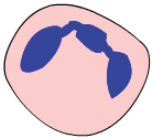
A NOVEL PROINFLAMMATORY ROLE FOR ANNEXIN A1 IN NEUTROPHIL TRANSENDOTHELIAL MIGRATION



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This thesis is submitted for the degree of

Doctor of Philosophy, University of Adelaide

January 2009

ABSTRACT

Neutrophil extravasation into tissues is an essential process required for the inflammatory response. Upon receiving an inflammatory cue, neutrophils begin accumulating on the luminal surface of the endothelium. Neutrophil recruitment is initiated by selectin-mediated tethering and rolling of neutrophils along the endothelial monolayer, followed by integrin-mediated firm adhesion. Adherent neutrophils then traverse the endothelium in a process known as transendothelial migration. The events mediating the rolling and adhesion steps are well characterised, but research into the molecular mechanisms regulating transendothelial migration is an area of intense focus. A previous study conducted in our laboratory found that the activation of endothelial extracellular signal-regulated kinase (ERK) 1/2 was required for neutrophil transmigration. Furthermore, it was found that endothelial ERK was activated in response to a soluble protein produced by fMLP- or IL-8-stimulated neutrophils.

In the present study, the soluble ERK-activating neutrophil protein was identified as annexin A1, which was selected as a possible candidate following mass spectrometry analysis of proteins secreted from activated neutrophils. Annexin A1 antibodies (Abs) were found to block endothelial ERK activation induced by conditioned medium harvested from stimulated neutrophils. Annexin A1 Abs were additionally able to inhibit neutrophil transmigration across human umbilical vein endothelial cell (HUVEC) monolayers in an *in vitro* transmigration assay. Following the purification of recombinant annexin A1, it was demonstrated that it could activate endothelial ERK in a similar manner to neutrophil conditioned medium. Upon further investigation, ERK activation was found to be induced by a truncated form of annexin A1 present in the protein preparation rather than the full length protein. Calpain I, a calcium dependent protease that is activated upon neutrophil stimulation and is known to cleave annexin A1 within the N-terminal domain, was shown to process full length inactive recombinant annexin A1 into an unidentified product that could activate endothelial ERK. A calpain I inhibitor was also found to prevent stimulated neutrophils from secreting an ERK-activating protein, thus further suggesting a role for calpain I in this process. As full length annexin A1 has been reported to signal through the formyl peptide receptor (FPR) family, a pan-FPR antagonist was incubated with endothelial cells and was found to inhibit ERK activation induced by neutrophil conditioned medium, indicating that pro-inflammatory annexin A1 is also a FPR ligand.

Endothelial projections termed “transmigratory cups” form around neutrophils during extravasation, of which ICAM-1 is a major component. Using an assay that examined transmigratory cups during neutrophil transmigration, it was found that annexin A1 Abs could inhibit neutrophil adhesion and transmigration through HUVEC monolayers by interfering with transmigratory cup formation around neutrophils, as shown by monitoring ICAM-1 during the process. Quantification of transmigrating neutrophils highlighted that the majority of neutrophils were emigrating via a transcellular pathway, which is in opposition to many *in vitro* studies where paracellular transmigration predominates.

The results generated from this study identified a novel pro-inflammatory role for annexin A1 in neutrophil transendothelial migration. Preliminary experiments suggested that the pro-inflammatory annexin A1 responsible for endothelial ERK activation was a truncated form. Calpain I appears to be a likely candidate responsible for the generation of this uncharacterised, truncated annexin A1 product, however further experiments are required to confirm this hypothesis. Pro-inflammatory annexin A1 represents a new target for the treatment of inflammatory disorders.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Samantha Williams.

ACKNOWLEDGEMENTS

I wouldn't be composing this acknowledgements section if it wasn't for my supervisor Yeesim Khew-Goodall. You are a remarkable scientist and thank you for being such a fantastic supervisor. Thank you for dealing with my ups and downs and thank you for basically letting me do whatever the hell I wanted to do. Although I didn't realise it at the time, you were showing me that you had confidence in me and in my ideas. There are only a small number of supervisors who are relaxed enough to allow their PhD students determine the direction of their own projects and luckily for me, you are one of them. Although I sometimes went about my experiments in a convoluted way, I got there in the end and the process of making mistakes has been an invaluable learning experience for me. That instant of realisation where one recognises a whole lot of time has been wasted because something wasn't considered or thought out properly is a gut-wrenching feeling, but experiencing those moments has made me an infinitely better scientist. You never made me feel stupid and always listened to my ideas and I cannot put into words how much I valued that. Until writing this thesis (combined with the benefit of 1.5 years off!) I didn't fully appreciate how good the results actually are. I'm finally proud of what I achieved during my PhD and I cannot thank you enough Yeesim for your guidance, patience and for being an all-round awesome lady.

I would also like to thank my co-supervisor, now boss, Stuart Pitson. Your teachings into the realms of protein chemistry were pivotal to many of the results gained during my PhD. Despite having your own lab to run, you were always willing to help, sometimes with great haste if there was an FPLC malfunction! Thank you for employing me before my thesis was completed- working in a different area has been a very welcome change of scenery and has made this thesis a much more positive, readable document. Thank you also for providing the subtle reminders about finishing my thesis, however your not-so-subtle declaration that my continued employment hinged on its completion before the end of the year really did the trick. I also appreciate your patience in tolerating my zombie-like state at work for the past few months, although I suspect you probably enjoyed the unfamiliar silence.

Thank you to my wonderful friend Leila. You are one of the most dedicated, diligent people I know and having you in the lab was a constant reminder of what I should aim to be. Thank you for suffering through my incessant chatter, loud music, whinging, stealing your equipment and bugging you to come to the pub when you wanted to write your lab book up, amongst the many other things I'm not aware of because you were too nice to say anything.

Thank you also to the staff and students in the Division of Human Immunology- I am convinced it is the best department ever to work in. I thoroughly enjoy the tea room conversations, which are always entertaining, lively and guaranteed to put at least one person off their food. Thank you to Jennifer Gamble and Mathew Vadas from the now extinct Vascular Biology Laboratory for their collaboration, which allowed me to obtain HUVEC every week to perform my experiments. Following on from that, I would like to thank Jenny Drew and Anna Sapa for passing on your expertise on the fine art of culturing of HUVEC, for doing the icky extraction of HUVEC from umbilical cords and for sneaking me extra lines when you could. You are both champions. A big thank you also to Hayley Ramshaw for offering to proof-read this thesis (no amount of chocolate could numb that pain!) and Midge for her little contributions.

I must also acknowledge Douwe Egberts for the assistance that their product, Moccona Espresso Blend Coffee, has given me in writing this thesis. Without a half-decent tasting brand of instant coffee, I would have had no other alternative than to drink the real stuff and consequently would have died from caffeine poisoning.

Finally to my husband Craig: Told you I'd finish it! :P 1337 +3 chair buff to thesis writing FTW! Now let teh pwnage begin!!~1!

“When you’re going through hell, keep going.”

-Winston Churchill

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ABBREVIATIONS

Ab	Antibody
Ac2-26	Acetylated N-terminal annexin A1 residues 2-26
ANOVA	Analysis of the variance
AnxA1	Annexin A1
BALF	Bronchoalveolar lavage fluid
Boc2	Boc-Phe-Leu-Phe-Leu-Phe
BSA	Bovine serum albumin
CHO	Chinese Hamster Ovary
CM	Conditioned medium
CMV	Cytomegalovirus
DAPI	4'-6-Diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
EM	Electron microscope
ERK	Extracellular signal-regulated kinase
ERM	Ezrin radixin moesin
ESAM	Endothelial cell-selective adhesion molecule
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Met-Leu-Phe
FPLC	Fast protein liquid chromatography
FPR	Formyl peptide receptor
FPRL	Formyl peptide receptor like
GFP	Green fluorescent protein
HEK293	Human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic Pituitary Adrenal
HRP	Horse Radish Peroxidise

HSA	Human serum albumin
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
JAM	Junctional adhesion molecule
KLH	Keyhole limpet hemocyanin
KO	Knock out
LAD	Leukocyte Adhesion Deficiency
LB	Luria Bertani
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LXA4	Lipoxin A4
mAb	Monoclonal antibody
Mac-1	Macrophage antigen-1
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MEK	Mitogen-activated protein kinase kinase
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloprotease
MNEI	Monocyte/Neutrophil Elastase Inhibitor
MOPS	3-(N-morpholino) propanesulfonic acid
MPO	Myeloperoxidase
MQ	Milli Q
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NET	Neutrophil Extracellular Traps
O.D.	Optical density
pAb	Polyclonal antibody
PAF	Platelet activating factor
PBS	Phosphate buffered saline

PBS-T	PBS/0.1% Tween 20
PCR	Polymerase chain reaction
PDZ	Post-synaptic density-95/discs large/zonula occludens-1
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PSGL-1	P-selectin glycoprotein ligand-1
PKC	Protein kinase C
PMA	Phorbyl myristate acetate
PMS	Phenazine methosulfate
PVDF	Polyvinylidene fluoride
ROS	Reactive Oxygen Species
SAA	Serum Amyloid A
SEM	Standard error of the mean
SMP	Skim Milk Powder
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Chapter 1

INTRODUCTION

INTRODUCTION

1.1. The immune system

Blood is comprised of many different cells that perform a wide range of functions. Leukocytes play a vital role in constantly patrolling the body for the early detection of invading pathogens and foreign antigen and are the core component of the body's immune response. In order to perform their specific functions, leukocytes must traverse the walls of blood vessels into tissues.

The human immune system can be divided into two branches: the acquired and the innate immune system. The acquired immune response is mediated by T- and B-lymphocytes, which are leukocytes that possess the capacity for specific antigen recognition. T-lymphocytes directly eliminate infected cells and influence the activity of other leukocytes, whereas B-lymphocytes produce antigen-specific antibodies and are responsible for the "memory" of antigen exhibited by the acquired immune response. The acquired immune response is a sophisticated process that is specific to the antigen that generated its activation and consequently is not an instantaneous process. Therefore to rapidly respond to invading pathogens, the body is dependent on the innate immune system.

The innate immune system is the body's first line of defence against pathogens and lacks the specificity of the acquired immune response. The innate immune response is rapid, non-specific and acts against a broad range of targets. Leukocytes of the innate immune system include monocytes and granulocytes. The activity of these cells is crucial for the innate immune response, as they non-specifically attack foreign material and subsequently activate the cells of the acquired immune response [1, 2]. Monocytes develop into macrophages upon leaving the blood stream and are one of the principle cell types involved in phagocytosing and destroying microbes. Granulocytes are involved in mediating inflammatory responses and are characterised by the presence of cytoplasmic inclusions or "granules", such as lysosomes and secretory vesicles, which contain anti-microbial substances [3]. Based on the staining properties and morphology of these granules, granulocytes can be subdivided into three classes: basophils, eosinophils and neutrophils. Whilst basophils and eosinophils are important in mediating inflammatory responses and allergic reactions, neutrophils far outnumber both of these cell types and have a critical role in primary host-defence.

1.2. Neutrophils

Neutrophils (also known as polymorphonuclear leukocytes due to their multi-lobed nucleus) are the most common class of granulocyte and occur at a concentration of approximately $5x10^9$/litre blood. Neutrophils predominate in the cellular infiltrate at sites of acute inflammation and are activated in response to signals generated from the activated endothelium and/or molecules emitted from the source of the inflammation [4]. Neutrophils are notably absent in uninflamed tissue and are located only in the blood stream in healthy subjects [5]. The role for neutrophils at inflamed sites is complex. Like macrophages, a fundamental task is to eliminate invading microbes by phagocytosis. During phagocytosis activated neutrophils engulf their target, upon which the pathogen-containing phagocytic vacuole then fuses with cytoplasmic granules within the neutrophil. These granules harbour an arsenal of cytotoxic substances and reactive oxygen species that eliminate the target [3, 6]. The cytotoxic substances contained within neutrophil granules are also released in a controlled manner upon stimulation and therefore additionally possess extracellular microbicidal activity. The process of releasing such factors can result in tissue damage however, which highlights the importance of the clearance of cells by the host after the pathogenic threat has been controlled. Circulating neutrophils exhibit a short half-life of approximately 6-10 h, whereupon they undergo apoptosis and are cleared by the spleen [7]. However neutrophils activated by inflammatory cues display a delay in apoptosis, presumably to allow for their accumulation and enhanced function at sites of infection [8]. After they have performed their tasks, senescent neutrophils containing phagocytosed material are removed by macrophages [9]. This systematic elimination is vital to ensure tissue damage does not occur through leakage of cytotoxic substances from necrotic neutrophils. Neutrophils also secrete classical mediators of inflammation at these sites, such as eicosanoids and cytokines. These mediators are responsible for a variety of features characteristic of an inflammation, such as prostaglandins that cause vascular dilation and increased vascular permeability, resulting in erythema and swelling. Neutrophils can also amplify the inflammatory response by releasing cytokines, such as TNF-α and interleukin (IL)-8, that attracts additional leukocytes [10, 11]. Therefore along with their anti-microbial phagocytic activity, neutrophils also actively participate in the development and continuation of the inflammatory response. The crucial role of neutrophils in the innate immune response is emphasised by the profound incidence of infection observed in patients displaying neutrophil dysfunction and impaired neutrophil trafficking [12].

1.2.1. Neutrophil migration

Neutrophils possess an extraordinary mechanism to "sense" chemotactic stimuli at extremely low concentrations, towards which they can migrate [13]. Neutrophils can detect a wide range of chemoattractants through receptors located on the plasma membrane including *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), lipid mediators such as platelet activating factor (PAF) and leukotriene (LT)B_4, complement component C5a and chemokines such as IL-8. The response of neutrophils to chemoattractants is initially manifested by polarisation of the cell and the projection of pseudopods, which are actin-rich cytoplasmic protrusions [14]. Chemotactic peptide receptors can be localised on pseudopods, therefore sensing and locomotion towards chemotactic stimuli occurs. The polarisation and locomotion of neutrophils is dependent on the cytoskeletal rearrangements that may follow a flux in intracellular calcium (Ca^{2+}) upon binding of chemotactic stimuli [15]. Desensitisation of chemotactic responses can also occur following prior exposure to the same chemotactic stimulus. Generally desensitisation is specific, providing that the exposure to agonist is moderate, however high concentrations of agonist can lead to non-specific desensitisation and may affect similar members within a receptor family [16].

1.2.2. Neutrophil activation and degranulation

Along with cytotoxic and immune-modulating contents, neutrophil vesicles and granules also contain adhesion molecules and receptors. Neutrophils possess four main granule types; secretory vesicles, tertiary (or gelatinase) granules, specific granules and azurophil (or primary) granules. Neutrophil granules are released in a hierarchical manner and require different levels of Ca2+ to initiate their secretion from the neutrophil [17]. Granules that are formed in the later stage of neutrophil maturation, such as secretory vesicles and gelatinase granules, are generally released first following neutrophil stimulation. Azurophil granules are more resistant to mobilisation and contain the cytotoxic enzymes myeloperoxidase (MPO) and neutrophil elastase [3]. It has been shown that neutrophils release a small proportion of total elastase upon activation (~2%), therefore the detection of elastase in inflamed tissues is probably due to neutrophil necrosis as opposed to specific mobilisation and secretion of granules [18]. Neutrophils also discharge neutrophil extracellular traps (NETs), which are a dense mixture of granule components and chromatin, following loss of nuclear integrity. NETs can ensnare and kill bacteria and fungi, even following the death of the neutrophil [19].

1.3. The neutrophil-mediated inflammatory response

In order for neutrophils to reach inflamed tissues and perform their antimicrobial functions, they must emigrate from blood vessels in a process known as transendothelial migration, which occurs primarily in the postcapillary venules of the affected tissue. The process of transendothelial migration during the inflammatory response is truly remarkable, as entire immune cells move through the endothelial monolayer without substantial disruption to the barrier function of the endothelium. It is crucial that the integrity of the endothelium is maintained during leukocyte extravasation so that uncontrolled movement of molecules in and out of the blood stream does not occur. The passage of an entire cell through the endothelial sheet is a significant event, as substantial alteration of endothelial cell-cell adhesion molecules and cytoskeletal remodelling are required to allow for the entry of the leukocyte. The leukocyte itself also upregulates a number of adhesion molecules and requires extensive cytoskeletal remodelling so its shape can be altered to assist in luminal crawling and squeezing through the endothelium.

