with no IL-6, supporting a role for IL-6 in regulating the neutrophil recruitment. Furthermore, IL-6 blocking antibodies reversed the reduction in neutrophil binding. These observations are consistent with previous reports where IL-6 was shown to be responsible for the inhibition of neutrophil recruitment to EnC co-cultured with dermal fibroblasts from individuals with rheumatoid arthritis (Helen et al., 2009; Lally et al., 2005). In vivo studies have demonstrated the attenuation of LPS induced pulmonary neutrophil infiltration in the presence of exogenous IL-6 and also complete abolishment of the over reactivity of cytokine responses (Ulich et al., 1991b; Xing et al., 1998). Interestingly, IL-6 has also been shown to have opposing effects *in vitro* and so its effects may be dependent on the exact milieu (McGettrick et al.).

Podocytes express IL-6R and transduces the IL-6 signal (Moutabarrik et al., 1994b), in contrast, there is no evidence that GEnCs express IL-6R on their surface. IL-6 interaction with EnCs that express only the gp130 signal transducing chain occurs via the involvement of soluble IL-6R and subsequent negative trans-signalling (Heinrich et al., 1998; Taga et al., 1989). It is notable that the soluble IL-6R (sIL-6R) was not detectable in the podocyte culture supernatants harvested from either unstimulated or cytokine-stimulated cultures, findings that were confirmed in the present study (Moutabarrik et al., 1994b). However, the

presence of sIL-6R (data not shown) in the supernatants of the cultures following the addition of neutrophils suggests that sIL-6R from neutrophils consequently mediates signalling that might contribute to the previously described effects of IL-6 on neutrophil recruitment (Ulich et al., 1991b; Xing et al., 1998).

Thus, sIL-6R along with IL-6 in the cultures may have induced the reduction in neutrophil recruitment observed in our studies (Modur et al., 1997). Alternatively, IL-6 may have stimulated IL-6 receptors expressed by podocytes with generation of secondary mediators responsible for inhibiting neutrophil recruitment. Previously, IL-6 has been demonstrated to suppress LPS induced TNF-α and IL-1 in macrophages both *in vivo* and *in vitro* (Aderka et al., 1989; Bode et al., 1999; Ulich et al., 1991a). Thus, it is conceivable that the low release of soluble mediators from GEnC that were observed following treatment with podocyte conditioned medium may reflect direct suppressive properties of IL-6. Further studies are needed to establish the pathophysiological implications of IL-6 in inhibition of inflammatory responses by TNF-α in co-cultures and signalling events involved in the process.

There was enhanced neutrophil transmigration across the layers in both mono and co-cultures. The chemotactic and chemokinetic signals which regulate neutrophil recruitment by the GEnC were explored in the co-cultures and mono-cultures. Here, we show that blockade of CXCR2 (IL8RB) was sufficient to reduce neutrophil recruitment in co-cultures while blocking of both CXCR1 (IL8RA) and CXCR2 affected neutrophil recruitment on GEnC in monocultures. This suggested the possible involvement of chemokines other than IL-8 in co-cultures since CXCR1 only binds IL-8 while CXCR2 binds all the ELR chemokines. (Ahuja and Murphy, 1996; Imaizumi et al., 1997; Luster, 1998). In co-cultures, interactions of ENA-78 with CXCR2 seems to be playing a dominant role despite

the increased levels of IL-8 and GRO- α expression in the cultures, since recruitment was significantly reduced by blocking ENA-78 activity. Previous studies have shown that trans-signalling of IL-6 not only blocks pro-inflammatory cytokines and suppresses IL-8 and GRO- α, but also promotes release of ENA-78 and CXCL-6 (Hurst et al., 2001; McLoughlin et al., 2004; McLoughlin et al., 2003; Modur et al., 1997), which is analogous to the findings with the co-cultures shown here. The increased transmigration in the co-culture system may be due to the hydrostatic pressure with in the system and the dedifferentiated appearance of podocytes. Coers *et al.,* investigated the effect of basolateral hydrostatic pressure on podocyte biology *in vitro* and found cell-cell contacts were often found more basally and intercellular spaces are widened (Coers et al., 1996). These spaces might have supported enhanced transmigration. However, when considering total neutrophil recruitment, the percentage of neutrophil recruitment in co-cultures is significantly reduced compared to mono-cultures. Thus, we conclude that TNF-α treated GEnCs in co-culture capture neutrophils through CXCR2 to ligate to CXC-chemokine(s) and there by induce neutrophil retention in these cultures.

The model described here appears relevant to the healthy glomerular environment and offer a means to study physiological processes regulating inflammation in human tissues. In conclusion, these studies reveal that podocytes can protect and modulate the inflammatory response mediated by GEnCs particularly during inflammatory insults via mechanisms that involve cell-cell contact and soluble mediators. These observations once again highlight the anti-inflammatory paracrine and autocrine actions of IL-6. The importance of organ specific cross talk is well documented within these renal cell populations.

Chapter 5: Superimposing a disease inflammatory insult on co-cultures of glomerular cells

5.1 Introduction

Infiltration of phagocytic leukocytes in kidney glomeruli is a pathological finding in many kidney diseases (Eddy et al., 1990). Both circulating inflammatory cells and resident glomerular cells can mediate acute glomerular injury. The acute injury can transition towards a chronic state by the release of various cytokines and growth factors which result in deposition of extracellular matrix leading to scarring and sclerosis of the kidneys (reviewed in (Couser, 1998)).

During VGN, neutrophils activated by ANCA interact with glomerular endothelium and sometimes leads to its damage. Neutrophils are the major cell type involved in the antibody-induced glomerular injury and ANCA were shown to promote neutrophil recruitment to cytokine activated endothelium provoking endothelial damage within glomerular capillaries (Charles et al., 1991; Ewert et al., 1995; Ewert BH, 1992; Falk et al., 1990b; Keogan et al., 1992; Little et al., 2005b). The ANCA activated neutrophils localize in glomeruli and undergo degranulation, respiratory burst followed by the release of SP and toxic oxygen metabolites, particularly hydrogen peroxide which reacts with MPO (Johnson et al., 1988; Johnson et al., 1987; Shah, 1989) inducing glomerular damage and our previous work stands as a possible proof (Kuravi and Bevins publication in press) (Lu et al., 2006; Lu et al., 2004). The recruitment of ANCA activated neutrophils could promote

a dynamic interplay between resident tissue cells which ultimately contributes to the pathophysiology of renal inflammation.

Very little is known about podocyte involvement in immune mediated injury and about their active involvement in regulation of inflammation. Podocyte apoptosis and their detachment from basement membrane was shown to contribute to glomerular injury and podocytes were also shown to be part of the formation of glomerular crescents in crescentic GN (Thorner et al., 2008). Evidences highlight the importance and contribution of podocytes in normal physiological functions, as well in disease states. Under pathological conditions, failure of the podocytes certainly results in the initiation of progressive renal diseases leading to proteinuria and progressing to end-stage renal failure. The inability of terminally differentiated podocytes to proliferate and repair or replace damaged podocytes in an injured glomerulus may be pivotal in chronic and end-stage renal disease. Foot process effacement is a hallmark of glomerular injury leading to proteinuria in the vast majority of human glomerulopathies and animal models of proteinuric glomerular disease, with rare exceptions (reviewed by shankland (Shankland, 2006)). Podocyte dysfunction can be due to genetic, congenital and acquired causes leading to disruption of the slit diaphragm and filtration barrier (Debiec et al., 2004; Kestilä et al., 1998; Kim et al., 2003; Ronco and Debiec, 2005; Tryggvason K, 1999). Podocyte effacement as described by Farquhar et al in 1957 (Farquhar, 1957), is an extensive process observed in biopsies of patients with nephritic syndrome. Effacement constitutes fusion or retraction and is a stereotypical reaction of podocytes to injury or damage caused by disruption of components of the slit diaphragm, deregulation of the actin cytoskeleton and alteration of the negatively charged apical domain of podocytes (Reidy and Kaskel, 2007). This dramatic alteration of podocyte complex cellular architecture is followed by cell hypertrophy, vacuolisation and detachment

from the basement membrane leading to the apoptosis of the podocytes and thus progression of glomerular diseases.