The process of neutrophil adhesion and transendothelial migration requires a highly controlled sequence of events that involves the activation and interaction of many molecules located on both the neutrophil and endothelial cells [20]. Neutrophils circulating at high velocity in the blood stream initially slow and begin rolling along the endothelium through transient binding mediated by the selectin family of proteins. Neutrophils then firmly adhere to the endothelium through the up-regulation of integrins and immunoglobulin (Ig)-like cell adhesion molecules, whereupon they flatten and migrate laterally across the endothelial surface, presumably to establish a suitable position for transmigration. Neutrophils then squeeze through or between endothelial cells and migrate through the endothelial sheet in a primarily adhesion-molecule dependent manner. The final step involves neutrophil penetration through the basal lamina and into the interstitial tissue. This complex process occurs within minutes [21], where the disturbance of a given protein involved at any of the distinct phases can be sufficient to compromise the degree of neutrophil transendothelial migration that would ordinarily occur.

1.4. Neutrophil transendothelial migration *in vitro*

Despite the apparent complexity of leukocyte recruitment, many relatively simple *in vitro* systems have been established to investigate the process and have been shown to faithfully mirror *in vivo* events with surprising accuracy. Neutrophil transmigration across endothelial monolayers can be induced *in vitro* by a range of different stimuli. Generally, unstimulated neutrophils will

not transmigrate across a resting endothelium that has not been activated, however if a chemotactic gradient is established by the addition of a chemoattractant on the abluminal surface of the monolayer, chemotaxis and transmigration towards this stimulus will occur. Stimulating endothelial cells with cytokines such as TNF-α or IL-1 can also induce transmigration without the presence of a chemoattractant. The addition of such cytokines results in the up-regulation of endothelial adhesion molecules and secretion of chemoattractants such as IL-8 [22], which facilitates transendothelial migration by activating neutrophils. Endothelial stimulation *in vivo* most likely originates from cytokines released during tissue injury and microbial products. Many of these *in vitro* experimental systems are performed under static conditions however, and are therefore lacking in important factors that are induced or upregulated by the shear force of blood flow and the process leukocyte rolling on the endothelium [23-25]. Consequently the results gained using static *in vitro* assays should be confirmed additionally through using flow chamber models.

1.5. The leukocyte recruitment cascade

It is essential that leukocyte infiltration to inflammatory sites is highly regulated so that the inflammatory response is specific and efficient. Naturally, extravasation must only occur at sites where there is need for the infiltration of immune cells. This is achieved by the local production of inflammatory mediators which stimulates the leukocyte to extravasate at a defined area. It is also important that leukocyte transmigration is transient, so that tissue damage does not occur due to the uncontrolled trafficking of leukocytes. To this end, anti-inflammatory mediators and negative feed-back mechanisms are critical in protecting the host organism from tissue damage arising from the sustained presence of leukocytes at an inflammatory lesion. It is also important that signalling is directed at the appropriate leukocyte subset, depending on the type of inflammatory response required, which is often governed temporally by distinct mechanisms. This section describes the suite of molecules involved in the leukocyte recruitment cascade (Figure 1.1) with a particular focus on neutrophil recruitment, which together function to achieve a rapid, yet specific immune response.

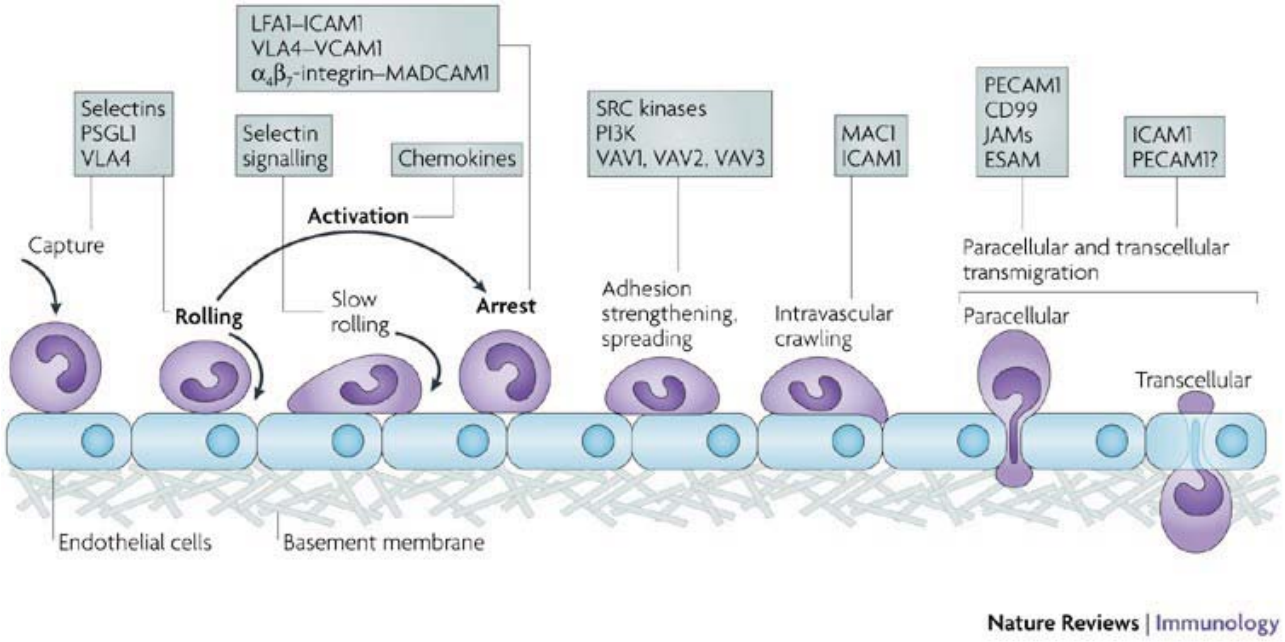


Figure 1.1: Current model of the leukocyte adhesion cascade.

Initial leukocyte tethering to the endothelium is then followed by slow rolling and strengthening of adhesive bonds. Following firm adhesion, leukocytes begin to spread and crawl on the luminal surface. Finally, paracellular and/or transcellular transmigration occurs. Key adhesion and signalling molecules involved in each step are highlighted in boxes. Abbreviations: ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1 or CD-11a/CD-18; MAC1, macrophage antigen 1 or CD-11b/CD-18 ; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 or $\alpha_4\beta_1$-integrin.

Sourced from: Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. **7** (9): p. 678-89.

1.5.1. Tethering and rolling: the selectins

Under normal conditions, circulating neutrophils do not adhere to the endothelium; however this rapidly changes in the context of inflammation. Before circulating neutrophils can migrate between the cells of the endothelium, the shear-force of blood flow within the vessel must first be overcome in order for neutrophils to initiate firm contact with the vessel wall. This process is principally mediated by a family of carbohydrate binding molecules known as selectins, which are present on both neutrophils and the activated endothelium to function as a primary adhesion mechanism between the two cell types [26]. Selectins are calcium-dependent, transmembrane glycoproteins that display a c-type lectin domain in the protein's extracellular region and bind sialylated and fucosylated oligosaccharides located on the plasma membrane. L-selectin is constitutively expressed on leukocytes and P- and E-selectin are upregulated on cytokine-stimulated endothelial cells [27]. Endothelial P- and E-selectin are lengthy molecules of approximately 30-40 nm that extend into the vessel to capture leukocytes by reversibly binding to their respective carbohydrate ligands, which decreases the velocity of circulating neutrophils through postcapillary venules [28]. The low affinity of selectin binding is not sufficient to halt circulating neutrophils, but it is able to decrease their velocity and gives rise to the rolling phenomenon along the endothelium. This has an important purpose as it allows locally produced inflammatory stimuli, such as IL-8 from the endothelium or products from pathogens, to reach the neutrophils in high enough concentration to initiate their activation. Selectin-induced neutrophil rolling also permits additional molecules to bind neutrophils with greater affinity, resulting in firm adhesion to the endothelium in preparation for transendothelial migration. The inhibition of selectin binding between neutrophils and endothelial cells decreases the total amount of transmigrating leukocytes [29]. P-selectin is constitutively expressed and is stored in Weibel-Palade bodies in the endothelium, which are rapidly mobilised to the plasma membrane upon stimulation with mediators such as histamine or thrombin to increase surface expression. The rate at which P-selectin is upregulated on the surface of the endothelium is consistent with its involvement in the early stages of leukocyte recruitment. It has been found that TNF-α, LPS and IL-1 induce the transcription of endothelial selectins, however while P-selectin is upregulated in mice, there appears to be a preference for E-selectin synthesis in human endothelial cells following stimulation [30]. The reason for this variation is unknown, however it has been shown that the leukocyte rolling velocity is profoundly decreased on E-selectin compared to P-selectin, which was postulated to increase the likelihood of adhesion to the endothelium [31]. Unlike P-selectin, E-selectin is not constitutively expressed in endothelial cells, but cell surface expression

is observed in as little as 2h after stimulation with TNF-α, where the simultaneous upregulation of both selectins enhances leukocyte recruitment [26].

L-selectin is present on leukocytes and is restricted to microvilli to facilitate interactions with the endothelium *via* binding to P-selectin glycoprotein ligand-1 (PSGL-1), however its role in leukocyte extravasation is not as apparent as that for P- and E-selectin [32]. *In vitro* it has been shown that circulating leukocytes bind to leukocytes already rolling on the endothelial surface in a L-selectin and PSGL-1 dependent manner [33]. This process has been termed secondary tethering and is believed to assist in the recruitment of leukocytes that do not express ligands for P- or E-selectin. L-selectin mediated rolling can also activate leukocytes, potentially through signalling to adjacent G-protein-coupled receptors, however the mechanisms of this activation are unclear [34]. The selectins involved in leukocyte emigration display "catch-bond" characteristics, where the shear-forces exerted by blood flow counter-intuitively increase the affinity of the molecular bonding of selectins to their ligands to support leukocyte adhesion [35].

1.5.2. Firm Adhesion: the integrins

It has been clearly demonstrated that after selectin-mediated neutrophil rolling, β_2-integrins on neutrophils are rapidly activated and bind to endothelial adhesion molecules. This is an essential step for neutrophils to slow and firmly adhere to the endothelium in preparation for transmigration [36]. The events regulating the transition of neutrophil rolling to integrin activation and adhesion are complex. Traditionally it was thought that chemokines and molecules presented on the surface of the activated endothelium were responsible for β_2-integrin activation on neutrophils [37, 38]. However it has also been shown that E-selectin and L-selectin binding can trigger signalling pathways in the endothelium [39] and neutrophils respectively [40, 41] and that E-selectin has the capacity to bind L-selectin in humans, although this is not observed in mice [42]. Most recently it has been demonstrated that E-selectin binding to L-selectin and PSGL-1 resulted in the clustering of these molecules on the surface of neutrophils by signalling through the p38 and p42/44 mitogen-activated protein kinase (MAPK) cascades [43]. Inhibitors of the MAPKs blocked this clustering and inhibited the activation of high-affinity β_2-integrins and subsequent firm adhesion of neutrophils to the endothelium. Therefore it appears that β_2-integrin activation and neutrophil arrest on the endothelium is the result of both chemoattractant and selectin mediated events.

Integrins are adhesive molecules that are composed of non-covalently linked α and β subunits. The key β_2-integrins involved in firm adhesion of neutrophils to the endothelium are lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) and macrophage antigen-1 (Mac-1 or CD11b/CD18), both of which consist of a unique alpha subunit but possess the same β_2-integrin chain (CD18). A unique feature of integrins is the ability to activate the extracellular portion of the molecule in <1 second *via* signalling pathways within the cell, coined as "inside-out signalling" [44]. This property has particular relevance in the inflammatory setting, as circulating neutrophils need to be efficiently decelerated and arrested on the area of the endothelium from where inflammatory signals are being generated. When activated, the β_2-integrins undergo a conformational change to dramatically increase their ligand-binding capacity. LFA-1 appears to be primarily involved in neutrophil adhesion to the endothelium, whilst Mac-1 activity is important in neutrophil spreading and locomotion to sites of transmigration [45, 46]. Both LFA-1 and Mac-1 bind to endothelial intercellular adhesion molecule-1 (ICAM-1), however this occurs through ligation to different domains [47, 48]. Depending on the inflammatory setting, Mac-1 can additionally bind other ligands, such as fibrinogen and junctional adhesion molecule (JAM) C [49, 50]. The involvement of LFA-1 and Mac-1 in neutrophil adhesion and crawling were convincingly shown in a study using LFA-1 and Mac-1 deficient neutrophils, using time-lapse microscopy *in vivo* [51]. The majority of LFA-$1^{-/-}$ neutrophils failed to adhere to inflamed venules, but those that did make contact were able to crawl efficiently on inflamed venules in a similar manner to wild-type neutrophils. Conversely, adhesion was unaffected in Mac-$1^{-/-}$ neutrophils; however intraluminal crawling to transmigration sites was significantly attenuated, causing neutrophils to transmigrate at the initial point of adhesion to the endothelium [51]. Furthermore, blocking Abs to ICAM-1 and ICAM-2 demonstrated that Abs to ICAM-1 alone were sufficient to inhibit Mac-1-mediated locomotion, however neither Ab could prevent neutrophil adhesion [51]. This study indicated that whilst LFA-1 and Mac-1 were important in neutrophil adhesion, they appear to mediate distinct sequential events.

The crucial role of β_2-integrins in neutrophil adhesion is highlighted in the inherited immunodeficiency disease leukocyte adhesion deficiency 1 (LAD) [52]. LAD1 is characterised by a deficiency in the expression of LFA-1, Mac-1 and other integrins expressing the β_2-integrin chain, usually due to a defect in the β-subunit or from point mutations that prevent the α and β subunits from associating [53]. Patients afflicted with the disease suffer from life-threatening recurrent bacterial and fungal infections, delayed wound healing and severe gingivitis. Because the β_2-integrins are not available to arrest neutrophils to the inflamed endothelium in response to

the usual stimuli, they cannot migrate into inflammatory sites to initiate an immune response. It has been shown through the use of animal models that antibodies (Abs) to CD18 confer a phenotype similar to that of LAD1 patients and that Abs to CD18 can inhibit neutrophil transendothelial migration *in vitro* [54]. Interestingly, neutrophils isolated from a child displaying LAD1 were able to migrate across endothelial monolayers *in vitro* using a static transmigration assay, but transmigration could only be induced using a chemotactic gradient of IL-8 and not fMLP [55]. Increased adhesion of these neutrophils to LPS-activated endothelium was also observed and thought to be a result of selectin-mediated mechanisms. This indicated that whilst β_2-integrin-mediated firm adhesion to endothelial cells is a critical step in the leukocyte recruitment cascade, other β_2-integrin independent mechanisms also exist and have been observed in disease states such as cystic fibrosis [56].

Other integrins have also been shown to be important for leukocyte rolling on the endothelium. Ablation of $\alpha_4\beta_1$ integrins in LFA-1-deficient mice using blocking Abs significantly increased the rolling velocity of leukocytes compared to the already reduced level of rolling detected in the knockout mice alone [45]. This indicates that both LFA-1 and the α_4-integrins are involved in leukocyte rolling to varying extents, which confirmed previous studies also highlighting a role for the α_4- integrins in leukocyte rolling [57-59]. Slow rolling induced by integrins is generally detected following initial selectin-mediated contact of the leukocyte with the endothelium and is characterised by decreased velocity arising from selectin signalling.

1.5.3. Intraluminal crawling

Following leukocyte arrest on the endothelium, leukocytes crawl laterally across the endothelium to locate a suitable site for transmigration (Figure 1.2). Luminal crawling or “locomotion” is mediated by Mac-1 and ICAM-1 [46, 51]. Mac-$1^{-/-}$ neutrophils were virtually unable to crawl on endothelial cells, where those cells that did displayed a dramatic reduction in crawling velocity. Mac-$1^{-/-}$ neutrophils were observed transmigrating at sites near initial adhesion, however this process was delayed compared to wild-type mice and was postulated to be due to emigration at “sub-optimal” areas [51]. These studies highlighted that intraluminal crawling is an important process required for efficient leukocyte extravasation.

Figure 1.2: Neutrophil intraluminal crawling *in vivo*.

Following leukocyte arrest on the endothelium, leukocytes crawl laterally across the endothelium to locate a suitable site to traverse the endothelial barrier. This neutrophil is projecting lamellopodium as it crawls across the endothelial surface. Magnification x8000.

Sourced from: Hoshi, O. and Ushiki, T. (1999) Scanning electron microscopic studies on the route of neutrophil extravasation in the mouse after exposure to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). *Arch Histol Cytol*. **62**(3): p. 253-60.

1.5.4. Neutrophil transendothelial migration

Up until the early 2000s, leukocytes were generally thought to transmigrate in a paracellular manner by squeezing through the intercellular junctions between endothelial cells. Leukocytes emigrating *via* the paracellular route transiently alter the organisation of lateral cell adherence molecules, which rapidly returned following extravasation to maintain the integrity of the endothelium [60-62]. Recently, the subject has become rather contentious after a number of groups have challenged the concept of paracellular migration and have presented data showing leukocytes traversing the endothelial barrier by passing transcellularly through the body of individual endothelial cells (Figure 1.3). Transcellular migration of leukocytes across the endothelium is not a new concept however and has been documented *in vivo* by a number of groups, with seminal studies describing this phenomenon being conducted nearly 50 years ago [63-67]. Another study also identified that fMLP- and leukotriene B4-stimulated neutrophils and monocytes extended pseudopods into the apical surface of endothelial cells in preparation for transmigration *in vitro*, but this rarely occurred at endothelial cell junctions [68].