Thus, our studies explored the role displayed by podocytes in regulating inflammation and we have investigated ANCA associated inflammation in a co-culture system to further explore the importance of podocytes in the resolution of inflammation. We also injured podocytes and studied the effect on the inflammatory response which might be a leading cause of chronic and intensive inflammatory states as in VGN.

5.2. Methods:

1. Isolation of ANCA from plasma exchange fluid of patients.

2. Endotoxin removal from ANCA IgG.

3. Co-culturing of podocytes and GEnCs and neutrophil recruitment assay introducing ANCA activated neutrophils.

4. MTT assay on PAN treated podocytes.

5. Co-culturing of PAN treated podocytes and GEnCs and neutrophil recruitment assay.

5.3 Results:

From previous chapter, it was clear that the presence of podocytes along with GEnC modulate the inflammatory stimuli with reduced neutrophil retention. This current chapter further elucidates the protective role displayed by podocytes under disease conditions, by adding ANCA activated neutrophils to TNF-α stimulated GEnC in mono-cultures and in co-culture with podocytes that were in healthy state or otherwise injured by PAN.

5.3.1 Effects of ANCA activated neutrophils in co-culture with podocytes in contact with ciGEnC and HUVEC

EnCs co-cultured with podocytes that had been differentiated for 14 days were subjected to treatment with TNF (2 Units/ml). Static neutrophil recruitment assays were performed, adding neutrophils activated by ANCA in the cultures for 1hr. As observed with 14-day podocyte co-cultures, ciGEnCs showed reduced recruitment of ANCA activated neutrophil recruitment in the presence of podocytes, when compared to ciGEnC mono-cultures in cultures stimulated even with lower concentrations of TNF-α (2U/ml) (Figure 37 A). The data shows that normal IgG activate neutrophils in the cultures whilst reduction in neutrophil recruitment seems to be prominent in co-cultures.

Following 2U/ml TNF-α treatment, ciGEnC co-cultured with podocytes showed a significant reduction in total neutrophil recruitment of $\geq$50% of neutrophil recruitment observed with ciGEnC mono-cultures and MPO ANCA activated neutrophils. In the presence of PR3 ANCA, the co-cultures presented reduced neutrophil recruitment up to 50% which did not

reach significance (Figure 45A). The neutrophils that were adherent, transmigrated away from the endothelium more in co-cultures than in mono-cultures (Figure 45B).

In contrast, HUVEC co-cultured with podocytes did not show any reduction in neutrophil recruitment with the presence of PR3 ANCA compared to mono-cultures (Figure 45C). The majority ($\geq$80%) of total adherent neutrophils were observed to be transmigrated through the cell layers and only a small sub-population were left firmly adherent at the EnC surface at the time the assay was terminated similar to GEnC cultures (Figure 45D).

These findings also suggest that podocytes have the ability to suppress neutrophil recruitment even at low concentrations of TNF stimulation in the presence of ANCA by up to 60% via direct contact. The cross talk was again shown to be specific to glomerular cells, as HUVEC in co-culture did not present similar response. Thus, close juxtaposition of podocytes with ciGEnC may promote an anti-inflammatory phenotype that suppresses neutrophil recruitment to a certain extent.

Figure 45: Recruitment of ANCA activated neutrophils in co-cultures of ciGEnC and podocytes

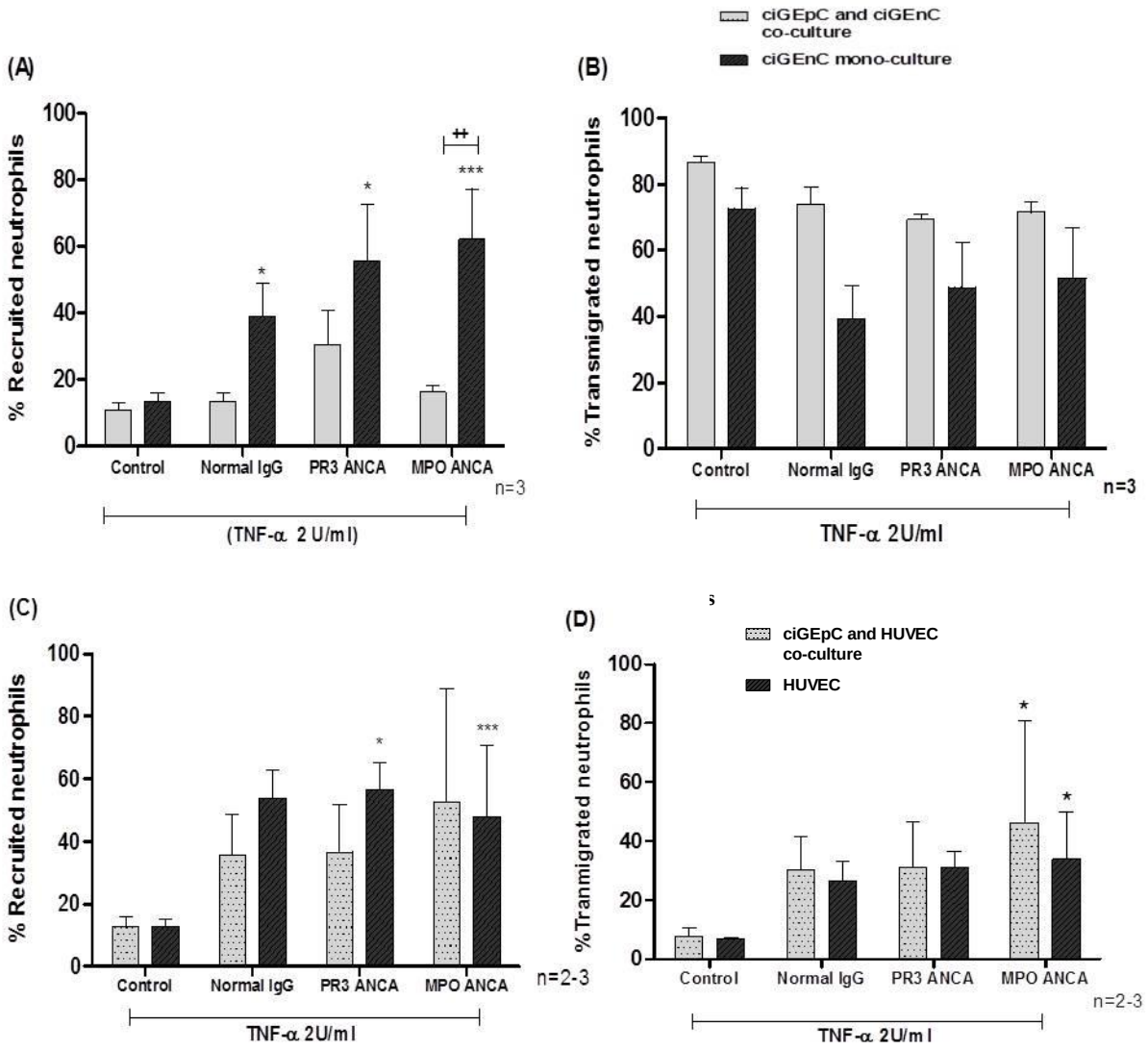


Figure 45: ANCA activated neutrophil recruitment by endothelium in co-culture with podocytes stimulated with TNF-α (2 U/ml). (A) Percentage neutrophil recruitment by ciGEnC co-cultured with podocytes or cultured alone in the presence and absence of ANCA IgG. (B) Percentage transmigrated neutrophils across ciGEnC co-cultured with podocytes or cultured alone. (C) Percentage neutrophil recruitment by HUVEC co-cultured with podocytes or cultured alone in the presence and absence of ANCA IgG. (D) Percentage transmigrated neutrophils across HUVEC co-cultured with podocytes or cultured alone. Statistics: The significance by one way ANOVA, comparing to the control in co-cultures.

5.3.2 Effects of ANCA activated neutrophils in co-culture of podocytes in contact with ciGEnC (using IgG without endotoxin)

As previous studies from section 5.3.1 had shown responses with normal IgG, we suspected the activity of endotoxin in the IgG. Endotoxins are unique glycoproteins such as lipopolysaccharides (LPS) of gram negative bacteria which can induce pathophysiological effects by inducing cytokine release and are thought to trigger flares of vasculitis (Mandell, 1998; Young, 1995). These endotoxins were shown to be contaminating some serum samples previously from our lab (unpublished data) and removal of endotoxin from the IgG samples provided a clear role of ANCA in activating neutrophils. Thus, previous results from 5.3.1 were repeated with endotoxin removed IgG, both normal and ANCA IgG as discussed in methods sections previously in chapter 2.

GEnC co-cultured with podocytes that had been differentiated for 14 days were subjected to treatment with TNF (2, 10 &100 Units/ml). Static neutrophil recruitment assays were performed, adding neutrophils activated by ANCA (without endotoxin) to the cultures. As observed with 14-day podocytes co-cultures, EnCs showed reduced ANCA activated neutrophil recruitment in the presence of podocytes when compared to EnC mono-cultures, but only in cultures stimulated with 2U/ml TNF-α (Figure 46A). With increasing concentration of TNF-α (10 & 100U/ml) in culture, the neutrophil recruitment was equal or even greater than in mono-cultures (Figure 46C&E).

Following 2U/ml TNF-α treatment, we have observed that ciGEnC co-cultured with podocytes showed significant reduction in total neutrophil recruitment of >50% of the neutrophil recruitment by ciGEnC mono-cultures with MPO ANCA activated neutrophils similar to previous findings of section 5.3.1 with the presence of endotoxins. In the presence of PR3 ANCA, the co-cultures presented reduced neutrophil recruitment of up to

30% which did not reach significance (Figure 46A). Increasing the TNF concentration to 10U/ml has altered the protective barrier thereby increasing the neutrophil recruitment in co-cultures to a similar extent as with ciGEnC mono-cultures. Further increasing the TNF concentration to 100U/ml resulted in upregulated neutrophil recruitment by ciGEnC in co-cultures by up to 24% compared to ciGEnC mono-cultures especially in the presence of ANCA.

The neutrophils that were adherent, transmigrated away from the endothelium more so in co-cultures than in mono-cultures, using cells stimulated with TNF 2 & 10 U/ml (Figure 46 B, D). When stimulated with TNF 100U/ml, the transmigration of neutrophils was less in co-cultures when compared to ciGEnC monocultures in the presence of ANCA (Figure 46F).

Figure 46: Recruitment of ANCA activated neutrophils in co-cultures of ciGEnCs and podocytes - IgG without endotoxin

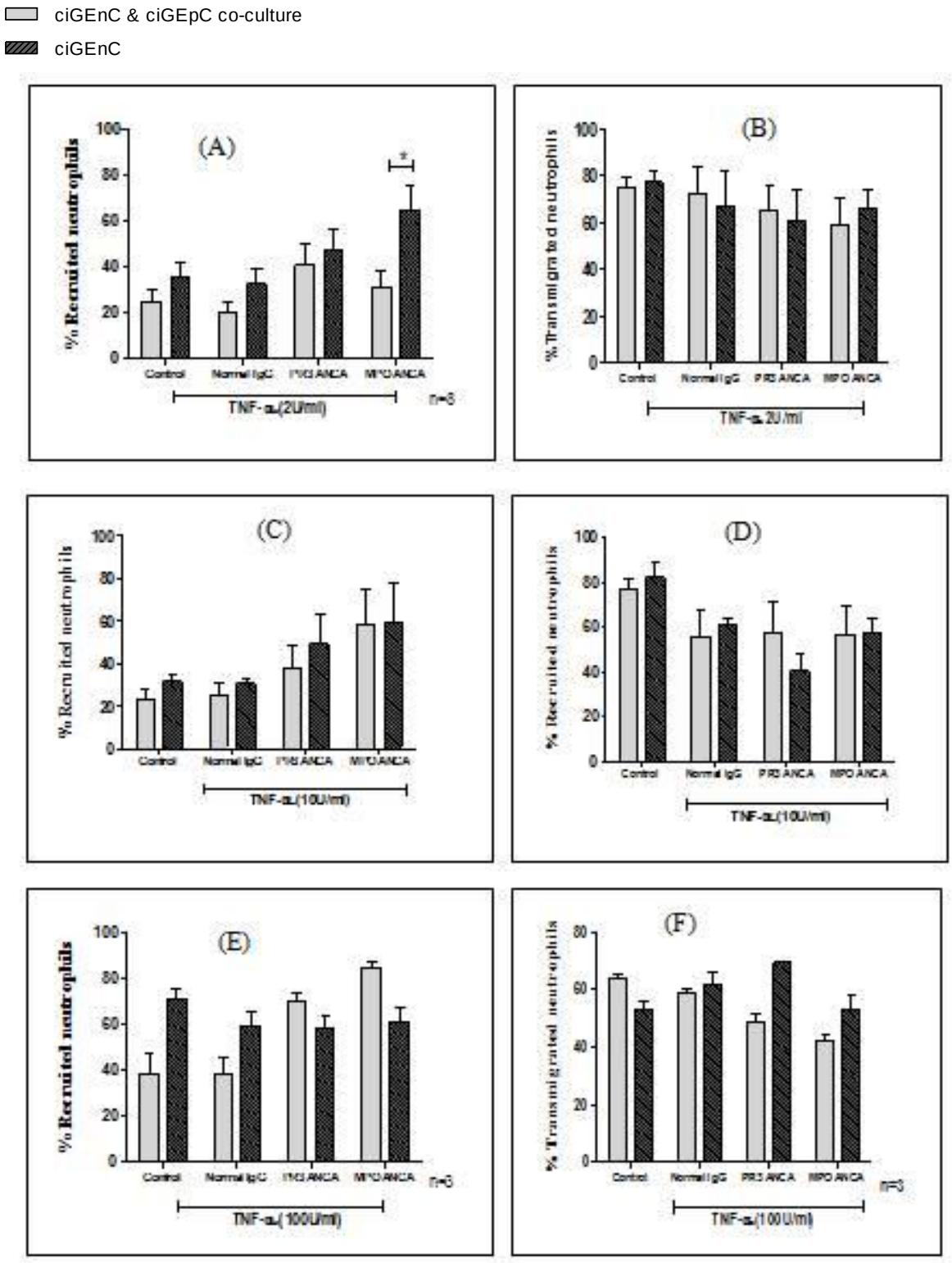


Figure 46: ANCA activated neutrophil recruitment by endothelium in co-culture with podocytes stimulated with different concentrations of TNF-α (2, 10 & 100 U/ml). (A, B) Percentage neutrophil recruitment and transmigration in co-cultures or mono-culture of ciGEnC stimulated with 2U/ml TNF-α concentration in the presence and absence of ANCA IgG. (C, D) Percentage neutrophil recruitment and transmigration in co-cultures or mono-cultures of ciGEnC stimulated with 10U/ml TNF-α concentration in the presence and absence of ANCA IgG. (E, F) Percentage neutrophil recruitment and transmigration in co-cultures or mono-cultures of ciGEnC stimulated with 100U/ml TNF-α in the presence and absence of ANCA IgG.
Statistics: The significance by two way ANOVA, comparing the treatment between the cultures.

5.3.3 Viability of podocytes treated with puromycin aminonucleoside (PAN)-determined by measuring mitochondrial activity

Podocytes were exposed to PAN of increasing concentrations (5 - 500 µg/ml) for 6 -24 hrs to identify the point where viability slips away by testing the mitochondrial activity using the MTT assay, as described previously. The results revealed that the treatment with PAN (500 µg/ml) is toxic even after only 6hrs, whereas 100 µg/ml PAN treatments of podocytes were toxic after 24hours of incubation (Fig. 47 A-C). The concentrations 50, 10 & 5 µg/ml of PAN treatment did not affect viability of podocytes even with incubation times up to 24 hrs.

The determination of PAN toxicity by MTT assay was confirmed by studying changes in cell morphology observed by inverted and fluorescent microscopy. The results revealed PAN induced dysregulation of the actin cytoskeleton with morphological changes like podocyte foot process effacement and cell elongation, induced by PAN, progressing in a concentration and time dependent manner. The progression of foot process effacement is gradual and progressive with concentrations at 16 hours treatment (Fig. 48).