Paracellular and transcellular migration are not mutually exclusive and both appear to take place under inflammatory conditions. However the preference for a particular mode of transmigration may be determined by the type of leukocyte, the vascular bed, the degree of endothelial activation and the type of inflammatory stimuli. Using an *in vitro* flow chamber model, Cinamon *et al* have demonstrated the transcellular transmigration of neutrophils, however the degree of transmigration was dependent on the level of β_2-integrin activation, the shear-stress applied to the system, along with the extent of endothelial activation and subsequent production of chemoattractant [69]. In a similar flow chamber model using TNF-α-activated human umbilical vein endothelial cells (HUVECs), neutrophils and lymphocytes were shown to transmigrate using different mechanisms under the same conditions [70]. Lymphocytes were detected transmigrating *via* the transcellular pathway, whereas neutrophils were found to emigrate preferentially at endothelial junctions. An additional study also highlighted the importance of endothelial activation, where it was found that following the stimulation of HUVEC with TNF-α for 4h, paracellular migration of human neutrophils was predominantly observed, however the amount of transcellular migration increased by 20% following 24h stimulation [71]. This study also suggested that different signalling pathways may also be involved in determining the type of transmigration that occurs, as inhibiting ICAM-1 signalling by removing the cytoplasmic portion of the protein could prevent the transcellular, but not paracellular, migration of neutrophils, [71].

Figure 1.3: Neutrophil transmigration *in vivo*.

Electron micrographs of neutrophils at different stages of transendothelial migration.
A) 5 neutrophils in the process of transmigration. Arrows indicate neutrophils migrating transcellularly and arrow heads show neutrophils transmigrating through paracellular junctions. Yellow region indicates the area of a single endothelial cell. Magnification x3000.
B) Closer view of A). L indicates raised endothelial surface due part of the transmigrated neutrophil located under the endothelial monolayer. Magnification x8000.
C) A neutrophil in the process of transendothelial migration (arrow) and two neutrophils adherent to the endothelial surface. N indicates nucleus of endothelial cell and * denotes platelets attached to the neutrophils. The arrowhead shows a membranous projection on the surface an adherent neutrophil. Magnification x5000.

Sourced from: Hoshi, O. and Ushiki, T. (1999) Scanning electron microscopic studies on the route of neutrophil extravasation in the mouse after exposure to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). *Arch Histol Cytol*. **62**(3): p. 253-60.

The mode of transmigration may also depend on the characteristics of the endothelium. In sensitive areas such as the brain, the endothelium possesses tight junctions between individual cells of the endothelial sheet, which are points of close contact that are critical in maintaining the integrity of the endothelium. However these also pose as an additional obstruction for extravasating leukocytes. Consequently it was found that transcellular migration of lymphocytes predominates across the inflamed blood-brain-barrier, which was postulated to occur so that the tight junctions remained intact [72].

Although many of the components implicated in transcellular and paracellular migration are common to both methods, the level of involvement appears to vary to different extents. The following sections describe the activity of the different adhesion molecules with important functions in the leukocyte recruitment cascade.

1.6. Adhesion molecules involved in leukocyte transmigration

1.6.1. ICAM-1 and ICAM-2

ICAM-1 is involved in the adhesion and transmigration phases of the leukocyte recruitment cascade. Although ICAM-1 is consitutively expressed on endothelial cells, the stimulation of cultured micro- and macrovascular endothelial cells with pro-inflammatory mediators such as TNF-α and IL-1 has been shown to dramatically upregulate ICAM-1 expression [73]. *In vivo*, increased endothelial ICAM-1 expression has also been observed in inflamed lung tissue [74] and in areas prone to atherosclerotic plaque formation [75]. The critical role for ICAM-1 was further emphasised when neutrophil transmigration was promoted following the overexpression of ICAM-1 in Chinese Hamster Ovary (CHO) cells, which do not normally synthesise endogenous ICAM-1, highlighting that ICAM-1 alone was sufficient to induce transmigration *in vitro* [76]. Blocking Abs to ICAM-1 have been shown to significantly impair leukocyte adhesion and transmigration in several animal models [77]. Consistent with these results, neutrophil infiltration into the peritoneal cavity was found to be significantly diminished in a model of peritonitis using ICAM-1 deficient mice [78]. These mice also displayed abnormal lymphocyte recruitment to the ear in a contact hypersensitivity model [78], which very convincingly shows ICAM-1 to be indispensible for the transendothelial migration of a number of leukocyte subsets.

ICAM-1 interaction with the leukocyte integrin LFA-1 jointly depends on ICAM-1 dimerisation [79] and the degree of LFA-1 activation [80]. The integrin-mediated clustering of endothelial

ICAM-1 induces the formation of docking structures that have been termed “transmigratory cups”, which are essential for leukocyte transendothelial migration to occur (Figure 1.4). Transmigratory cups are endothelial projections rich in ICAM-1, vascular cell adhesion molecule (VCAM)-1 and LFA-1 that extend from endothelial cells to capture and guide transmigrating leukocytes [81-83]. Transmigratory cup formation is dependent on actin polymerisation, microtubule reorganisation and modulation of intracellular Ca^{2+} [84]. In addition to adhesion proteins, transmigratory cups also contain high levels of cytoskeletal molecules [83]. These endothelial projections resemble microvilli and were initially observed forming ring-like clusters around adherent leukocytes, however it has since been shown that the transmigratory cups can further extend up and over transmigrating leukocytes to envelop them in “dome” structures [85]. It was postulated that the formation of endothelial transmigratory cups/domes over emigrating leukocytes assists in maintaining endothelial barrier function during the process of transmigration [85]. Transmigratory cups have been found to participate in both transcellular and paracellular migration events and are connected to the actin-cytoskeleton through ERM (ezrin, radixin, moesin) adaptor proteins [83]. Consequently the clustering of ICAM-1 and VCAM-1 can lead to reorganisation of the cytoskeleton through anchorage of these adaptor proteins with proteins such as vinculin and α-actinin [86]. Following the adhesion of leukocytes to the endothelium and ICAM-1 ligation, apical ICAM-1 has been shown to translocate to the basolateral membrane by association with regions concentrated in F-actin and caveolae [72]. Furthermore, it was found that this feature resulted in the formation of endothelial channels, stabilised by actin and vimentin [70, 72], through which leukocytes could migrate in order to traverse the endothelial barrier.

Despite the number of studies detailing the effects of ICAM-1 signalling, a signal transduction pathway downstream of its activation is still yet to be identified. The C-terminus of ICAM-1 appears to be important for its signalling, as an ICAM-1 mutant devoid of the C-terminus is inefficient at inducing the adhesion and transmigration of lymphocytes [87]. Furthermore, the expression ICAM-1 lacking its C-terminus failed to promote transmigration in the same manner as wild-type ICAM-1 expressed in CHO cells [76].

In comparison to ICAM-1, ICAM-2 activity does not appear to be as essential for leukocyte transendothelial migration in the inflammatory setting. ICAM-2 shows restricted tissue distribution and is present in substantial quantities in the vascular endothelium. ICAM-2 does not require cytokine treatment to induce expression like ICAM-1 and is actually down-regulated following treatment with inflammatory cytokines, indicating ICAM-2 may be important in basal

Figure 1.4: Transmigratory cup formation around extravasating monocytes.

Endothelial projections rich in ICAM-1 and VCAM-1 surround monocytes during transendothelial migration.
A) Apical image of a monocyte during early stage transmigration. Image is a composite image of ICAM-1 (green) and LFA (red).
B) Lateral view of a transmigrating monocyte. α4-integrin (green-monocyte), VCAM-1 (red -endothelium).
C) Apical and lateral view of endothelial projections surrounding a transmigrating monocyte. ICAM-1 (IC1, green), VCAM-1 (VC1, red), β_2-integrin (β2, blue). Transmigrating monocyte is not stained. Last panel shows a merged image of the first three panels.

Sourced from: Carman, C.V. and Springer, T.A. (2004) A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol*. **167**(2): p. 377-88.

leukocyte emigration into non-inflamed tissues as required for general immune surveillance [88]. In line with these observations, T-lymphocyte transendothelial migration across ICAM-1$^{-/-}$ ICAM-2 $^{-/-}$ immortalised brain endothelioma cells can be fully restored following expression of ICAM-1, however ICAM-2 expression could only partially reconstitute transmigration [89].

1.6.2. VCAM-1

VCAM-1 is upregulated at inflamed sites by pro-inflammatory cytokines and has an important role in the leukocyte recruitment cascade as it is an adhesion receptor for leukocytes and other inflammatory cells bearing β_1-integrins [90]. As alluded to above, VCAM-1 is also important in the extravasation of leukocytes as it is a major component of endothelial transmigratory cups and associates with the cytoskeletal adaptor proteins ezrin and moesin [81, 83]. Unlike ICAM-1, there are no reports investigating the effect of C-terminally truncated VCAM-1 on leukocyte transmigration and endothelial signalling. Analysis of the C-terminal domain has found that VCAM-1 possesses a PDZ binding motif, but noticeably its juxtamembrane domain lacks the basic region that is present in ICAM-1 and ICAM-2 [91, 92]. These areas have been ascribed as important for binding to ERM proteins for molecules such as CD43 and CD44 [92], however VCAM-1 can still associate with ezrin and moesin *via* its cytoplasmic domain [83]. Although no such interaction of VCAM-1 and components of the cytoskeletal through the PDZ binding motif has been indentified to date, this region could also be important for cytoskeletal signalling.

Although the mechanism has not been defined, VCAM-1 clustering can activate Rac-1 and Rho in endothelial cells, induce the generation of reactive oxygen species (ROS), activate p38 MAPK, and cause alterations in the actin cytoskeleton and stress fibre formation [93, 94]. Furthermore, VCAM-1-induced ROS production was implicated in the activation of PKCα, an event essential for the transmigration of spleen cells across lymph node endothelial cells in mice [95]. The significance of this signalling in transcellular leukocyte transmigration is unclear however, as it involved increasing endothelial permeability by the temporary disruption of endothelial junctions and consequently may only be relevant to paracellular migration [94, 95]. It was also shown that leukocyte binding/Ab cross-linking of VCAM-1 or the addition of exogenous ROS induced the rapid activation of matrix metalloproteases (MMP) from endothelial cells [96]. MMP production was proposed to alter vascular permeability by degradation of components of the adherens junction or the extracellular matrix [96].

It has also been shown that shear stress is important for VCAM-1 signalling in endothelial cells during neutrophil and eosinophil adhesion [97]. Ligation of VCAM-1 under shear conditions results in the activation of the extracellular signal-regulated kinase (ERK) pathway by signalling through focal adhesion proteins and the actin cytoskeleton, where ERK activation been shown to be essential for both eosinophil and neutrophil transendothelial migration [98, 99].

1.6.3. PECAM-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1 , CD31) is a well-characterised cell adhesion molecule involved in the emigration of neutrophils [100]. PECAM-1 is a transmembrane glycoprotein of the Ig superfamily and is expressed on endothelial cells and leukocytes. PECAM-1 clearly concentrates at lateral cell-cell borders of the endothelium and more diffusely on the surface of leukocytes and platelets [101, 102], where it functions as both a cell adhesion molecule and a signal transducer through homophilic or heterophilic binding. The extracellular domain of PECAM-1 contains 6 Ig-like domains, while the cytoplasmic tail is long and complex, containing a number of tyrosine and serine residues [103]. Phosphorylation of these residues occurs in response to various forms of cellular stimulation, which in turn leads to the regulation of signalling complexes and PECAM-1 interaction with the cytoskeleton of endothelial cells.

The importance of PECAM-1 in inflammation has been established both *in vitro* and *in vivo*, through the use of soluble isoforms of PECAM-1 [104] and functional blocking Abs [105-107] that prevent neutrophil pseudopodia from entering the junctions between endothelial cells, which consequently inhibits transmigration. Binding of neutrophil PECAM-1 to Abs has been shown to activate neutrophils, trigger the activation of the Mac-1 and down-regulate L-selectin expression [108], highlighting its function in neutrophil adhesion to endothelial cells. PECAM-1 has also been shown to facilitate neutrophil trafficking across the basement membrane following passage through endothelial cell junctions [109-111], a function generally thought to be performed by proteases such as neutrophil elastase [112-114].

The movement of endothelial PECAM-1 in response to the paracellular transendothelial migration of neutrophils has been traced in real-time [62]. Homophilic endothelial PECAM-1 interactions appeared to open and surround the border of the migrating neutrophils, which then reformed approximately two minutes after the neutrophils had passed between the endothelial cells. This was in contrast to the movements of VE-cadherin [61, 62], which was transiently

displaced from the endothelial cell junction. The presence of PECAM-1 on the surface of neutrophils could explain this difference, where endothelial PECAM-1 may bind to its neutrophil counterpart to guide its movement between endothelial cells [115]. A large proportion of PECAM-1 is localised to a stretch of vesicle-like structures located immediately below the plasma membrane of endothelial cells, which regularly recycle between the plasma membrane and the sub-membrane compartment [116]. It was found that these recycling vesicles were targeted to and surrounded transmigrating neutrophils and monocytes that, when inhibited by interrupting microtubule activity, could also inhibit leukocyte extravasation [117].

Despite the obvious importance of PECAM-1 in mediating leukocyte extravasation, it is interesting that PECAM-1-deficient mice do not display a significant impairment of leukocyte recruitment in response to inflammatory stimuli and leukocytes still possess the means to traverse the endothelial barrier [110, 118]. This has been shown to be partly dependent on the genetic background of the mice, as some strains of PECAM-1-deficient mice show decreased leukocyte infiltration whilst others do not [119]. These studies did highlight that both PECAM-dependent and independent mechanisms exist for leukocyte extravasation.

1.6.4. CD99

Although PECAM-1 has been shown to significantly contribute to transmigration, the blocking of PECAM-1 function with Abs never resulted in complete inhibition of neutrophil extravasation, indicating that other molecules are involved in the process. CD99, a highly O-glycosylated molecule mainly thought to be involved in the cell-cell adhesion and activation of T-cells, thymocytes and other haemopoietic cells, was found expressed at the borders of confluent endothelial cells and on the surface of leukocytes, including neutrophils [120]. Where PECAM-1 Abs blocked leukocyte emigration at the luminal surface of endothelial cells, blocking of CD99 resulted in the suspension of migrating monocytes partially through the endothelial cell junctions. This indicated that the function of CD99 is subsequent to PECAM-1 binding of leukocytes and that each molecule mediates distinct steps during transendothelial migration through paracellular junctions. Blocking Abs to CD99 resulted in more than a 90% inhibition of monocyte transmigration *in vitro* and, when combined with PECAM Abs, the result was additive with essentially 100% inhibition of transmigration [120]. Since the discovery of its involvement in monocyte transmigration, CD99 and CD99L2, a murine CD99-related protein, have also been shown to also participate in the process of neutrophil extravasation [121, 122]. In contrast to

CD99, CD99L2 only mediated neutrophil emigration in mice and lymphocyte recruitment to inflamed tissue was unaffected following the treatment with CD99L2-blocking Abs [121].

1.6.5. JAMs

Junctional adhesion molecules (JAMs) are Ig-like proteins with varying tissue distribution, but localise to tight junctions and cell-cell borders [123]. The JAM family consists of the classical members JAM-A, JAM-B and JAM-C, in addition to other related proteins (see review [124] for the current nomenclature), which have been implicated in the transendothelial and transepithelial migration of leukocytes [125, 126]. Following treatment with pro-inflammatory cytokines, JAM-A translocates from lateral intercellular junctions and redistributes to the surface of endothelial cells [127]. This redistribution was found to facilitate JAM-A binding to the leukocyte integrin LFA-1 and consequently increased firm adhesion and transmigration of neutrophils and T-cells [128]. It is unclear whether this binding is required for specific signalling events or functions as a leukocyte-endothelial seal during leukocyte emigration. JAM-C expression has been detected in vascular endothelial cells, including high endothelial venules and in lymphatic vessels, but is also expressed by human leukocytes [129]. JAM-C can participate in homophilic interactions, however it has also been shown to specifically interact with the β_2-integrin Mac-1, but can not bind to LFA-1 in a similar manner as JAM-A [130]. The role for JAM-C in leukocyte extravasation was further strengthened by a report showing that JAM-C neutralising Abs could effectively prevent leukocyte infiltration in mice, whilst its targeted over-expression in endothelial cells could significantly increase leukocyte accumulation to inflamed sites [131]. The activity of JAM-C appears to be primarily involved in leukocyte adhesion and transmigration, where rolling events on the endothelium are unaffected by JAM-C Abs [131]. Endothelial JAM-B has been shown to interact with JAM-C, highlighting that this interaction may also be important in leukocyte adhesion to endothelial cells [132]. In fact, the inhibition of JAM-B and JAM-C interaction has been shown to increase monocyte reverse-transmigration, where decreased levels of infiltrating cells were observed at inflamed sites combined with elevated monocyte concentrations with a reverse-transmigratory phenotype detected in peripheral blood [133]. Interestingly, neutrophils have also been shown to reverse-transmigrate and display a reduced capacity for re-transmigration following this event [134].