Figure 47: Effect of PAN on podocyte viability

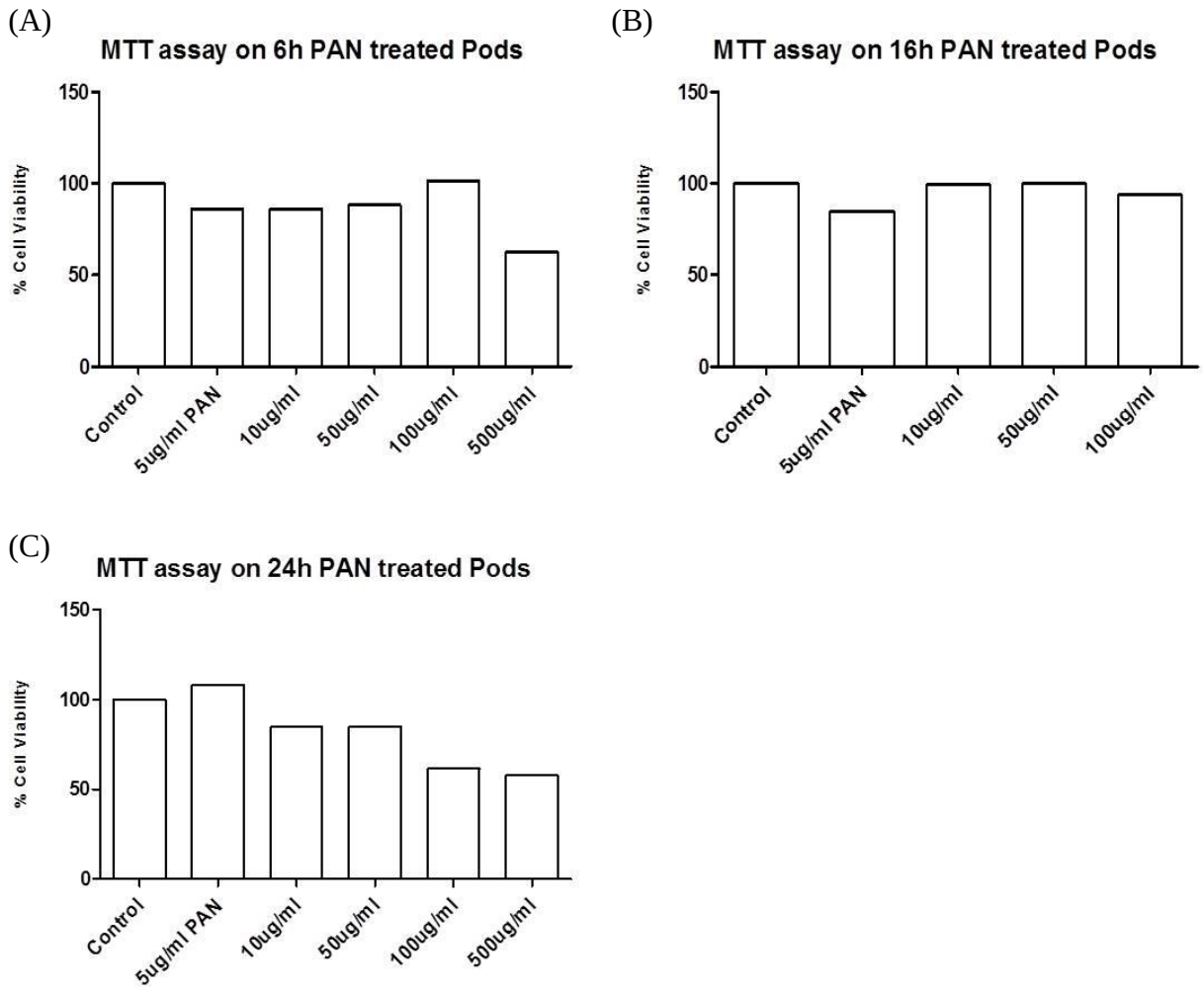


Figure 47: Effect of PAN treatment on podocyte mitochondrial activity. Podocyte cell viability was assessed using the MTT assay treated with concentrations of PAN ranging from 5 to 500 µg/ml for 6 hours (A), 16 hours(B) and 24hours (C).

Figure 48: Effect of PAN on podocyte cell morphology

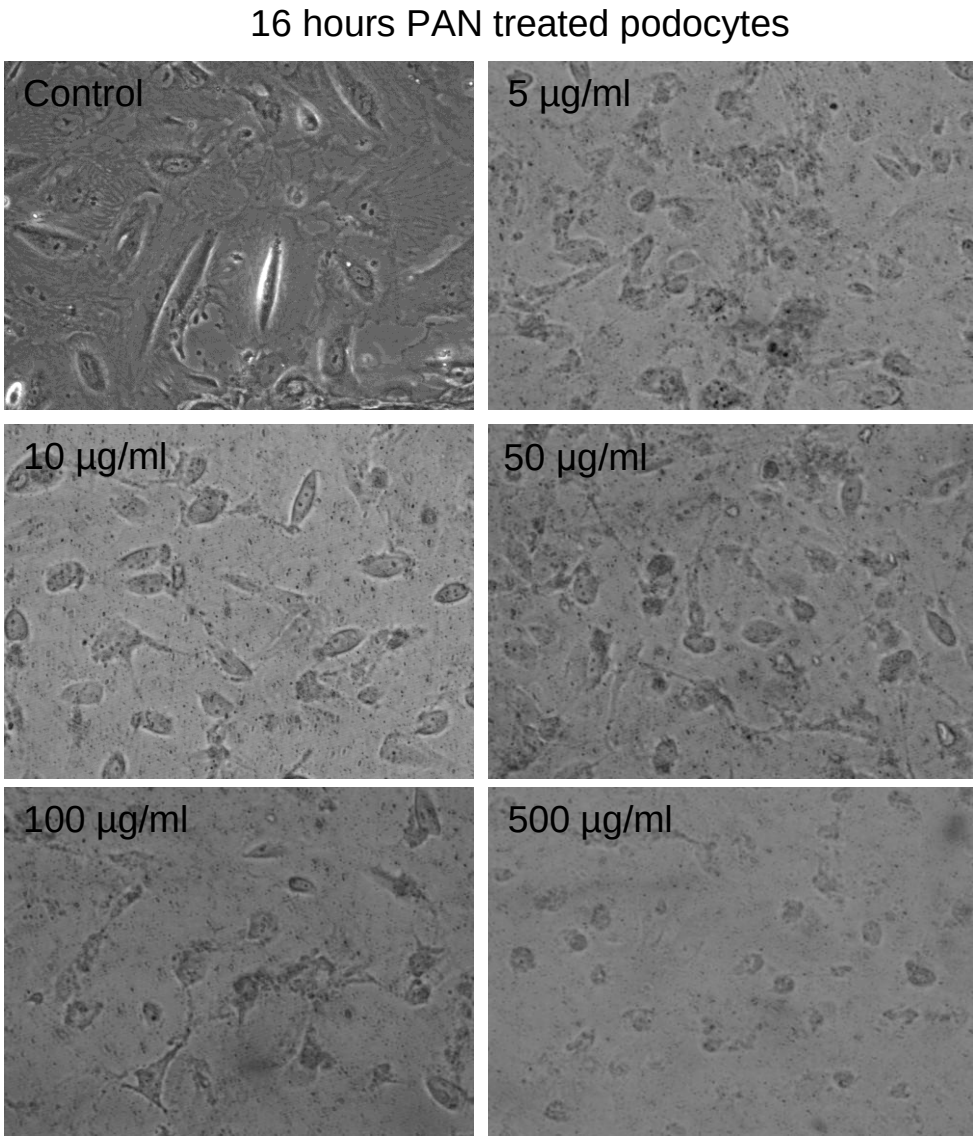


Fig 48: Effect of PAN treatment on podocyte cell morphology. Podocytes treated for 16 hours with a range of concentrations of PAN, present a changing morphology with increasing concentrations of PAN and severe damage at 500µg/ml.

5.3.4 Effect of PAN injured podocytes on neutrophil recruitment by ciGEnC in co-culture

Following the previous studies involving PAN treatment of podocytes, injured podocytes were used in co-cultures along with ciGEnC and TNF-α, so that the effect on neutrophil recruitment could be studied. The results revealed that presence of injured podocytes in the co-culture shows no protection towards cytokine stimulation and increased neutrophil recruitment occurred over and above that observed with ciGEnC mono-cultures, with increasing concentrations of PAN (Fig. 49). The results were compared to neutrophil recruitment by ciGEnC co-cultured with healthy podocytes that were subjected to TNF-α 100U/ml treatment. The neutrophil recruitment in co-cultures with injured podocytes treated with 10, 50, 100μg/ml PAN, was significantly different from co-cultures with healthy podocytes. Co-cultures with 10μg/ml PAN treated podocytes showed increased neutrophil recruitment over ciGEnC mono-culture on stimulation with 100U/ml TNF-α (Fig.49).

Figure 49: Effect of PAN injury to podocyte on neutrophil recruitment by ciGEnC in co-culture

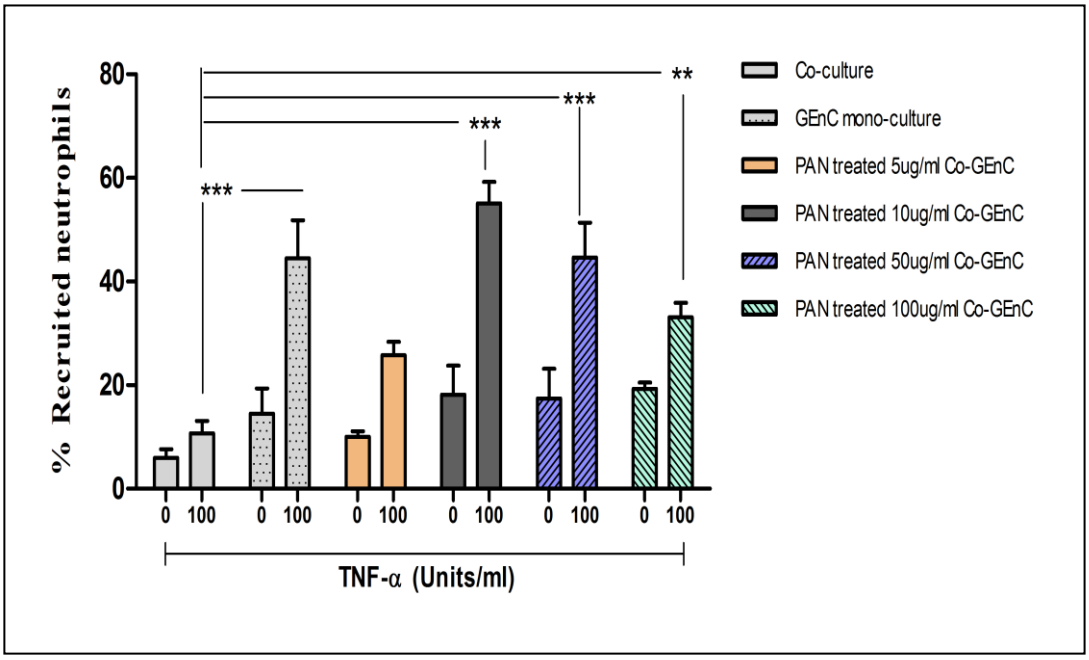


Figure 49: Neutrophil recruitment by TNF-α (100U/ml) stimulated and unstimulated co-cultures of ciGEnC with PAN injured podocytes. Podocytes were injured using different concentrations of PAN – 5, 10, 50, 100 µg/ml treated for 16hrs. The data expressed is a mean of 5 individual experiments. Statistics: One way ANOVA with bonferroni post tests compared to the healthy co-cultures stimulated with TNF-α.

5.3.5 Recruitment of ANCA activated neutrophils by GEnC in co-culture with injured podocytes

To determine the effect of podocyte injury on ANCA activated neutrophil recruitment by ciGEnC stimulated with TNF (2 &100 Units/ml), podocytes were injured by treating with 10µg/ml PAN and co-cultured with ciGEnC on transwell inserts. Static neutrophil recruitment assays were performed by adding neutrophils activated by ANCA (without endotoxins) in the cultures.

As seen in the previous studies, the results revealed that presence of injured podocytes in the co-culture shows no protection towards cytokine stimulation and increase the ANCA activated neutrophil recruitment over and above that observed with ciGEnC mono-cultures with increasing concentrations of TNF-α (Figure 50). The results were compared to ANCA activated neutrophil recruitment by ciGEnC co-cultured with healthy podocytes that were subjected to TNF-α treatment. The neutrophil recruitment in co-cultures with injured podocytes treated with 10µg/ml PAN, in the presence and absence of ANCA, was significantly different from co-cultures with healthy podocytes. Co-cultures with injured podocytes showed an increase in neutrophil recruitment over ciGEnC mono-culture on stimulation with 100U/ml TNF-α (Figure 50).

Figure 50: ANCA activated neutrophils in the co-culture of ciGEnC with PAN injured podocytes

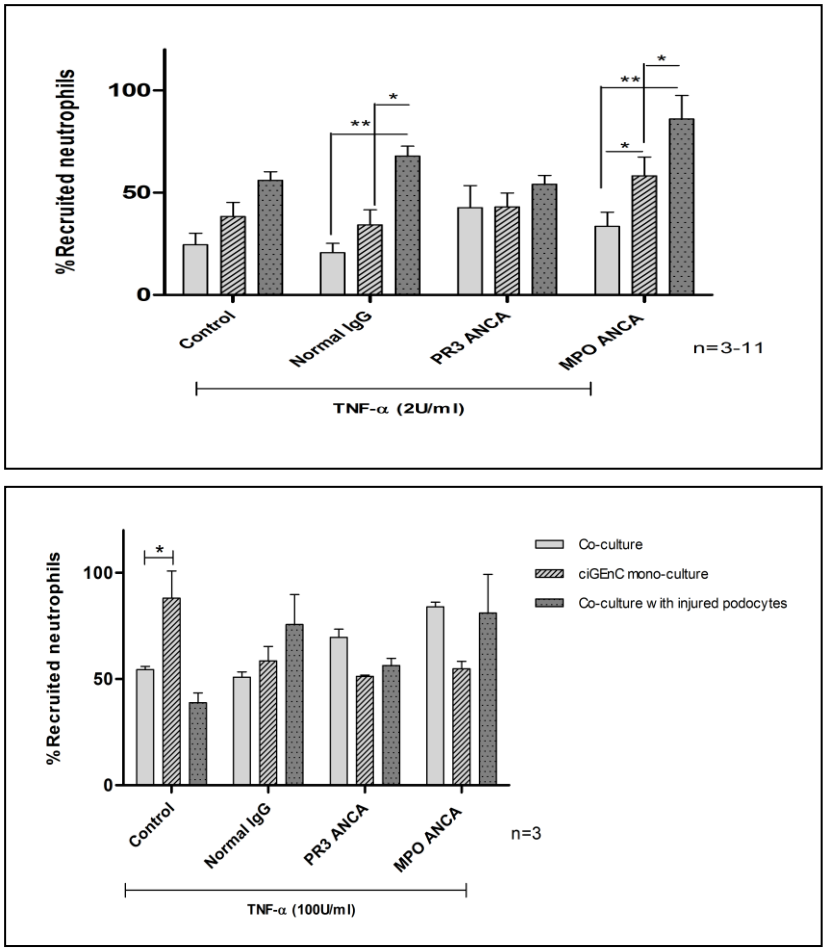


Figure 50: ANCA activated neutrophil recruitment by endothelium in co-culture with PAN injured podocytes stimulated with different TNF-α concentrations (2 & 100 U/ml). (A) Percentage neutrophil recruitment in co-cultures with healthy or PAN injured (10µg/ml) podocytes or mono-cultures of ciGEnC stimulated with TNF-α concentration (2 U/ml) in the presence and absence of ANCA IgG. (B) Percentage neutrophil recruitment in co-cultures with healthy or PAN injured (10µg/ml) podocytes or mono-cultures of ciGEnC stimulated with TNF-α (100 U/ml) in the presence and absence of ANCA IgG. Statistics: The significance by one and two way ANOVA comparing treatments and between the cultures.

5.4 Discussion:

Glomeruli of kidneys are frequent targets of inflammatory and immune processes which threaten their functional ability to clear the body of toxins, and often resulting in kidney failure. ANCA are involved in inappropriate activation of the neutrophils in renal vasculitis and infiltration of these immune cells is well documented within the glomerulus (Suh et al., 1999). The role of glomerular cells during inflammatory episodes and how they regulate the inflammatory cell influx has been explored in this study. This work demonstrates novel findings about the protective role displayed by podocytes by dramatically reducing ANCA activated neutrophil recruitment by GEnC with mild TNF stimulation of the cultures. The balance between health and disease is well documented by increasing TNF concentration which induced elevated levels of neutrophil recruitment in co-cultures. Further, the cross-talk between acute and chronic inflammation is being explored. This work also highlighted the importance of the state of podocytes in maintaining a subtle transition from health to disease and demonstrating that injured podocytes lose their protective ability and cannot play a role in resolving the inflammatory episodes.