A JAM-related protein, endothelial cell-selective adhesion molecule (ESAM), has recently been shown to promote leukocyte extravasation through the use of ESAM$^{-/-}$ mice. Neutrophil, but not

lymphocyte, accumulation in the peritoneal cavity was significantly impaired in ESAM$^{-/-}$ mice, however this was not through interefering with rolling or adhesion events [135]. As ESAM is consitutively and specifically expressed in tight junctions of endothelial cells, it was postulated that the protein could be involved in promoting neutrophil extravasation through endothelial paracellular junctions. This was supported by the finding that the absence of endothelial ESAM results in reduced levels of activated Rho, a GTPase involved in destabilising tight junctions, and the degree of permeability induced by vascular endothelial growth factor (VEGF) was reduced in ESAM$^{-/-}$ endothelial cells [135].

Although the JAMs have a clear role in cell-cell signalling and in leukocyte extravasation, it is unclear how these molecules signal to endothelial cells to promote transmigration. JAMs do however possess a C-terminal PDZ binding motif, which has been shown to mediate binding to cytosolic scaffolding proteins such as ZO-1, MUPP1, AF6 and Par 3 [136], and therefore may provide a mechanism by which JAMs provide outside-in signalling to endothelial cells.

1.6.6. VE-cadherin

The role of vascular endothelial-cadherin (VE-cadherin) in neutrophil transendothelial migration has been controversial. VE-cadherin is located on the lateral membrane of endothelial cells in the adherens junction complex. It primarily functions as an adhesion molecule between endothelial cells to maintain the integrity of the endothelium and is anchored to the cytoskeleton through interactions with α- and β-catenin. VE-cadherin was initially found to be a regulator of leukocyte transmigration when Abs to the protein increased the rate of neutrophils reaching sites of inflammation [137]. This logically suggested that the adherens junction serves as a barrier to migrating leukocytes. A number of early reports found that VE-cadherin and associated proteins were degraded in response to neutrophil migration between endothelial cells, therefore resulting in paracellular gap formation through which neutrophils could move [138, 139]. This was subsequently found to be the result of post-preparation artefacts generated from fixation methods [140], which proved to be a problem for a number of following investigations. The issue was finally resolved using a VE-cadherin-GFP construct, where the movements of VE-cadherin in response to neutrophil transmigration was visualised in real-time [61]. This study showed that *de novo* paracellular gaps formed in response to migrating neutrophils appeared to transiently displace VE-cadherin molecules whilst migrating between endothelial cells. The paracellular gaps were then found to reseal within five minutes. This VE-cadherin displacement from the

endothelial cell junction was confirmed in a later study, which employed Abs to visualise the real-time movement of VE-cadherin and PECAM-1 in response to emigrating neutrophils [62]. Neutrophils were also found to migrate at tri-cellular junctions where VE-cadherin is essentially not expressed [61], a feature that was also identified in previous studies [141, 142].

Therefore VE-cadherin does not appear to have a function in neutrophil transmigration *per se*, but its displacement from endothelial junctions is required for emigration to occur, as it would otherwise pose as a barrier.

1.7. Endothelial signalling during leukocyte extravasation

In light of the formation of ICAM-1 and VCAM-1 enriched endothelial transmigratory cup structures and the dynamic movement of endothelial PECAM-1, JAMs and VE-cadherin during neutrophil transendothelial migration, it stands to reason that endothelial intracellular signalling is required for these events. Early work conducted by the Silverstein laboratory highlighted that the endothelium was proactively involved during the transendothelial passage of leukocytes and was not simply an inert barrier [143]. It was reported that neutrophil adhesion to endothelial cells and transmigration in response to a fMLP gradient results in a transient increase in endothelial cytosolic free calcium Ca^{2+}, which was essential for transendothelial migration to occur [143, 144]. The rise in endothelial Ca^{2+} occurs just before or concurrently with transmigration and blocking the Ca^{2+} flux results in decreased neutrophil migration, but not adhesion. Unstimulated neutrophils were found to have no effect on endothelial Ca^{2+}. It was postulated that the rise in Ca^{2+} was involved in endothelial signalling pathways that "open" endothelial cell junctions to emigrating neutrophils. These studies stimulated a field of research into identifying endothelial signalling events that were involved in leukocyte extravasation, however much of the work was initially focussed on paracellular transendothelial migration as opposed to transcellular migration, probably because transcellular migration was not routinely observed *in vitro*. However a number of recent studies have investigated signalling pathways activated during transcellular migration since the identification of endothelial transmigratory cup formation and the detection of transcellular migration *in vitro* [81].

The concept that endothelial junctions could be disrupted by the active contraction of endothelial cells was first proposed over 50 years ago in the context of oedema [145]. It has since been shown that the phosphorylation of myosin light chains by myosin light-chain kinase (MLCK) mediates endothelial cell contraction [146]. Studies investigating the role of the cytoskeleton

during neutrophil extravasation found that phosphorylation of endothelial myosin II light chain by MLCK, a Ca^{2+}/calmodulin-dependent kinase, was essential for neutrophil transendothelial migration [147] and could be induced by activated neutrophils [148]. Whether this results in increased endothelial permeability to enable leukocyte emigration is a subject of contention, as some studies have reported that there no change in endothelial permeability associated with leukocyte transmigration [85, 149]. Thus Ca^{2+}-dependent endothelial cytoskeletal remodelling is an important event in neutrophil transmigration, but the effects of this process appear are varied.

Activation of ERK also appears to be an important endothelial signalling target during leukocyte transendothelial migration, as shown for both neutrophils and eosinophils [97, 99]. E-selectin ligation and clustering results in its association with the actin cytoskeleton and with Ras, Raf and MEK through the intracellular domain [97, 150]. This triggers ERK signalling in vascular endothelial cells and consequently activates transcription factors such as c-fos [150]. ERK activation was later found to be the result of association with SHP2 [151] and, in a similar manner to E-selectin, PECAM-1 ligation can also activate ERK through association with SHP2 [152]. Transmigratory cup components ICAM-1 and VCAM-1 have also been shown to mediate effects *via* the ERK signalling pathway. Ab cross-linking of endothelial ICAM-1 (to mimic ligand binding) was found to induce the production of chemokines by human umbilical vein endothelial cells (HUVECs), which was dependent on the activation of ERK1 and ERK2 [153]. VCAM-1 ligation under shear stress has additionally been shown to induce the activation of ERK in endothelial cells [97]. These studies highlight an important role for ERK signalling in the transendothelial migration of leukocytes, as the engagement of many of the major proteins involved in this process induce the activation ERK to mediate their effects. The ultimate downstream effects of ERK activation by these adhesion molecules are still yet to be determined.

Signalling to endothelial cells can be induced by many surface adhesion molecules and endothelial receptors following leukocyte contact and shear stress. Despite the range of proteins involved in this process, the trend appears to involve signalling *via* the cytoskeleton and MAPKs to upregulate additional pathways that modulate efficient leukocyte extravasation and amplification of the inflammatory response. Other molecules such as ROS, small GTPases, transcription factors and proteolytic enzymes have also been implicated in endothelial signalling events [152].

1.8. Endothelial ERK activation is required for neutrophil transmigration

In a previous study conducted in the laboratory, a range of inhibitors were screened to identify endothelial signalling pathways that were required for neutrophil transendothelial migration. Inhibitors of the ERK signalling pathway, PD98059 and UO126, significantly inhibited neutrophil transmigration, indicating that ERK activation was essential for neutrophil transmigration [99]. Furthermore, it was demonstrated that endothelial ERK was activated in response to a soluble protein produced by fMLP- or IL-8-stimulated neutrophils and the release of this protein was not dependent on neutrophil adhesion to endothelial cells.

This study provided the basis for the work outlined in this PhD thesis, where the initial aim was to identify and characterise the ERK-activating neutrophil protein. Additional investigations were also carried out to determine how the soluble neutrophil protein modulated transmigration by examining its effects on the endothelium.

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Reagents and antibodies

Antibodies (Abs) to active ERK1/2, which recognise dually phosphorylated residues Thr202/Tyr204 and Thr185/Tyr187 on ERK 1 and ERK2 respectively, were from either Promega (Madison, WI, USA) or Cell Signalling Technologies (Danvars, MA, USA) and anti-ERK Ab was from Promega. Mouse anti-annexin A1 was from BD Pharmingen (San Diego, CA, USA) and rabbit anti-annexin A1 was from AbCam (Cambridge, MA, USA). Anti-β-tubulin was from BD Biosciences (Franklin Lakes, NJ, USA). Anti-calprotectin mAb (clone 27E10) was from Hycult Biotechnology (Netherlands). Anti-calgranulin A and anti-calgranulin B pAb were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ICAM-1 and VCAM-1 monoclonal Abs (mAbs) were generated in-house [154]. fMLP was from Sigma Aldrich (St. Louis, WA, USA) and was diluted to 1 x 10^{-3}M in DMSO. PMA was from Sigma Aldrich and was diluted in Milli-Q H20 to 0.1mg/ml. Chemically synthesized IL-8 was a gift of I. Clark-Lewis (Centre for Biomedical Research, Vancouver, Canada) and was produced as a 72-aa form using automated solid-phase methods. Purified human fibronectin (Boehringer Mannheim, Indianapolis, IN, USA) was diluted in PBS, pH 7.3, to 50 µg/ml. Ac2-26 (acetyl-AMVSEFLKQARFLENQEQEYVQ AVK) was synthesised by Auspep (Parkville, Australia) and solubilised in 100mM ammonia. Boc2 (N-t-Boc-Phe-Leu-Phe-Leu-Phe) was from Phoenix Pharmaceuticals (Burlingame, CA, USA) and was diluted to 1mM concentration in 50% methanol. 4'-6-Diamidino-2-phenylindole (DAPI) was prepared in 100% methanol and used at a concentration of 1µg/ml.

2.2 Mammalian cell culture

2.2.1. Culture of HUVEC

HUVEC were extracted from umbilical veins by collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) treatment according to a modified protocol of Wall *et al.* [155] Cells were grown in gelatin-coated (Sigma Aldrich) tissue culture flasks (Costar, Cambridge, MA) in endotoxin-free M199 medium (JRH, Lenexa, KS, USA) supplemented with 20% foetal bovine serum (FBS, JRH), 20mM HEPES, sodium pyruvate and non-essential amino

acids at 37°C in a humidified 5% CO_2 atmosphere. Cells were replated 2-5 days after establishment of culture by harvesting with 0.05% trypsin, 0.02% EDTA. 0.1 mg/ml endothelial cell growth supplement (BD Biosciences) and 0.1mg/ml heparin (sodium salt, Sigma Aldrich) were added to cells passaged twice or more. Only cells between passages 2 and 4 were used for experiments.

2.2.2. Culture of HEK293 cells

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagles Medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 50µg penicillin/streptomycin (Invitrogen) and grown at 37°C in a humidified 5% CO_2 atmosphere. HEK293 cells were split every 3-4 days after reaching 80-90% confluency by harvesting with 0.05% trypsin, 0.02% EDTA.

2.2.3. Generation of HEK293 stable cell lines

15µl Lipofectamine 2000 (Invitrogen) with diluted into 250µl Opti-MEM (Invitrogen) and incubated at room temperature for 5 min. 4µg DNA was diluted into 250µl Opti-MEM, mixed with the diluted Lipofectamine 2000 and incubated for 20 min at room temperature. HEK293 growth medium was exchanged with 1.5ml Opti-MEM, then the above mixture added per 10 cm^2 ~90% confluent HEK293 cells. Cells were incubated at 37 ºC for ~15h before the transfection mixture was removed and replaced with growth medium. 24 later, the cells were trypsinised and plated into two 60cm^2 dishes in growth medium containing 300µg/ml G418 (geneticin). G418-containing medium was replaced every 1-2 days for ~7 days until mock-transfected cells were no longer viable. When isolated colonies of transfected cells were observable, individual colonies were picked and propagated separately. Expression of recombinant protein was compared between the clones by western blotting whole cell lysates with an Ab relevant to the recombinant protein.

2.3. Molecular biology methods

Standard cloning methods, unless otherwise stated, were performed as described in Sambrook and Russell [156].

2.3.1. Generation of neutrophil RNA

Neutrophils from 50ml fresh human blood were prepared as per section 2.5.1 and contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were resuspended in 5ml TRIzol reagent (Invitrogen) and incubated at room temperature for 5 min. 1ml chloroform was then added, mixed well and incubated for a further 3 min at room temperature. Following centrifugation at 4000 x g for 15 min at 4ºC, the aqueous phase was transferred to a fresh tube and an equal volume of phenol added. The total volume was made up to 10ml with chloroform, mixed and centrifuged 4000 x g for 15 min at 4ºC. The aqueous phase was transferred to a fresh tube then 2.5ml isopropanol added, mixed and then incubated for 10 min at room temperature. Following centrifugation at 4000 x g for 30 min at 4ºC, the supernatant was removed and the RNA pellet carefully washed with 5ml 75% ethanol prepared in DEPC-treated H_2O. The RNA pellet was dried at room temperature, then resuspended in 100µl RNase-free 10mM Tris, 0.1 mM EDTA, pH 8 and briefly heated at 65ºC to assist with dissolution.

2.3.2. Synthesis of neutrophil cDNA

Reverse transcription reactions from neutrophil RNA were carried out using Omniscript RT Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. This generated cDNA to clone S100A8, S100A9 and annexin A1 by polymerase chain reaction (PCR).

2.3.3. S100A8, S100A9 and annexin A1 cloning protocol

HA-tagged S100A8, Myc-tagged S100A9 and untagged annexin A1 were amplified by PCR from neutrophil cDNA using primers shown in Table 2.1. The PCR fragments were ligated into pGEM-T Easy (Promega) as described in the manufacturer's protocol and clones containing inserts were identified by blue-white colour selection. Plasmids were isolated from white colonies as described in section 2.3.10 and the S100A8/S100A9 or annexin A1 were excised using BamHI/XhoI (restriction sites engineered into the primer sequences), gel purified and inserted directionally into pcDNA3 (Invitrogen) (Figures 2.1, 2.2 and 2.3). The clones were sequenced using T7 (5'-ATTTAGGTGACACTATAG-3') and SP6 (5'-TAATACGACTCACTATAGGG-3') primers, which recognised sequences flanking the pcDNA3 multiple cloning site (MCS).

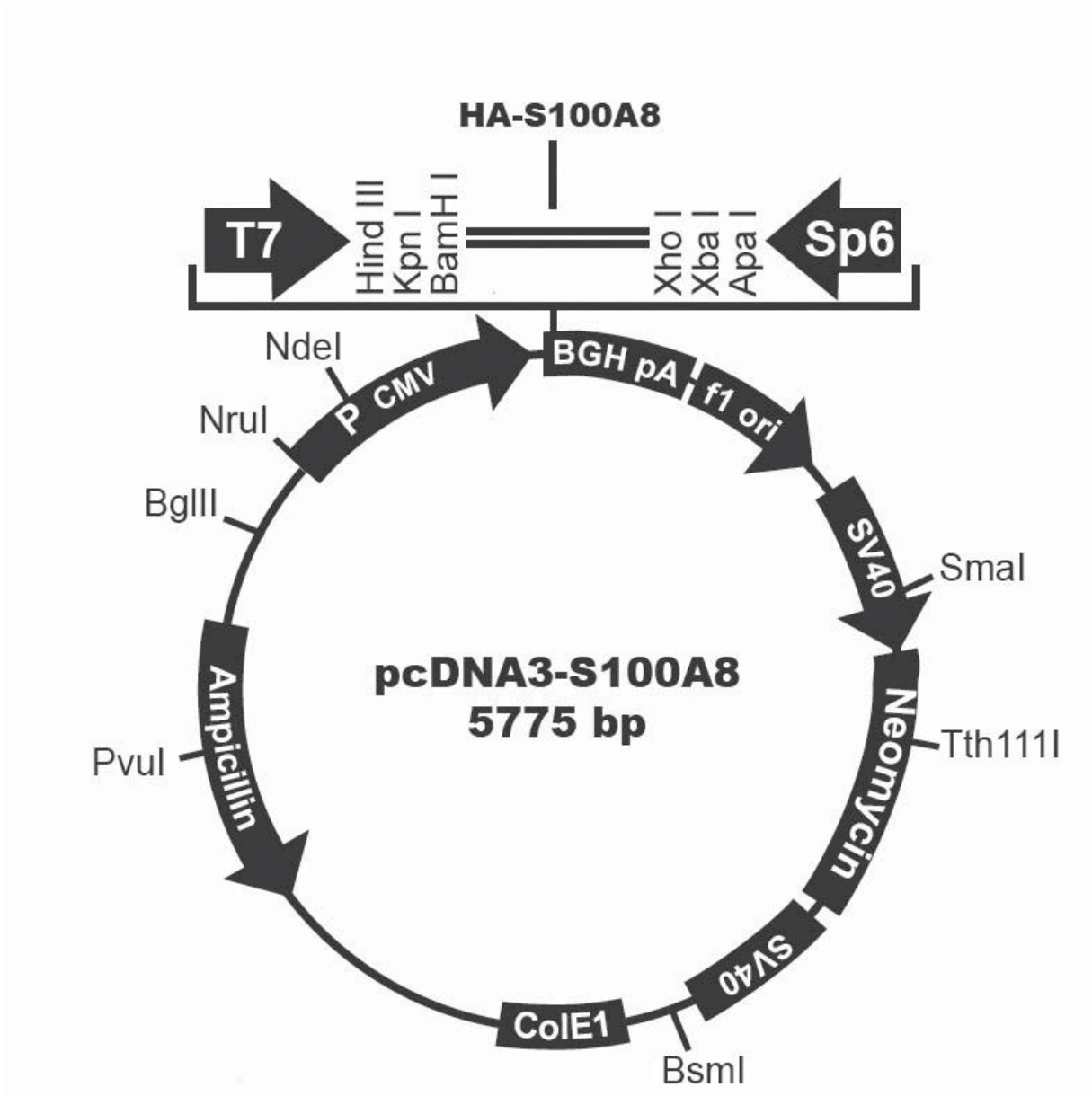


Figure 2.1: pcDNA3-S100A8.

Following ligation of the S100A8 PCR product into pGEM-T Easy, it was excised using BamHI/XhoI and ligated directionally into pcDNA3.

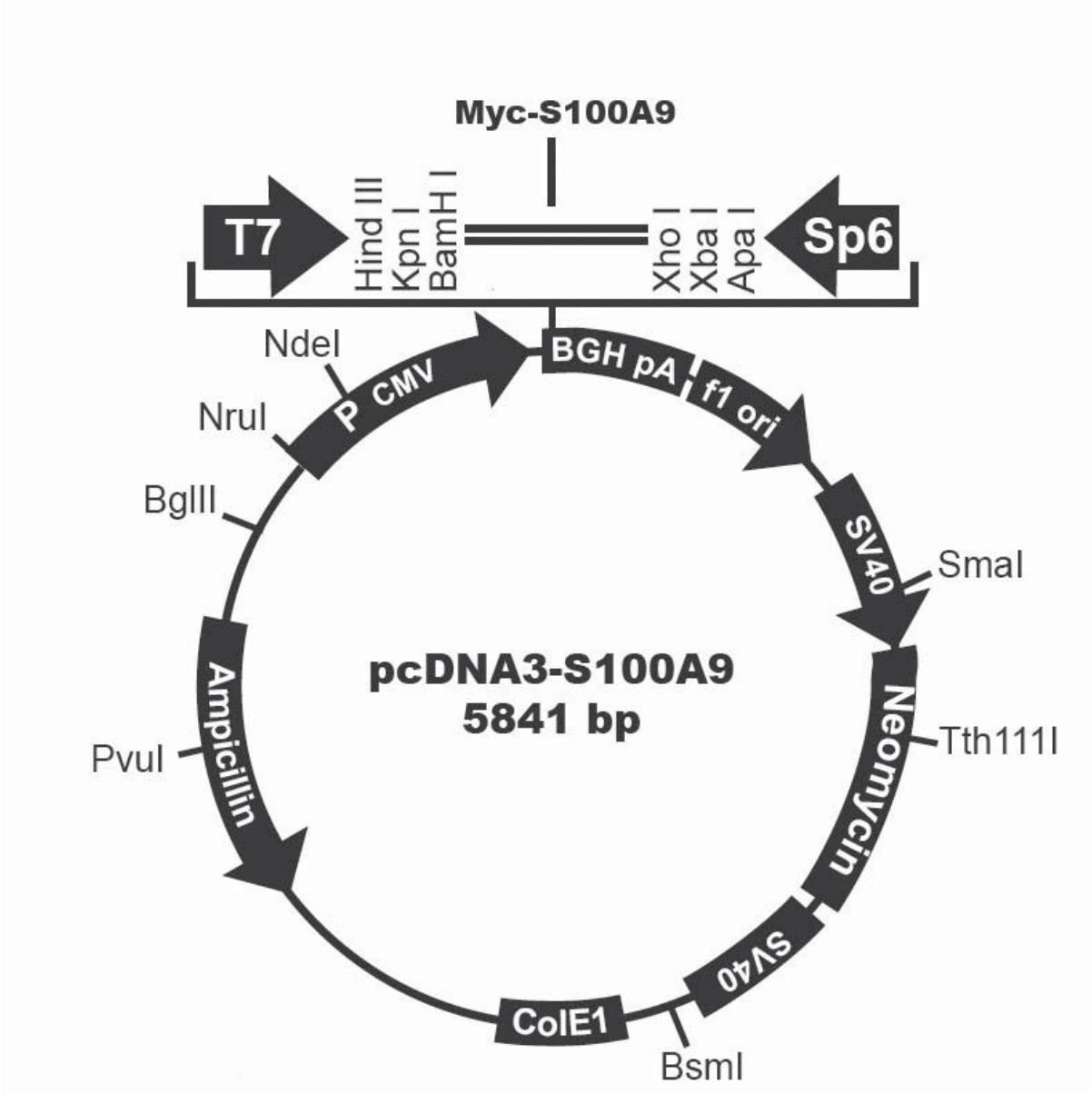


Figure 2.2: pcDNA3-S100A9.

Following ligation of the S100A9 PCR product into pGEM-T Easy, it was excised using BamHI/XhoI and ligated directionally into pcDNA3.

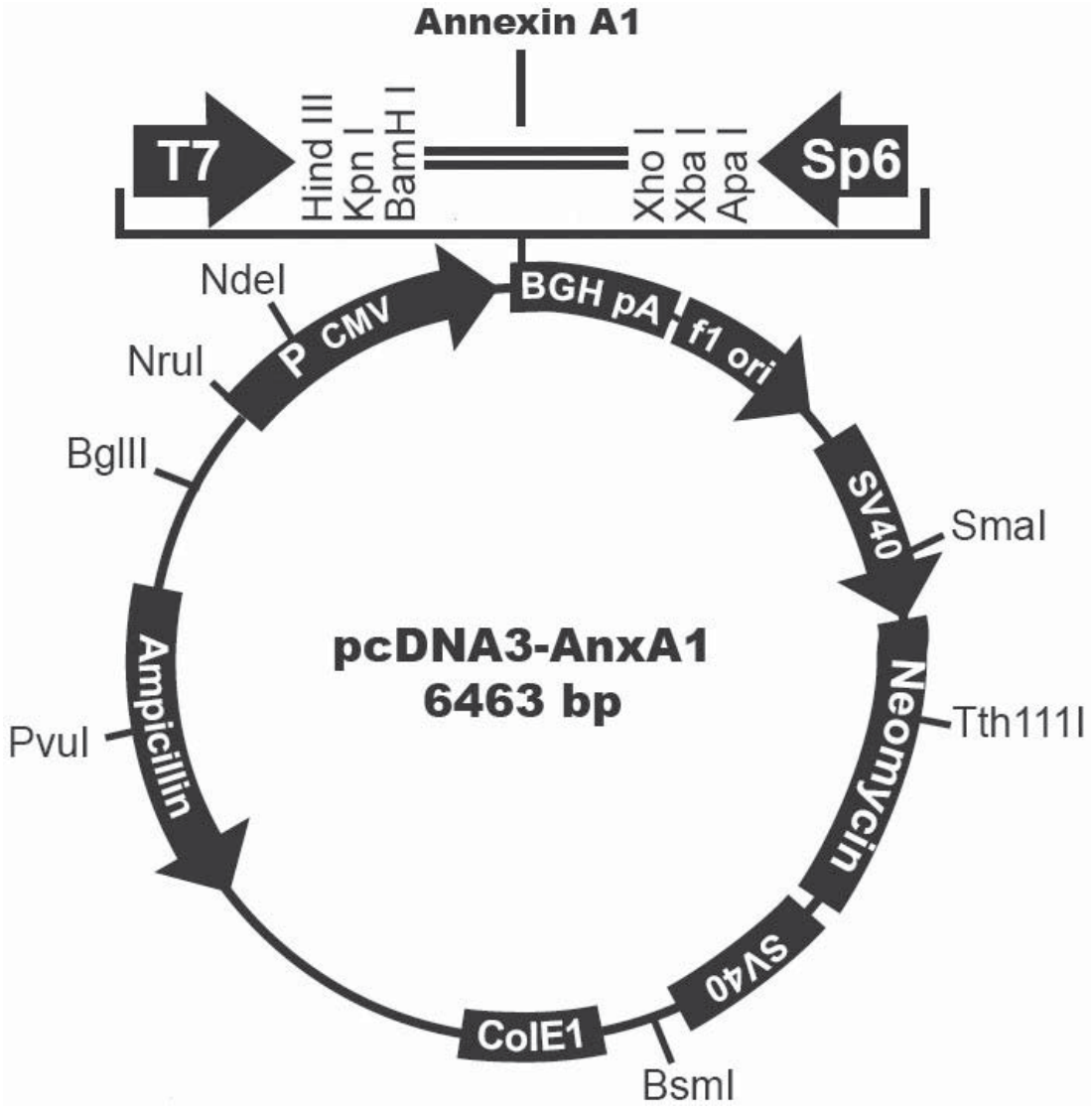


Figure 2.3: pcDNA3-AnxA1.

Following ligation of the annexin A1 PCR product into pGEM-T Easy, it was excised using BamHI/XhoI and ligated directionally into pcDNA3.

	Forward Primer (5' => 3')	Reverse Primer (5' => 3')
HA-tagged S100A8	TAGGATCCATGTACCCATACGACGTCCCAGACT ACGCTTTGACCGAGCTGGAGAAAGC	TTACTCGAGCTACTCTTTGTGGC TTTCTTCATGGC
Myc-tagged S100A9	TAGGATCCATGGAACAAAAACTTATTTCGAAGA AGATCTGACTTGCAAAATGTCGCAGCTGG	TTACTCGAGTTAGGGGGTGCCC TCCCCGA
Annexin A1	GGATCCATGTTTATTGAAAATGAAGAGCAGGAA	CTCGAGTTAGTTTCCTCCACAA AGAGC

Table 2.1. PCR primers.
Primers used to amplify HA-S100A8, Myc-S100A9 and annexin A1 by PCR from neutrophil cDNA.

2.3.4. Preparation of heat-shock competent DH5-α

Escherichia coli DH5α cells were streaked onto a LB plates and grown at 37ºC overnight. A single colony was picked and used to inoculate 2.5ml LB broth, which was incubated at 37ºC on a shaking rotor overnight. The overnight culture was used to inoculate 250ml LB broth and incubated at 37ºC on a shaking rotor until $O.D_{600}$ reached between 0.4 and 0.5. Cells were chilled on ice for 10 min, then centrifuged at 3800 x g for 10 min at 4ºC. The supernatant was poured off and the cells gently resuspended in 100ml TFBI solution (30mM potassium acetate, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% (v/v) glycerol, pH 5.8). Cells were incubated on ice for 5 min then centrifuged at previous conditions. The supernatant was poured off, the cells gently resuspended in 10ml TFBII solution (10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% (v/v) glycerol, pH 6.5) and incubated on ice for 15 min. Cells were aliquoted into pre-chilled eppendorf tubes and snap frozen in liquid nitrogen. Cells were stored at –80ºC.

2.3.5. Restriction enzyme digestions

Restriction enzymes were sourced from either New England Biolabs (Ipswich, MA, USA) or Promega and digestions were performed according to the manufacturer's recommendations.

2.3.6. Ligation reactions

Ligation reactions were performed using Rapid Ligation Buffer and T4 DNA ligase (Promega) according to the manufacturer's recommendations.

2.3.7. Transformation of heat-shock competent DH5-α

20ng of plasmid DNA or 2µl of ligation reaction was added to 50µl heat-shock competent DH5-α thawed on ice, then mixed gently and incubated on ice for 30-45 min. Samples were heat-shocked for 5 min at 37ºC and then returned to ice for a further 10 min. 1ml antibiotic-free LB broth was added and the samples were incubated on a shaking platform for 1h at 37ºC. All or a portion of the transformation reaction was plated onto LB plates + 100µg/ml ampicillin and incubated overnight at 37ºC.

2.3.8. Plasmid DNA purification

Plasmid mini-preps were used to purify plasmids from transformed bacteria. Isolated colonies were used to inoculate 2ml of LB broth, which was incubated overnight at 37ºC on a shaking platform. The culture was pulse-centrifuged at 17000 x g for 10 sec at 4ºC and the supernatant removed. The pellet was resuspended in 100µl ice-cold Solution I (50mM glucose, 25mM Tris pH 8, 10mM EDTA) by vortexing. 200µl freshly prepared Solution II (0.2M NaOH, 1% SDS) was added and mixed thoroughly by inversion. 150µl Solution III (3M sodium acetate, 5M glacial acetic acid) was added, mixed by inversion and incubated on ice for 20 min. The sample was centrifuged at 17000 x g for 10 min at 4ºC and the supernatant transferred to a new tube. 150µl TE-buffered phenol and 150µl 24:1 chloroform: isoamyl alcohol was added, the sample vortexed well and centrifuged at 16000 x g for 4 min at 4ºC. The top aqueous layer was transferred to a fresh tube, 300µl 24:1 chloroform:isoamyl alchohol added, vortexed and centrifuged at 17000 x g for 3 min. The top aqueous layer was transferred to a fresh tube, 2.5 volumes of 100% (v/v) ethanol added and incubated at 25ºC for 2 min. The sample was centrifuged at 17000 x g for 10 min, the ethanol decanted and the DNA pellet air dried. The pellet was resuspended in 10mM Tris pH 8.5, 20µg/ml RNase A (Promega) and stored at –20ºC.

2.3.9. Preparation of DNA for sequencing

Plasmid DNA was sequenced using BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Following the sequencing reaction, samples were subjected to an isopropanol clean up method. 10µl MQ H_2O)

was added to 10µl sequencing reaction, then 80µl 75% isopropanol was added, vortexed and incubated at room temperature for 15 min. The samples were centrifuged at 17000 x g for 20 min at room temperature and the supernatant decanted. Samples were then washed with 250µl 75% isopropanol and air-dried. Sequencing was performed by the Institute of Medical and Veterinary Science Sequencing Department.

2.4. Protein detection methods

2.4.1. Bradford protein assay for protein quantification

Bradford protein reagent (Biorad, Hercules, CA, USA) was diluted 1:5 in MQ H_2O. Protein samples and a 0.5-10 mg/ml titration of bovine serum albumin (BSA) were pipetted into a flat-bottomed 96-well plate in triplicate, and 100µl diluted Bradford reagent was added to each well. Following incubation at room temperature for 5 min, the absorbance was measured at 595nm. The concentration of protein samples was determined following the construction of a standard curve generated from the BSA readings.

2.4.2. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared as outlined in Sambrook and Russell [156] and run under reducing conditions. All samples were run on 10% SDS-PAGE gels, with the exception of samples to be used for mass spectrometry analysis, which were run on 15% gels. Sample buffer [157] was added to protein samples and boiled for 5 min, then loaded onto SDS-PAGE gels. Gels were run in SDS running buffer (25mM Tris, 192 mM glycine, 0.1% SDS) at 200V for 50 min.

2.4.3. Electrophoretic transfer of proteins to PVDF membrane

Proteins resolved by SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membrane. PVDF membrane was wet briefly in 100% methanol, then soaked in transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) for 45 min. Proteins were transferred to PVDF membrane in transfer buffer at 250mA for 1.5h.

2.4.4. Western blotting

Proteins of interest transferred to PVDF membranes were identified by western blotting using Abs and conditions outlined in Table 2.2.