Bacterial infection is often observed prior to the onset of vasculitis and is accompanied by elevated TNF levels which play a role in activating the endothelium, while the presence of circulating ANCA escalates neutrophil recruitment by the endothelium (Grau, 1989). Previous studies from our lab have already shown the pathogenicity of ANCA and upregulated neutrophil recruitment by EnCs in the presence of secondary stimulus with ANCA (Calderwood et al., 2005; Radford DJ, 2001). In an effort to create disease inflammatory stimuli, ANCA activated neutrophils were added to cytokine stimulated cultures. Minimally activated co-cultures (TNF-α 2 U/ml) did not support ANCA- activated neutrophil recruitment and reduced it by > 50% compared to mono-cultures, but when the

cultures were highly activated (TNF-α 100U/ml), there was increased neutrophil recruitment with untreated and ANCA treated neutrophils. Previous results from our lab demonstrated similar results where minimally activated endothelium in mono-culture presented increased ANCA activated neutrophil recruitment similar to the current findings and were contrary in co-cultures (Calderwood et al., 2005; Radford DJ, 2001). The presence of ANCA in the cultures stimulated with higher concentrations of TNF-α (10 & 100U/ml), neutrophil recruitment in co-cultures was similar or even more to that of mono-cultures and in all cases, rapid transmigration of neutrophils away from the endothelium was observed. The ANCA activated neutrophil response in co-cultures demonstrates that these effects could be relevant to injury in VGN.

The podocyte is a complex cell with a critical role in maintaining glomerular architecture and function. There is an increasing body of evidence that loss or damage to this terminally differentiated cell will contribute to development of proteinuria, leading to loss of glomerular function. However, it appears that podocytes associated with chronic inflammation cannot defend themselves, as they get injured due to ROS and other mediators released, thereby further promoting an inflammatory environment. The involvement of ROS in podocyte damage is well documented . Non inflammatory lesions such as minimal change nephropathy/focal sclerosis or membranous nephropathies result from podocyte disorders, whereas inflammatory lesions are generally induced by circulating inflammatory cells. There is a possibility that these inflammatory lesions could also be included with podocyte disorders and damage. ANCA induced neutrophil-EnC interactions were considered to be important in the disease process involving inappropriate molecular processes resulting in untimely secretion of superoxide and proteases proceeding towards cellular destruction (Falk et al., 1990b). Podocyte injury has

been reported to be the initiating cause of many renal diseases progressing to end-stage renal failure with podocyte effacement being the initial response towards injury. We have thus hypothesized that podocyte injury could promote the disease process by enhancing ANCA mediated neutrophil responses.

Using a PAN induced podocyte injury model (Diamond et al., 1986; Fishman and Karnovsky, 1985; Lannigan et al., 1962), we have described podocyte injury and its effects neutrophil recruitment in the presence and absence of ANCA. PAN toxicity also involves overproduction of ROS (Marshall et al., 2006; Rincon et al., 2004; Vega-Warner et al., 2004). PAN was found to injure podocytes specifically leading to effacement of foot process, disorganization of actin cytoskeleton and affecting slit diaphragm proteins, nephrin and podocin (Guan et al., 2004; Mundel et al., 1997; Oh et al., 2004). We also investigated whether such injury to podocytes reduces their ability to cross talk with endothelial cells and affects podocyte regulation of neutrophil recruitment by glomerular endothelium during inflammatory episodes. Our injury model has shown that injury to podocytes result in increased neutrophil recruitment by GEnCs, while podocytes that are in healthy state can fight off minor inflammatory insults and can regulate the cytokine sensitivity of the glomerular endothelium. In contrast, podocytes associated with chronic inflammation bypass this and promote an inflammatory environment. Thus the model described here appears relevant to the glomerulus in both health and disease. Previous evidence has shown that injured podocytes were shown to be inefficient in mediating angiogenesis, proliferation and migration of GEnC (Liang et al., 2006).

In conclusion these studies reveal that podocytes can modulate the inflammatory response mediated by GEnC, and that this modulation may itself depend on the state of the podocytes. The exact molecular mechanisms and mediators involved in modulating the

glomerular diseases needs to be fully delineated. This will allow therapies to be focused towards repair mechanisms that would address the inflammatory insults targeting the glomerular cells.

Chapter 6: Discussion and Conclusions

VGN is a feature of ANCA associated vasculitis and the presence of ANCA activates neutrophils inappropriately resulting in increased infiltration and infiltrated immune cells along with the resident cells have been found in the crescents of crescentic GN (He et al., 2006; Thorner et al., 2008). During active vasculitis, increased levels of SP have been detected in patient's plasma (Haubitz et al., 1997; Timothy et al., 1994). The importance of SP in the GEnC activation and/or injury that occurs during the development of VGN and the role of glomerular epithelium in regulating the inflammatory episodes during both health and disease were presented clearly in this study. Targeting SP, as well as podocyte recovery from injury, could potentially be amenable to therapeutic manipulation. In this current study, we have investigated four models of inflammation that might mimic different inflammatory conditions. Firstly, the role of SP in activating the EnC mono cultures and looked at neutrophil recruitment under static and flow conditions. Secondly, the cultures were stimulated with TNF concentrations from 0-100U/ml, mimicking an inflamed state in which there is an elevated level of cytokine and chemokine release. Thirdly, in addition to an inflammatory stimulus, a secondary stimulus was added to the situation by adding ANCA representing the ANCA associated vasculitic condition. Lastly, an injury model was hypothesized on the basis of the state of podocytes, where they were injured by using PAN and recreated both inflammatory and disease states in an effort to model the inflammation occurring *in vivo* in vasculitis. The results revealed clear transition between health and disease.

Differences in SP effects on different EnCs:

We have used primary GEnC as well as immortalised cell line cultures in these studies as GEnCs are the target cells in VGN. We couldn't use primary cells all the time due to the complexity of their isolation process and/or cost involved in buying them commercially. The cell line cultures were developed from primary cells to over come similar problems and are successfully achieved by Dr Satchell group. The cell line GEnCs were shown to share similar morphological and phenotypic features compared to primary cells. We have also used HUVEC in our studies as a large vessel endothelial comparitive and primary GEnCs to confirm major findings.

Whilst investigating the mechanisms by which SP affected the endothelial cell phenotype, cell line GEnC were stable compared to primary cultures of GEnC or HUVEC following the SP treatments. Our findings also confirmed previous finding that HNE is more damaging compared to PR3. Previous findings have shown that HNE in particular was linked more towards EnC disruption (Carden et al., 1998; Nakatani et al., 2001; Smedly et al., 1985) and was found to be deposited on glomerular endothelium in renal biopsies of patients with crescentic GN (Kuzniar et al., 2007). The neutrophil adhesion pattern was also different between the cell types, where HUVEC and ciGEnC demonstrated significantly increased levels of neutrophil adhesion under static conditions, following 1µg/ml protease treatment, whilst primary GEnCs did not show any response. The primary GEnCs were more sensitive to the protease treatment despite them being viable as demonstrated by MTT assay. Under physiological flow conditions, significant increase in neutrophil capture was observed by HUVEC only with PR3 treatment and not with HNE and none by GEnC (both primary and cell line cultures), and they completely blocked neutrophil recruitment.

The reason could be that the endothelium might not be armed with adhesion molecules. The transient neutrophil interactions that were demonstrated under static conditions were not mediated by adhesion molecules as absence of classical adhesion molecules was demonstrated by a colleague (unpublished data). The lack of selectin or integrin upregulation in the cultures mitigated against capturing neutrophils under flow conditions. The presence of surface P-selectin (unpublished data) might have played a role in the neutrophil recruitment by HUVEC since PR3 induced P-selectin expression was significantly lower in GEnC than HUVEC.

The differences in the response with these cell types was previously documented in our laboratory by Dr Pankhurst while looking at neutrophil capture in the presence of ANCA. Her data also demonstrated the fragility of the primary GEnCs which could be due to differences in the environment *in vitro* and *in vivo*. The difference between HUVEC and GEnC is explainable as they are from completely different types of vessels and are functionally different as well.