Antibody	1º /2º	Dilution	Blocking	Host	Supplier
Anti-annexin A1	1º	1:1000	No blocking	Mouse	BD Pharmingen
Anti-annexin A1	1º	1:1000	5% SMP in PBS-T	Rabbit	AbCam
Anti-α-tubulin	1º	1:5000	5% SMP in PBS-T	Mouse	BD Biosciences
Anti-ERK	1º	1:5000	5% SMP in PBS-T	Rabbit	Promega
Anti-ACTIVE MAPK, (pTEpY)	1º	1:1000	5% SMP in PBS-T	Rabbit	Promega
Anti-phospho-p44/42 MAPK (Erk1/2)(Thr202 /Tyr204)	1 º	1:1000	5% SMP in PBS-T	Mouse	Cell Signalling
Anti-S100A8	1º	1:1000	5% SMP in PBS-T	Rabbit	Santa Cruz
Anti-S100A9	1º	1:1000	5% SMP in PBS-T	Rabbit	Santa Cruz
Anti-mouse-HRP	2º	1:10 000	5% SMP in PBS-T	Goat	Pierce
Anti-rabbit-HRP	2º	1:10 000	5% SMP in PBS-T	Goat	Pierce

Table 2.2: Antibodies and conditions used for western blotting.
Abbreviations: 1º, primary antibody; 2º, secondary antibody; HRP, horse radish peroxidise; PBS-T, phosphate buffered saline with 0.1% Tween-20; SMP, skim milk powder.

2.4.5. SDS-PAGE gel and PVDF membrane staining

Total protein in SDS-PAGE gels was visualised by staining with coomassie blue (0.1% Coomassie Brilliant Blue R250, 50% methanol, 10% glacial acetic acid) then destained in destain solution (40% methanol, 10% glacial acetic acid). Where required, PVDF membranes were also

stained with coomassie blue following western blotting. Membranes were stained in a solution of 0.02% Coomassie Brilliant Blue R250 in 10% glacial acetic acid for 30 min, then destained briefly (~20 seconds) in destain solution (40% methanol, 10% glacial acetic acid) and dried.

2.4.6. Membrane stripping

PVDF membranes probed with phospho-ERK Abs were stripped before reprobing with ERK Abs. Membranes were stripped in stripping buffer (100mM β-mercaptoethanol), 2% (w/v) SDS, 62.5 mM Tris, pH 6.7) for 30 min at 60ºC with gentle agitation. Membranes were then washed with repeated changes of PBS/0.1% Tween-20 for 1h.

2.5. Isolation of immune cells from human peripheral blood

2.5.1. Purification of human neutrophils from peripheral blood

Human neutrophils were prepared immediately from the fresh blood of healthy donors under endotoxin-free conditions. Blood was taken into syringes containing acid citrate dextrose solution (9:1 blood:acid citrate, kind gift from the Department of Haematology, Royal Adelaide Hospital) and mixed to prevent coagulation. 5% dextran T-500 (Pharmacia Biotech, Uppsala, Sweden) prepared in PBS was added to citrated blood to a final concentration of 1%, then mixed by gentle inversion. Erythrocytes were allowed to sediment following incubation at room temperature for 30 minutes. The top layer remaining after erythrocyte sedimentation, the buffy coat, was gently removed with a transfer pipette and layered carefully onto 25ml Lymphoprep (Nycomed, Oslo, Norway). Following centrifugation at 500 x g for 20 min, the supernatant (serum and Lymphoprep) was aspirated from the neutrophil pellet. If required, hypotonic lysis was then carried out to remove contaminating erythrocytes. Hypotonic lysis involved resuspending the neutrophil pellet in 10ml 0.2 % NaCl for 1 min, then a further 10ml of 1.6% NaCl was added to return the mixture to a physiologic salt concentration. The sample was then centrifuged at 500 x g for 5 min and the neutrophil pellet was resuspended in PBS for ERK-activation assays or serum-free HUVEC medium for transmigration assays. Cytological examination of stained neutrophil preparations showed they were 95% pure and trypan blue staining confirmed >98% of cells were viable.

2.5.2. Purification of human eosinophils from peripheral blood

Eosinophils from human blood were kindly purified by Dr Hayley Ramshaw (Cytokine Receptor Laboratory, Hanson Institute). Dextran sedimentation, density gradient separation and erythrocyte lysis of citrated blood were performed as described in section 2.5.1. Following centrifugation, the cell pellet containing neutrophils and eosinophils was first washed in PBS/0.1% albumin, then washed in HEPES buffer. Cells were then counted, cytospun and resuspended at a concentration of 1 x 10^9 cells/ml in HEPES buffer. An equal volume of CD16 microbeads (Miltenyi Biotec, Germany) was added and incubated on ice for 30 min. MACS columns were prepared by washing with 9mL PBS/HSA/acid citrate then the cells/microbeads mixture was added (200µl cells made up to 1ml with PBS/HSA/acid citrate). The flow-through containing eosinophils was collected, counted and determined to be 85% pure.

2.5.3. Purification of peripheral blood mononuclear cells

Dextran sedimentation, density gradient separation of citrated blood were performed as described in section 2.5.1. Mononuclear cells localised at the plasma-Lymphoprep interphase were withdrawn using a transfer pipette, centrifuged and resuspended in PBS at 1x10^8 cells/ml.

2.6. Protein Purification

2.6.1. Purification of S100A8 and S100A9 from human neutrophils

S100A8 and S100A9 were purified according to a protocol developed by van den Bos and colleagues [158]. Neutrophils were purified from 100ml human blood as described in section 2.5.1 and all purification steps were performed at 4ºC. Neutrophils were resuspended in 10ml Buffer A (50mM Tris, pH 8.5, 1 mM EDTA, 1mM EGTA, 1mM DTT, 1xP2714 (Sigma Aldrich) and sonicated. Cellular debris was removed by ultracentrifugation at 110 000 x g for 30 min. The supernatant was transferred to a fresh tube and solid ammonium sulphate was slowly added to a final concentration of 55% and stirred gently for 1h. Following centrifugation at 48000 x g for 30 min, the supernatant was dialysed using 5kDa molecular weight cut-off Spectra/Por dialysis tubing (Spectrum Laboratories, CA, USA) for ~18h against 4L Buffer A, incorporating two buffer changes during that time. S100A8 and S100A9 were purified from the supernatant by fast protein liquid chromatography (FPLC, Pharmacia Biotech, Uppsala, Sweden) performed at 4ºC. The dialysed material was injected on to a Mono Q HR 5/5 (Pharmacia Biotech) anion exchange

column at a flow rate of 1ml/min. After elution of unabsorbed material and washing with 5 column volumes of Buffer A, a linear gradient of Buffer B (50mM Tris, pH 8.5, 1 mM EDTA, 1mM EGTA, 1mM DTT, 1M NaCl) was applied to the column. Nil to 0.4M NaCl was applied over 35 min, then 0.4M to 1M over 15 min. 1ml fractions were collected and total protein elution was measured by absorbance at 280nm. Protein contained within each fraction were resolved on a 15% SDS-PAGE gel and coomassie stained as described in section 2.4.

2.6.2. Purification of recombinant annexin A1

HEK293 cells stably expressing human annexin A1 were established, from which annexin A1 was purified as outlined in [159]. Cells were resuspended in 10mM HEPES, 5mM EDTA, 100mM NaCl, 1 x P2714 protease inhibitor cocktail, pH 7.4 and sonicated. Cellular debris was centrifuged at 20 000 x g for 30 min at 4ºC. Proteins in the supernatant were precipitated with 6mM calcium for 30 min at 4ºC, then centrifuged at 39 000 x g for 30 min at 4ºC. The precipitate was washed with a buffer containing 10mM HEPES, 150mM NaCl, 1mM $CaCl_2$, pH 7.4 and centrifuged at 39 000 x g for 30 min at 4ºC. The pellet was resuspended in 10mM HEPES, 10mM EGTA, 1 x P2714 protease inhibitor cocktail, pH 7.6. A final centrifugation at 110000 x g for 30 min at 4ºC was performed and the supernatant containing annexin A1 was recovered. Annexin A1 was purified from the supernatant by fast protein liquid chromatography performed at 4ºC. The supernatant was adjusted to 10ml with Buffer A (20mM ethanolamine, pH 9.0) and injected on to a Mono Q HR 5/5 anion exchange column at a flow rate of 1ml/min. After elution of unabsorbed material and washing with 5 column volumes of Buffer A, a linear gradient of Buffer B (20mM ethanolamine, 1M NaCl, pH 9) was applied to the column. Nil to 0.4M NaCl was applied over 30 min, then 0.4M to 1M over 15 min. 1ml fractions were collected and total protein elution was measured by absorbance at 280nm.

2.6.3. Fractionation of neutrophil-conditioned medium

1 x 10^8/ml neutrophils resuspended in PBS were stimulated with 100nm fMLP for 15 min at 37°C. Neutrophils were centrifuged at 500 x g for 5 min, then the conditioned medium recovered and re-centrifuged to remove all neutrophils. The conditioned medium was concentrated to 500µl using a 10kDa molecular weight cut-off membrane (Centricon concentrator, Millipore, Billerica, MA, USA) and run through two Superdex 75 HR 10/30 gel filtration columns (Pharmacia Biotech) pre-equilibrated with PBS, pH 7.4, sequentially. Approximately thirty 1ml fractions

were collected and the molecular mass of eluted proteins was estimated by comparison of the elution rate of a protein mixture of known molecular weights (Biorad).

2.7. Mass spectrometry analysis

One-fifth of each fraction obtained after the fractionation of neutrophil conditioned medium (section 2.6.3) were run on a 15% SDS-PAGE gel and protein bands were visualised by staining with coomassie blue. Candidate bands in the ERK-activating fraction were excised, digested with trypsin and the resulting peptides were desalted into a $QTOF^2$ mass spectrometer through a C18 reverse phase silica column. Automated MSMS sequencing was carried out using data directed analysis techniques. The data was analysed using Protein Lynx Global Server and the sequence tags used to search against the SwissProt database, limited to human entries. Tryptic digestion and mass spectrometry was performed by Drs Ian Milne and Christopher Bagley of the Protein Core Facility (Hanson Institute, Adelaide).

2.8. Preparation of neutrophil conditioned medium

Stimulated neutrophil conditioned medium was prepared by stimulating neutrophils with 100nM fMLP or IL-8 for 15 min at 37°C. Neutrophils were pelleted at 1800 x g for 5 min, then the conditioned medium recovered and re-centrifuged to remove all neutrophils. Conditioned medium from 5 x 10^6/ml neutrophils was used to treat 10cm^2 of confluent, resting HUVEC. These amounts were scaled up or down accordingly.

2.9. HUVEC ERK-activation assay

HUVEC were seeded at high-density and grown for 24 hours to ensure confluence. Cells were treated with conditioned medium or as indicated, with the addition of 5% FCS, for 10 min at 37°C then washed and lysed in sample buffer. Equal volumes of cell lysate were loaded onto a 10% SDS polyacrylamide gel for analysis by western blotting. Membranes were probed with an Ab specific to phosphorylated ERK, and developed with ECL Plus (Amersham, Arlington Heights, IL, USA). ERK or β-tubulin in each sample was also determined as a loading control.

The HUVEC ERK-activation assay was adapted where needed using eosinophils or mononuclear cells in place of neutrophils.

2.10. Neutrophil transmigration assay

Transmigration assays were performed as previously described [160] using Transwells (6.5mm diameter, 3.0μm pore size) in 24-well tissue culture trays (Costar, Cambridge, MA, USA). Transwells were coated with 50μg/ml fibronectin (Roche Diagnostics, Basel, Switzerland) for 30 min and 5 x 10^4 HUVEC were plated in the apical chamber. HUVEC were grown for 4 days to ensure a confluent monolayer was established. Transmigration was initiated in two ways: activation of the endothelial monolayer using TNF-α (R&D Systems, Minneapolis, MN, USA) or by using the bacterial peptide fMLP to establish a chemoattractant gradient. TNF-α activation of endothelial cells involved treating HUVEC with 4ng/ml TNF-α for 4h, which results in the secretion of IL-8 from the endothelial cells into the apical and basolateral compartments of the Transwell. The medium in the apical compartment was replaced with fresh medium, therefore establishing a chemoattractant gradient across the HUVEC monolayer as IL-8 is a chemoattractant and remained in the basolateral chamber. For fMLP-induced transmigration, 10nM fMLP was added to the basolateral chamber to establish a chemoattractant gradient. Following the application of either inducing agents, 5 x 10^5 neutrophils were added to the apical chamber and incubated at 37°C for 1 hour. Transmigrated neutrophils in the basolateral chamber were counted and expressed as a percentage of total neutrophils added to the apical chamber. Neutrophils were counted using a MTS indirect colorimetric assay based on the conversion of the MTS tetrazolium salt to soluble formazon by viable neutrophils. Transmigrated neutrophils were collected and gently resuspended in 100μl PBS. 20μl 2mg/ml MTS + PMS (Promega) was added and incubated at room temperature for 1 hour, then the absorbance was read at 490nm. A standard curve was prepared by making serial dilutions of the neutrophil preparation, from which the percentage of migrated neutrophils was calculated. Figure 2.4 is a diagrammatical representation of the neutrophil transmigration assay.

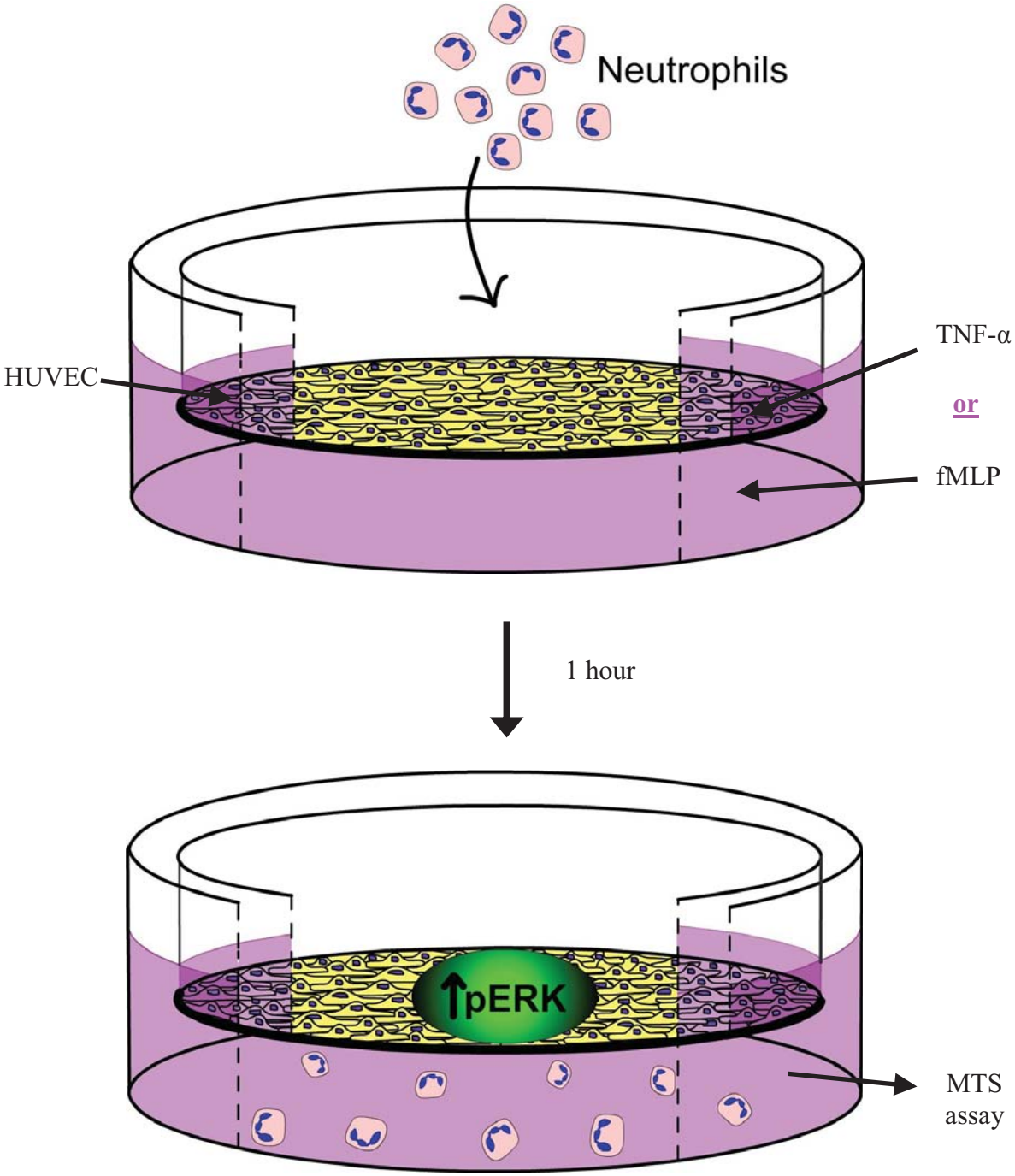


Figure 2.4: *In vitro* neutrophil transmigration assay.

Confluent HUVEC are either stimulated with TNF-a for 4 hours, or 10mM fMLP is added to the basolateral compartment to establish a chemoattractant gradient to initiate transmigration. All medium in the apical compartment is then removed before the addition of neutrophils and/or indicated treatments before the commencement of the assay. Neutrophils are allowed to transmigrate for 1h, then neutrophils that have migrated through the HUVEC monolayer into the basolateral compartment are quantified using a MTS assay.

2.11. Proteolysis of annexin A1 with calpain I

Recombinant annexin I as described in section 2.6.2., then 0.8 units of human calpain I (Sigma Aldrich) was added per 20µg total protein. 5mM $CaCl_2$ was added to initiate the reaction, which was then incubated for 20 min at room temperature. For samples where calpain inhibitor was used, calpain inhibitor was incubated with calpain I for 10 min at room temperature before adding to recombinant annexin A1. Calpain-cleaved annexin A1 was tested for activity using the HUVEC ERK activation assay described in section 2.8. Calpain I was not inactivated prior to usage in ERK-activation assays.

2.12. Neutrophil treatment with calpain inhibitor

Neutrophils were prepared as above and were immediately resuspended in either PBS alone or PBS with 1µM calpain inhibitor XII (EMD Biosciences, San Diego, CA, USA) and incubated at room temperature for 10 min. Neutrophils were then stimulated with 100ng/ml fMLP for 15 min, then the neutrophils removed by centrifugation as above to recover conditioned media. For experiments where HUVEC were treated with calpain inhibitor XII or vehicle, the additives were incubated on HUVEC monolayers for 10 min prior to the addition of neutrophil conditioned medium.

2.13. FITC-dextran permeability assay

HUVEC were established in Transwells as described in section 2.9 for the transmigration assay. Treatments were prepared in 150µl serum-free HUVEC medium. HUVEC were treated as indicated, then 1µl of fluorescein isothiocyanate (FITC)-conjugated dextran (40kDa, 0.003-0.02 mol FITC per mol of glucose) prepared in 20µl serum-free HUVEC medium was added to the apical compartment of each Transwell. 20µl was removed from the basolateral compartment at 10 min intervals for the duration of the assay and transferred to a 96-well plate. Each treatment was performed in triplicate. The absorbance of the samples was read at 530nm.

2.14. Inhibition of formyl peptide receptors with Boc2

Boc2 is a pan-formyl peptide receptor antagonist. To inhibit endothelial formyl peptide receptors, HUVEC were pre-treated for 15 min with either vehicle, 10µM or 20µM Boc2 in HUVEC

medium. fMLP-stimulated neutrophil conditioned medium was prepared as described in section 2.8, added to Boc2-treated HUVEC and phosphorylated ERK levels were detected as per HUVEC ERK-activation assay outlined in section 2.9.

2.15. Transmigratory cup assay

HUVEC were seeded into fibronectin-coated Lab-Tek chamber slides (Nunc/Thermo Fisher Scientific, Denmark) at high density and grown overnight. On the morning of the assay, the growth medium was changed to remove dead cells. Neutrophils were purified from human blood as described in section 2.5.1 with erythrocyte lysis and resuspended in HUVEC medium at 5 x 10^6 neutrophils/ml. Per chamber, 5 x 10^5 neutrophils were stimulated with 100nM fMLP for 2 min, with the addition of 5µg annexin A1 or KLH Ab where indicated. Neutrophils were then added to HUVEC and incubated at room temperature for 2 min, where they were then immediately removed and the HUVEC monolayers were gently washed 3 times with warm HUVEC medium. HUVEC were immediately fixed in warm 4% PBS-buffered paraformaldehyde for 15 min at 37ºC, then washed 4 times with PBS.

2.15.1. Silver-staining of fixed HUVEC monolayers

HUVEC were grown in fibronectin-coated Lab-Tek slides as described in section 2.15. HUVEC were either immediately stained with 1% $AgNO_3$ for 20 sec, or the neutrophil adhesion assay was first carried out then the monolayers were washed and stained with $AgNO_3$. Following staining with $AgNO_3$, HUVEC monolayers were fixed by the addition of 1% formaldehyde prepared in HUVEC medium for 30 min, and then washed with PBS.

2.15.2. ICAM-1/VCAM-1 staining of fixed HUVEC monolayers

HUVEC were blocked for 30min in PBS + 2% BSA. HUVEC were probed with 1:1000 anti-ICAM-1 or anti-VCAM-1 primary Abs for 1h at room temperature then washed 4 times with PBS. Monolayers were then probed with 1:1000 anti-mouse Alexa488 secondary Ab for 30 min protected from light and washed 4 times with PBS-T to permeabilise cells. Slides were mounted using 2:1 Fluorescence Mounting Medium (DAKO, Carpinteria, CA, USA):1µg/ml DAPI and stored protected from light at 4ºC until image capture with an Olympus IX81 microscope and Hamamatsu Orca digital camera (Olympus Corporation, Tokyo, Japan). Image capture and analysis was performed using cell^R software package (Olympus). For experiments where

transmigratory cup intensity was quantified, four points of grey-value intensity around the periphery of each neutrophil (0º, 90º, 180º, 270º) were taken and averaged to give transmigratory cup intensity per individual neutrophil.

2.16. Data analysis

Statistical analyses were performed using Prism 4.0 statistical software package. For experiments with 3 treatments, one-way analysis of the variance (ANOVA) was initially performed to determine if the means of the treatments significantly differed. If a difference was found, an unpaired t-test (two-tailed) assuming equal variance was performed to compare two individual treatments. For experiments with only two treatments, an unpaired t-test (two-tailed) assuming equal variance was used to determine if the means of the two treatment groups differed significantly. Linear regression was performed on data obtained from the FITC-dextran permeability assay and difference between the slopes were analysed using a one-way ANOVA, followed by Tukey's multiple comparison post test.

Chapter 3

IDENTIFICATION OF THE SOLUBLE NEUTROPHIL PROTEIN

INTRODUCTION

A previous study carried out in our laboratory investigated signalling pathways induced during neutrophil extravasation. It was found that the activation of endothelial extracellular signal-regulated kinase (ERK) 1/2, was essential for neutrophils to transmigrate across human umbilical vein endothelial cell monolayers in response to both fMLP and IL-8 chemotactic gradients [99]. This event was identified through the use of two MEK inhibitors; PD98059 and UO126, where MEK is a kinase in the MAPK family that directly phosphorylates and activates ERK1 and ERK2. It was revealed that transmigration was not inhibited through impairing neutrophil function, as chemotaxis towards fMLP or IL-8 was unaffected by the presence of the inhibitors, nor was neutrophil adhesion to activated endothelial monolayers. This suggested that it was the inhibition of endothelial ERK that prevented neutrophil transmigration. As firm adhesion of neutrophils to endothelial cells is a critical step in the transmigration process, a β_2 integrin blocking antibody was employed to determine whether ERK activation was dependent on integrin mediated neutrophil adhesion. Although the Ab effectively blocked stimulated neutrophil adhesion to HUVEC, ERK activation was observed regardless, indicating that neutrophil adhesion was not required. Further investigation showed that cell-free conditioned medium harvested from neutrophils stimulated with fMLP or IL-8 could activate ERK. This suggested that activation of endothelial ERK during neutrophil transmigration was mediated by a soluble factor produced by activated neutrophils. Very preliminary experiments conducted to characterise the soluble neutrophil factor identified it as a protein, as boiling the conditioned medium resulted in the loss of activity. The molecular weight was determined to be approximately 30-40 kDa following fractionation by gel filtration chromatography [99].

In a separate study, endothelial ERK activation was later found to also be essential in a shear-flow model of both neutrophil and eosinophil transmigration [97], where blood or fluid is perfused over HUVEC monolayers at a predetermined velocity to mimic the *in-vivo* effects of blood flow on the endothelium. Given the requirement of endothelial ERK activation in neutrophil and eosinophil transmigration, identifying the ERK-activating factor would provide important information regarding leukocyte extravasation and the proactive role of the endothelium during the process. The discovery of potential inhibitors to this protein may also

prove useful in treating pathologies where neutrophil infiltration is undesirable, such as in ischemia reperfusion injury [161].

This chapter describes the process taken to investigate a number of candidates for the soluble ERK-activating protein identified by mass spectrometry analysis of fMLP-stimulated neutrophil conditioned medium. Several selected proteins identified by mass spectrometry were evaluated for their ability to activate ERK in HUVEC monolayers in a similar manner to stimulated neutrophil conditioned medium. Although S100A8 and S100A9 appeared to be the most promising candidates, annexin A1 was ultimately found to be the protein in neutrophil conditioned medium that was activating endothelial ERK.

RESULTS

3.1. Preliminary characterisation of the neutrophil factor

While experiments were being conducted to identify the ERK-activating neutrophil protein, preliminary tests were carried out which did not require the identity of the protein to be known.

3.1.1. Red blood cell lysis does not contribute to ERK activation induced by neutrophil conditioned medium

During the purification of neutrophils from human blood, contaminating red blood cells are generally lysed to remove them from the neutrophil preparation [162]. To ensure that the ERK-activating factor was not a red blood cell product arising from lysis, neutrophils were prepared both with and without red blood cell lysis and then stimulated with fMLP to produce the ERK-activating factor. The conditioned media from both of these preparations were tested on HUVEC monolayers for ERK activation using the ERK-activation assay outlined in section 2.9 and it was found that both had the capacity to do so (Figure 3.1). Conditioned medium prepared from neutrophils without the red blood cell lysis produced more ERK activation compared to that with red blood cell lysis, however this was probably due to a proportion of neutrophils being lost during the lysis and re-centrifugation. This therefore confirmed that ERK activation was attributable to a factor specifically produced by neutrophils and was not an artefact of red blood cell lysis during neutrophil preparation.

3.1.2. fMLP-stimulated mononuclear cells can produce an ERK-activating factor

To determine if other immune cells are also able to produce an ERK-activating factor after stimulation, the buffy coat containing mononuclear cells generated after Lymphoprep separation was harvested, stimulated with fMLP and the conditioned medium from these cells was tested on HUVEC for ERK activation. It was found that fMLP-stimulated conditioned medium gave a greater degree of ERK activation when compared to unstimulated conditioned medium (Figure

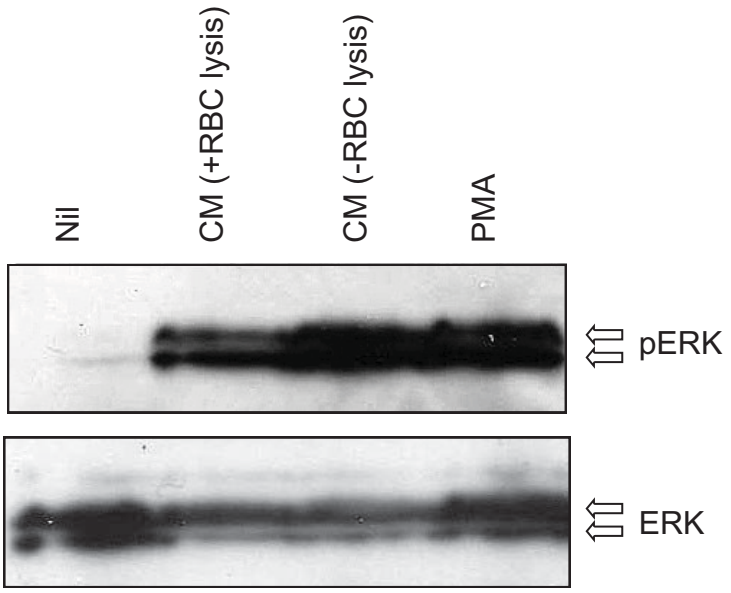


Figure 3.1: Erythrocyte lysis products do not induce endothelial ERK activation.

Upper panel- Neutrophils were prepared from fresh blood, and the red blood cells of one half of the preparation were lysed by hypotonic lysis. Conditioned medium from fMLP-stimulated neutrophils +/- RBC lysis were then assayed for phospho-ERK on HUVEC alongside a PBS negative control (Nil) and PMA (0.25μg/ml) positive control. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.

3.2). It has been previously shown that fMLP alone does not induce ERK activation in HUVEC [99], therefore this control treatment was omitted from the assay. This experiment indicated that a subset or all of the mononuclear cells were also capable of producing a soluble ERK-activating factor in a similar manner to neutrophils.

3.1.3. fMLP-stimulated eosinophils do not produce an ERK-activating factor

As neutrophils and one or more mononuclear cells could produce a factor or factors that could activate endothelial ERK, it was next investigated whether eosinophils, the main contaminating cell type present in the purified neutrophil cell pellet, could also activate ERK. Eosinophils were kindly prepared by Dr Hayley Ramshaw (Cytokine Receptor Laboratory, Hanson Institute) using CD16 magnetic beads and were determined to be 85% pure. Conditioned medium obtained from 2×10^7 unstimulated and fMLP-stimulated eosinophils were assayed on HUVEC monolayers for ERK activation. fMLP stimulation had no effect on the ability of eosinophils to produce a soluble ERK-activating factor, unlike neutrophils that could (Figure 3.3). This particular line of HUVEC had a high basal level of phosphorylated ERK, but both unstimulated and fMLP-stimulated eosinophil conditioned medium did not activate endothelial ERK beyond basal ERK activation, although PMA and neutrophil conditioned medium did. Therefore eosinophils do not appear to secrete an ERK-activating protein following fMLP stimulation in a manner similar to neutrophils.

3.1.4. Time course of ERK activation

The assay described in Stein *et al* [99] to monitor ERK-activation in HUVEC involved incubating neutrophils or conditioned medium on HUVEC monolayers for 15 min before lysing the cells to analyse levels of phosphorylated (active) ERK. To determine if 15 min incubation was optimal to observe ERK activation at its peak, fMLP-stimulated neutrophil conditioned medium was incubated on HUVEC for varying lengths of time. It was found that maximum ERK activation induced by conditioned medium was after 10-15 minutes (Figure 3.4), with phosphorylated ERK levels decreasing dramatically after 20 minutes incubation. A 10 min incubation period was therefore employed in all subsequent assays.

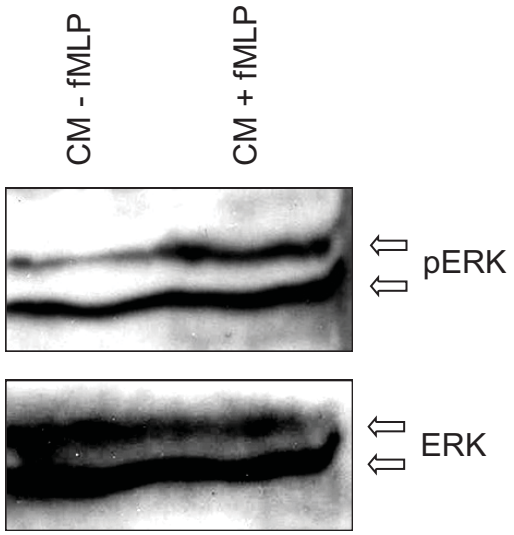


Figure 3.2: fMLP-stimulated mononuclear cell conditioned medium can activate endothelial ERK.

Mononuclear cells from human peripheral blood were prepared by taking the buffy coat at the lymphoprep-serum interphase, from which conditioned medium was isolated following stimulation with fMLP.
Upper panel- Resting HUVEC monolayers were treated with conditioned medium obtained from both unstimulated (CM-fMLP) and fMLP-stimulated mononuclear cells (CM+fMLP). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti-total ERK Ab after stripping.

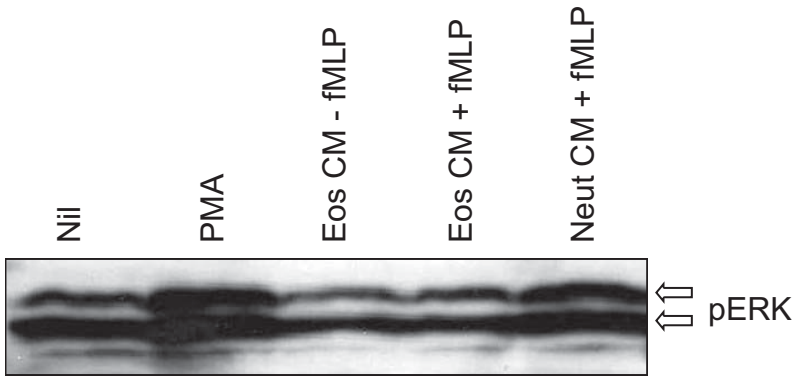


Figure 3.3: fMLP-stimulated eosinophil conditioned medium does not activate endothelial ERK.

Upper panel- Resting HUVEC monolayers were treated with conditioned medium obtained from both unstimulated (Eos CM-fMLP) and fMLP-stimulated eosinophils (Eos CM+fMLP), alongside fMLP-stimulated neutrophil conditioned medium (Neut CM + fMLP) and PMA (0.25μg/ml) positive controls. Basal phosopho-ERK levels can be seen in the PBS treated sample (Nil). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.