Importance of co-culture model:

Co-culture systems mimic *in vivo* tissues and allow the study of cell–cell interactions. This system is widely used currently to study different aspects of health and disease conditions which are found within different organ systems (Helen et al., 2009; Hermanns et al., 2004; Lally et al., 2005; Ma et al., 2005; McGettrick, 2010; Rainger and Nash, 2001; Wang and Takezawa, 2005). *In vitro* studies have previously been attempted to study glomerular inflammation and to characterise glomerular cell behavior in order to provide insights into the mechanism of interactions of neutrophils under different conditions. Availability of cell

line podocytes and GEnCs brought it a step close and putting these cells together in co-culture made it easy to interrogate the intricate details about glomeruli. At the same time, the system is limited and may not mimic the exact role of GEnC and the support or provocative effects they receive from neighboring cells, but it did enable us to study some mechanisms in greater detail than in their real environment. This system also provided the flexibility for the cells to behave similarly to *in vivo* conditions and also can overcome some of the limitations of single culture models. It allowed us to study interactions of the neighboring cells and to determine how the interactions help in mediating the functions of the system. Neutrophil recruitment by GEnCs and also interactions of ANCA were studied in the presence of podocytes and the recruitment pattern was directly observed.

Disadvantages of the co-cultures:

PET membrane:

PET (polyethylene terephthalate) is a carbon polymer based membrane which is a hydrophilic thin film and is translucent or microscopically transparent for cell visualization and monitoring of the cell monolayer using light microscopy techniques.

Many *in vitro* studies were successful in delivering knowledge about the intricate communication between cell types of different tissue systems (Hermanns et al., 2004; Ma et al., 2005). The transwell insert compared to glomerular filtration barrier functions is questionable as the mean GBM thickness is between 300-350 nm and alters in disease states. The thickness of the TES membrane on the insert is 10μm. The thickness of PET membrane is greater, but nevertheless, most of adherent neutrophils transmigrated rapidly. The neutrophil recruitment also increased with the presence of ANCA and injured

podocytes which demonstrates that the thickness of the membrane was not a critical factor for reduced neutrophil recruitment. However, having a thicker membrane might have affected the proximity of the cells and might have reduced the responses mediated, while at the same time the pore size might have promoted cell interactions. Despite the challenges with the membrane and efforts to generate alternative forms of porous support by other groups (Abbott et al., 1992; Daley et al., 2008; Matthews et al., 2002; Slater et al., ; Wang et al., 2008), the model is widely accepted and other forms of membrane structures might still need work to establish them as a successful model.

Differences in neutrophil recruitment in the co-culture of HUVEC with podocytes and GEC with podocytes:

HUVEC co-cultured with podocytes presented different findings to that of GEnC. The primary reason could be the fundamental differences in their origins. Physiologically GEnC and podocytes sit next to each other, whereas HUVEC were isolated from umbilical vein conduit vessels. Hence the cross talk that we have seen could be organ specific. Further, there are likely to be basic physiological and functional differences of EnCs from microvasculature and macrovascular EnCs. There is considerable evidence in the literature of differing phenotypes of endothelial cells in different vascular beds (Lim et al., 2003);(Franzen et al., 2003; Murakami et al., 2001; Vora et al., 1996). GEnC are of capillary origin and are exposed to increased numbers of circulating cells as the glomeruli are exposed to 180L of blood per day and thus armed to prevent inflammation. Despite these issues, both cell types were compared and were shown to be expressing selectins,

ICAM1 and P-selectin (unpublished data) and therefore to have a similar potential to recruit neutrophils.

The other difference that we observed in our cultures was the production of IL-6 in co-cultures of HUVEC or GEnC with podocytes as shown in figure 53. GEnC in mono-cultures did not release any IL-6 into culture supernatants. The IL-6 that was released in GEnC co-cultures was by the podocytes as shown previously. This was different with HUVEC cultures, where supernatants from HUVEC mono-cultures also had increased levels of IL-6 in response to TNF-α along with increased levels in co-cultures (upto1700 pg/ml). The levels in HUVEC cultures were lower to that GEnC co-culture supernatants (upto10,000 pg/ml).

The electrical resistance of filters with podocytes:

The ER of the membranes with monocultures and co-cultures were not significantly different. The integrity of the podocyte monolayer was tested by microscopy and its growth on the underside of the filter did not change the cell morphology or alter the growth of the second cell type inside of the filter. Despite formation of intact monolayers, the ER of podocytes was similar to that of a blank filter which could be due to the podocyte finger-like projections that spread around the cell body of the podocyte which might have allowed the electrical conductivity. Similar results were observed in other studies as well (Satchell et al., 2006; Slater et al.). This data also suggests that the reduction is not due to the two monolayers being in close proximity but is due to the involvement of soluble molecules released by podocytes. To prove this, we have replaced podocytes with other epithelial cells or with EnCs (shown in a previous chapter) and have observed increased neutrophil

recruitment in the case of GEnC with GEnC or AKN with GEnC or ARPE with GEnC. In all these cases, the electrical resistance was twice that of single cultures and could have reduced the neutrophil recruitment. Whilst in the co-cultures of GEnC with HKC, reduced neutrophil recruitment was observed as was with podocytes and also showed similar ER resistance. This could possibly be due to the fact that they are renal tubular cell line and are from same organ and thus could possibly be mediated in cross talk which could be another project and needs futher investigation.

ANCA responses in co-cultures:

Previous findings have shown the effect of ANCA in promoting neutrophil recruitment (Calderwood et al., 2005; Radford DJ, 2001; Radford et al., 1999). ANCA presents variable pathogenicity as observed in previous studies and some studies suggest that PR3 ANCA are less effective *in vitro* compared to MPO ANCA (Harper et al., 2001b). ANCA variability was not clearly explained and it is also unclear about underlining factors responsible for varying responses of ANCA in various assays and also the variability in inducing degranulation and respiratory burst of neutrophils from different donors. There is also the difficulty of quantifying the amount of specific ANCA IgG within the total IgG preparation. Differences inl ANCA concentration could vary between patients which would contribute to the differences in ANCA responses. Despite testing several ANCA IgG preparations and selecting the ones that worked in degranulation assays and superoxide assays, it was difficult to predict the effects of each ANCA preparation and such differences may also extend to real variation in ANCA pathogenicity. Furthermore, there

may be variability in pathogenicity related to subclasses of IgG. This concern led to the development of chimeric ANCA of different IgG subclasses in order to investigate the importance of Fc binding by different IgG subclasses in ANCA-IgG pathogenicity (Hussain et al., 2009; Rachel Colman, 2007). Pankhurst et al recently reported that IgG3 is the most potent IgG, followed by IgG1 and IgG2 and last IgG4, in terms of capturing flowing neutrophils (Pankhurst et al.).

Another problem in the IgG experiments was activation of neutrophils by control IgG. Again this was another problem with no clear explanation but could be the result of cross linkage of IgG resulting in neutrophil activation. Other technical issues include usage of the same columns for both patient and normal samples which might cross-contaminate the samples despite cleaning columns between samples by acid elution. The presence of endotoxin appeared to cause the activation of neutrophils by normal IgG in some cases. Bacterial lipopolysaccharides can activate immune cells and removal of the endotoxin resulted in reduced activation.

Effects of SP on neutrophil recruitment by endothelium in the presence of podocytes:

As primarily designed, my work could not proceed to demonstrate the effects of PR3 or HNE on GEnC in the presence of podocytes in the co-culture system. Our preliminary data on testing the viability of podocytes treated with SP did not show any reduction in viability at the lower concentrations (1µg/ml) following 7 day differentiation of podocyte cell line cultures (i.e. the same concentration as we observed increased neutrophil adhesion to

EnC under static conditions, as shown in the previous section). Viability was not checked for podocytes that were completely differentiated for 14 days. The fully differentiated podocytes were more sensitive to toxic treatments and could be sensitive to SP treatments as well. On the other hand, despite no loss in viability, there is a possibility for the podocytes to be injured by SP which will further activate the EnC there by promoting neutrophil recruitment in the cultures as was seen in my injury model. It will be difficult to dissect the details of SP effects on endothelial activation in the presence of podocytes. Also the complexity of the co-culture system and time and funding restrictions did not permit elucidation of the effects of SP and the molecules and processes that could be involved in mediating the effects. It would be interesting to find out how PR3 and HNE effect fully differentiated podocytes and their effect on the cells in co-culture. If the cells sustain the treatments in co-culture, then it would be interesting to further elucidate the role of podocytes in modulating the neutrophil recruitment by GEnCs and also investigating the mechanistic side of the effects. If the podocyte foot processes get injured as was observed with PAN, this will be most relevant to the *in vivo* situation, which would make endothelium sensitive to further SP treatments, thereby enhancing neutrophil retention and escalating the injury process which is relevant to the disease process of VGN.

Major conclusions of this study:

SP damage endothelium in a time and dose dependent manner. At the concentrations where they do not damage endothelium, they stimulate the endothelium in a way that recruits neutrophils, with PR3 than with HNE. HNE is more damaging compared to PR3. The presence of α1-AT along with the SP in the cultures nullified the effects of SP. This could be relevant to the disease process of VGN and thus SP are the potential therapeutic targets.

The vulnerability of the glomerulus to an inflammatory insult is due to its anatomical specialisation. Podocytes not only maintain vessel architecture, but also regulate the actions of glomerular endothelial cells, modulating the inflammatory conditions. Healthy podocytes mediate IL-6 dependent cross talk and regulate neutrophil recruitment by GEnC under stimulated conditions, thereby demonstrating an endogenous mechanism by which the glomerulus is protected against an inflammatory insult. This negative regulation could be organ specific as podocyte cross talk was not observed with HUVEC, thus demonstrating the specificity of the renal vasculature.

Most importantly we have also addressed the relevance of podocyte-glomerular endothelial cell crosstalk by superimposing two disease models on the co-culture microenvironment. Podocytes were able to dramatically reduce the endothelial recruitment of neutrophils activated with ANCA IgG, even at lower TNF-α concentration and increasing TNF-α stimulation escalated neutrophil recruitment. Finally, injury to the podocytes by PAN disturbs the balance. Thus the state of podocytes is important in regulating the glomerular responses.

Figure 52: Conclusions of co-culture work.

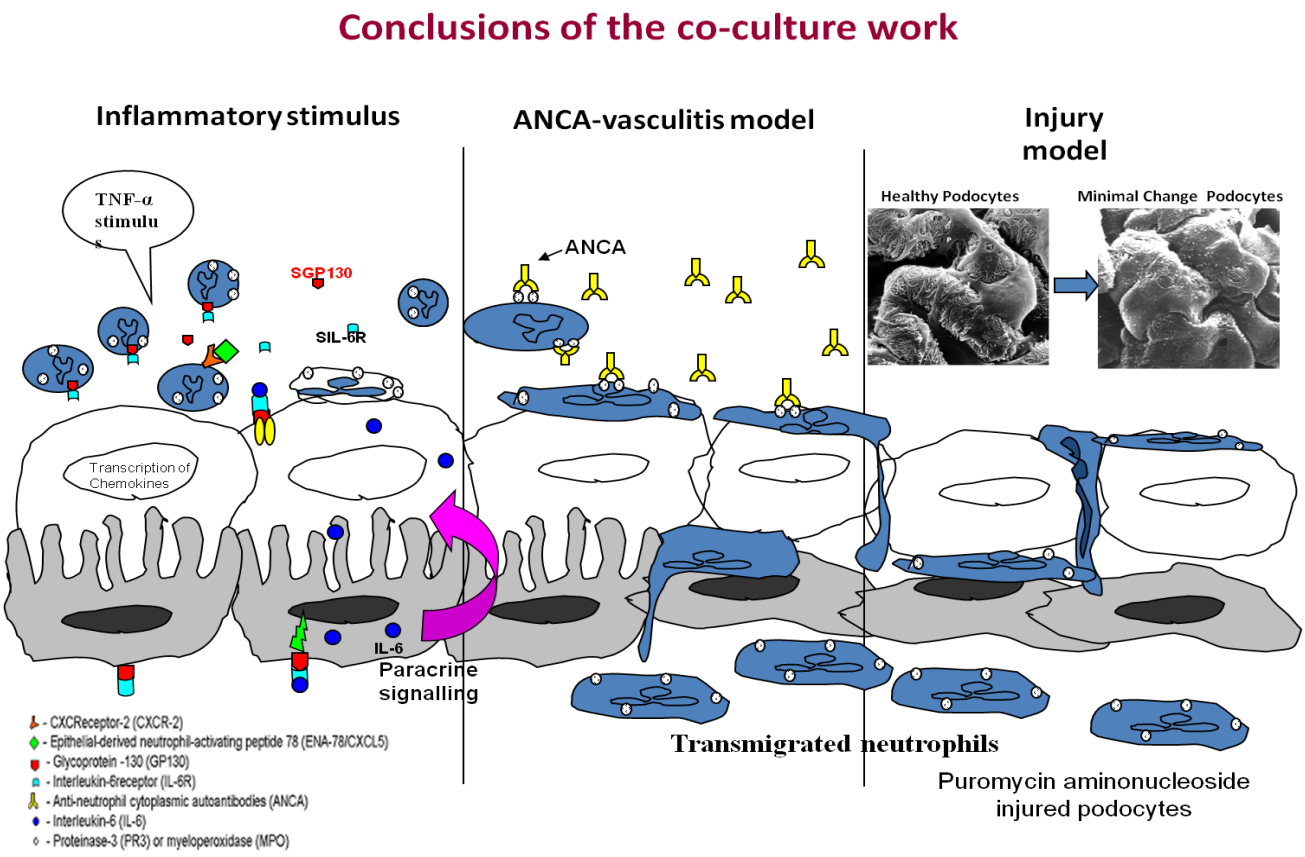


Figure legend: Illustration of complete Coculture work

Inflammatory stimuli: mechanism of neutrophil recruitment on GEnC in the presence of podocytes with an inflammatory stimulus (tumor necrosis factor TNF-α) and the interactions of CXCR2 and its ligand ENA-78 in the mechanism of neutrophil recruitment. Podocytes regulate the inflammatory process by IL-6 mediated cross talk with the GEnC and resolve the inflammation.

ANCA vasculitis model represents the inflammation in disease model, ANCA specific for PR3 or MPO bind to the molecules expressed on the surface of the neutrophil leading to the activation of neutrophil and resulting in intensive inflammation. The release of reactive

oxygen intermediates and other mediators results in the injury of the podocytes resulting in retraction of foot process of podocytes there by enhancing the neutrophil recruitment during inflammatory episodes.

Injury model to test the hypothesis that podocyte injury could be initiating point for the glomerular dysfunction and PAN injured podocytes resulted in increased neutrophil recruitment. It appears that podocytes from inflamed glomeruli cannot regulate the cytokine-sensitivity of vascular endothelium to other agonists.

Future work

1. SP in co-cultures.
2. Adding patient neutrophils with and without ANCA.
3. Investigating co-cultures under flow conditions.
4. Examining how presence of platelets in co-culture system attracts neutrophils, or other inflammatory cells such as monocytes.
5. Introducing PAN *in vivo* and looking at neutrophil adhesion by intravital microscopy.
6. Targeting podocyte recovery by adding steroids such as dexamethasone to the cultures with injured podocytes.

Solutions and Buffers

1. Reagents for Neutrophil preparation:

Acid Citrate Dextrose	4.2g Disodium hydrogen citrate 5.0g D-glucose 200ml pyrogen-free water
2% Dextran	9g NaCl 20g Dextran T500 1 Litre pyrogen-free water FILTER STERILISE Store 4^{o}C
HBH	500ml Hanks buffered salt solution 5ml 1M Hepes
1.5M Saline	

2. Reagents for western blotting:

Coomassie	400ml Methanol 500ml dH20 100ml Acetic acid 1g Brilliant Blue dye
10x Running buffer	30.2G Tris Base (Sigma Aldrich) 100ml 10% SDS 187.6g Glycine 1L dH2O
1x Running buffer	12.11G Tris Base 2g SDS 57.65g Glycine 2L dH2O
10X TBS - PH 7.6	24.2 G Tris Base 80g NaCl 1L dH2O
1 X TBS-T	100ml 10X TBS 900ml dH2O 1ml Tween 20

Transfer buffer	2.93g Glycine 5.81g Tris 0.375g SDS 200ml Methanol

6x SDS -page loading buffer	10ml
0.35M Tris 10.28% SDS 36% Glycerol 5% β-mercaptoetanol Bromophenol blue	3.5ml 1.28g 4.8ml 0.5ml Unitil blue

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