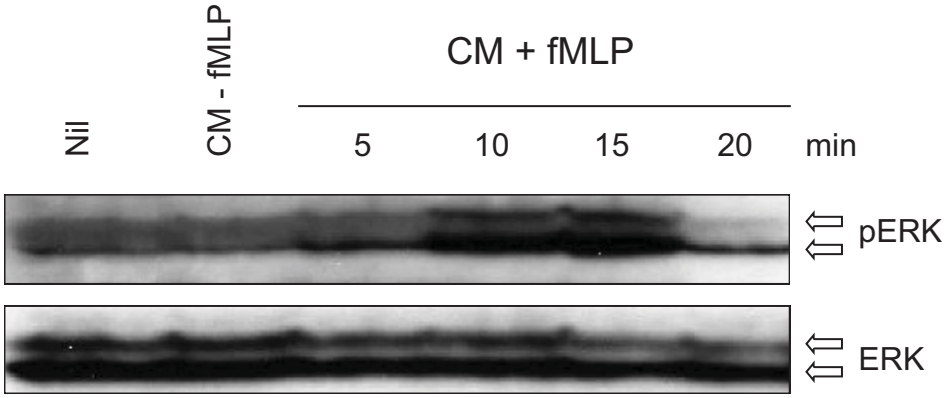


Figure 3.4: Maximum ERK activation is achieved after 10-15 minutes treatment with fMLP-stimulated neutrophil conditioned medium.

Upper panel- Resting HUVEC monolayers were treated with PBS (Nil), unstimulated neutrophil conditioned medium (CM-fMLP) for 10 min. fMLP-stimulated neutrophil conditioned medium was incubated on HUVEC for the indicated period of time before lysis. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti-total ERK Ab after stripping.

3.2. Mass spectrometry analysis of fMLP-stimulated neutrophil conditioned medium

To identify potential candidates for the ERK-activating factor, conditioned medium from neutrophils stimulated with fMLP was fractionated by gel filtration chromatography (Figure 3.5A). Fractions eluted from the column were assayed for the ERK-activating factor using the ERK activation assay, which was found to elute solely in fraction 10 (Figure 3.5B) and was estimated to be a protein of approximately 30 kDa following comparison to molecular weight standards run on the same column (Figure 3.5A, inset). Proteins comprising the active fraction were resolved by SDS-PAGE and four candidate proteins unique to the active fraction were selected for analysis by mass spectrometry (Figure 3.6, bands 1-4). Due to the high abundance of bands 5 and 6, these were also analysed despite their presence in the flanking inactive fractions. The selected proteins were subsequently identified as 1) monocyte/neutrophil elastase inhibitor (MNEI), 2) annexin A1, 3) annexin A3, 4) chloride intracellular channel protein, 5) S100A9 and 6) S100A8 through comparison to human entries in the SwissProt database (Appendix 1). It was determined that the presence of chloride intracellular channel protein was a membrane component of neutrophil granules that was most likely a by-product of neutrophil degranulation following fMLP stimulation and consequently was not pursued.

3.3. Monocyte/neutrophil elastase inhibitor can not activate endothelial ERK

The effect of recombinant MNEI on HUVEC was evaluated using the ERK-activation assay. Assuming MNEI was the ERK-activating factor, the amount of MNEI present in 4/5 of the ERK-activating fraction from purified neutrophil conditioned medium (fraction 10, Figure 3.6) was determined to be approximately 300ng after comparison to BSA standards run on the same SDS-PAGE gel (not shown). 0.2ml of this fraction (diluted to 0.5ml before assaying) could adequately activate ERK in 10 cm^2 of confluent HUVEC (Figure 3.5B), which corresponded to approximately 40-60ng/ml MNEI added. Treating HUVEC with recombinant MNEI (kind gift from P. Bird, Monash University, Australia) at concentrations comparable to and in excess to that present in fMLP-stimulated neutrophil conditioned medium was unable induce ERK activation (Figure 3.7A). Much lower concentrations, estimated to be closer to physiologically relevant levels *in vivo*, were also tested but these also had no effect on ERK activation (Figure 3.7B).

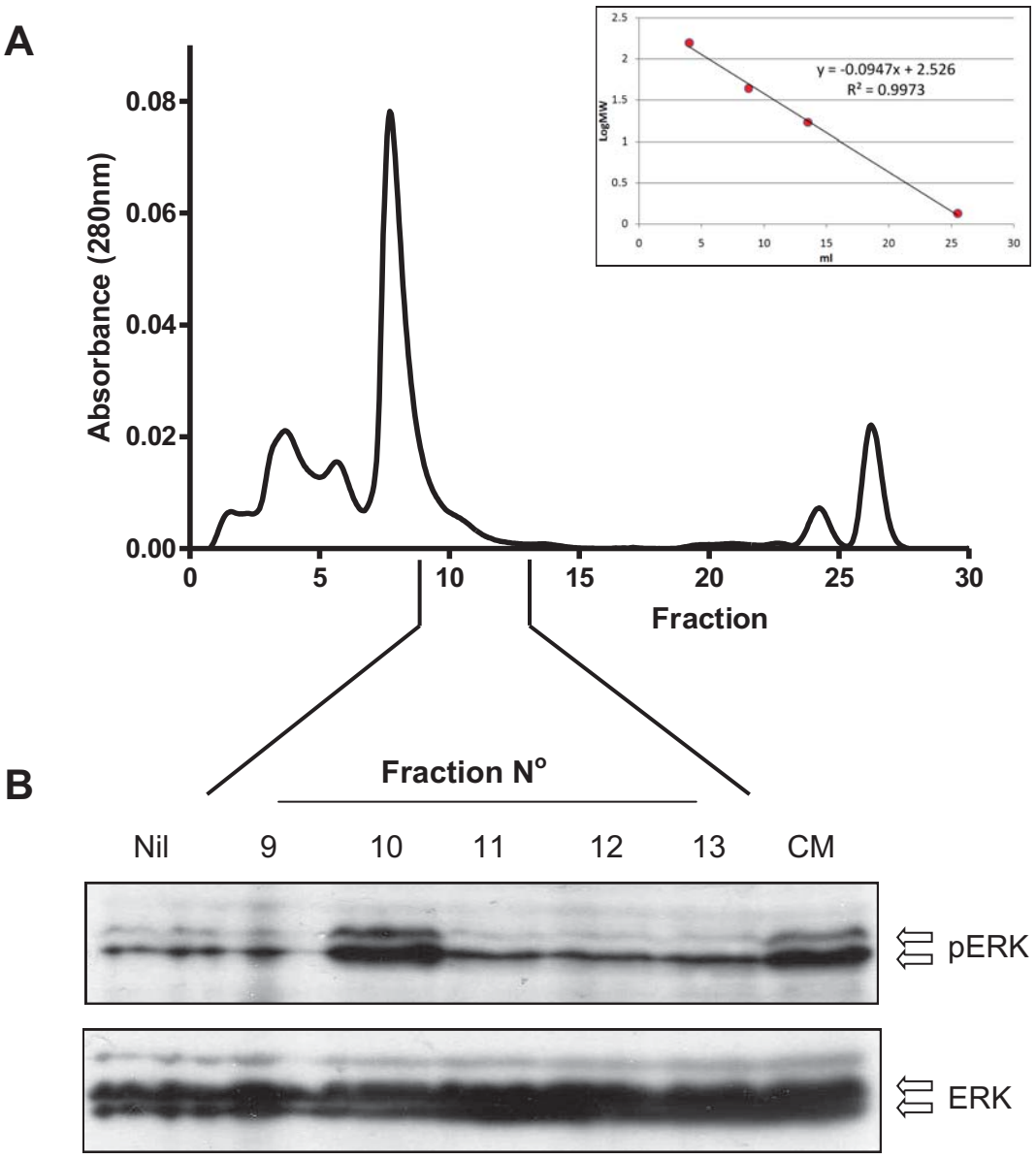


Figure 3.5: Partial purification of the ERK-activating neutrophil protein by gel filtration chromatography.

A) Total protein elution profile resulting from fractionation of fMLP-stimulated neutrophil-conditioned medium by gel filtration chromatography. ***Inset-*** Column calibration curve generated from known molecular weight standards.
B) ***Upper panel-*** One-fifth of each fraction was added to resting HUVEC monolayers for 10 minutes. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti-total ERK Ab after stripping.

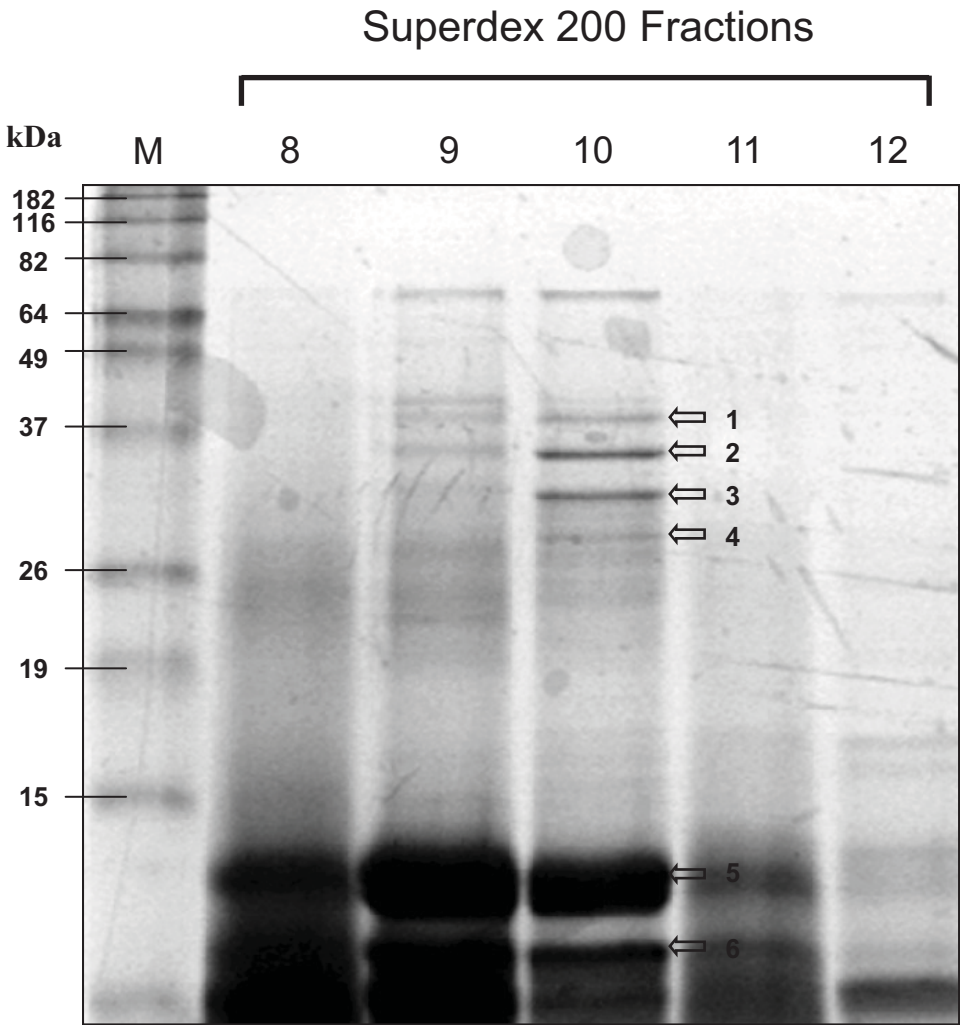


Figure 3.6: Visualisation of proteins present in fractions from gel filtration chromatography of neutrophil conditioned medium.

Four fifths of each fraction generated from gel filtration chromatography of neutrophil conditioned medium were TCA-precipitated and the protein components in each fraction separated on a 15% SDS-PAGE gel. Proteins were visualized by staining with Coomassie brilliant blue. Bands marked 1-6 in the active fraction 10 were excised and identified by mass spectrometry.

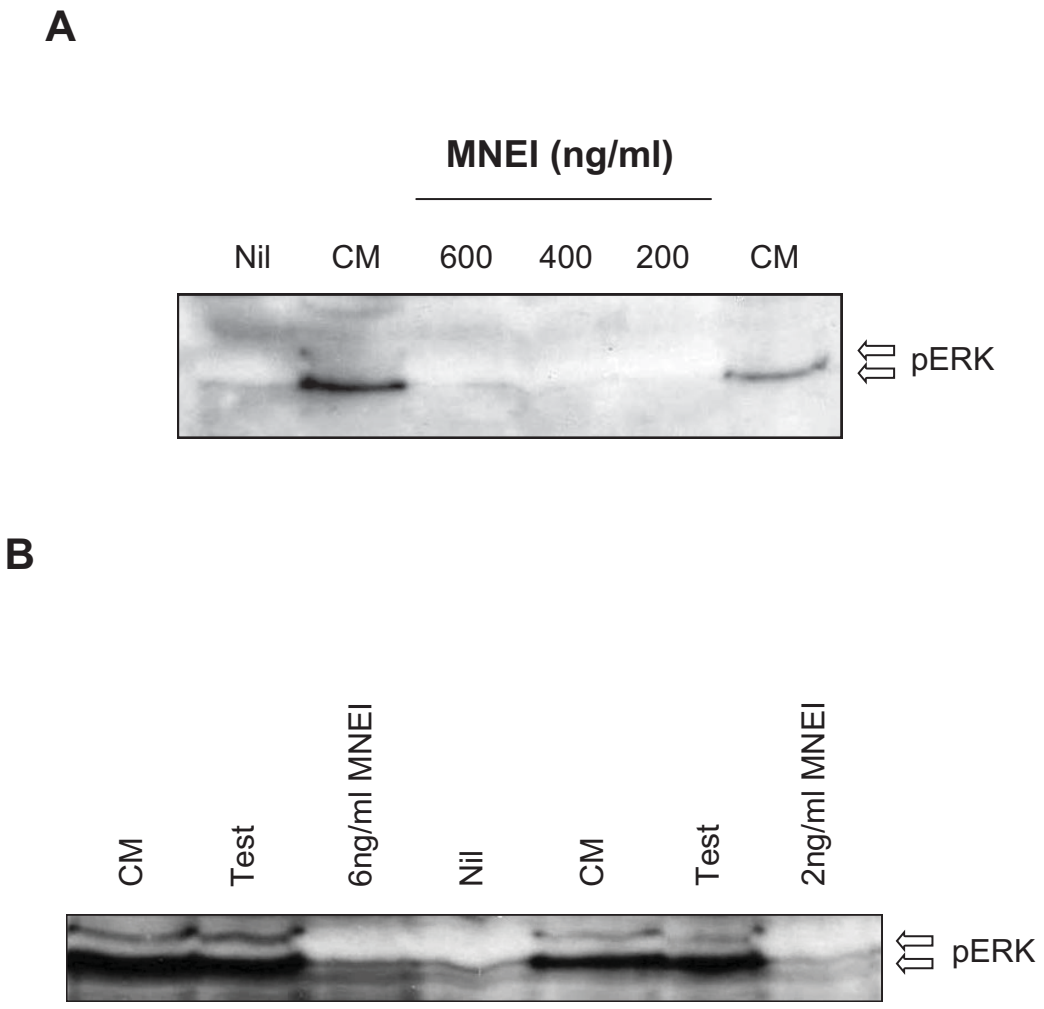


Figure 3.7: Monocyte/neutrophil elastase inhibitor cannot activate endothelial ERK.

A- Resting HUVEC monolayers were treated with PBS (Nil), fMLP-stimulated neutrophil conditioned medium (CM) or 600, 400 or 200 ng/ml monocyte/ neutrophil elastase inhibitor (MNEI), for 10 min. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK. **B-** Resting HUVEC monolayers were treated with fMLP-stimulated neutrophil conditioned medium (CM), or 6 or 2 ng/ml monocyte/neutrophil elastase inhibitor (MNEI). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK. Test indicates samples irrelevant to this experiment.

3.4. Investigation of S100A8 and S100A9

S100A8 and S100A9 heterodimerise to form calprotectin, a protein previously shown to be involved in leukocyte extravasation [163, 164]. In addition to the heterodimer calprotectin, S100A8 and A9 have each been shown to dimerise and form other multimeric complexes [165]. Although S100A8 and A9 were present in inactive fractions (Figure 3.6), it is probable that the S100A8 and A9 in these fractions arose from multimers different to that present in the active fraction 10. S100A8 and A9 were therefore investigated further for endothelial ERK-activation activity, despite being observed in inactive fractions.

3.4.1. Effect of calprotectin Ab on neutrophil factor-induced ERK activation

To determine if the S100A8/A9 heterodimer, calprotectin, could activate ERK in HUVEC, a calprotectin mAb was added with stimulated neutrophil conditioned medium to resting HUVEC. The rationale behind this experiment was that the mAb could bind calprotectin in neutrophil conditioned medium and prevent ERK activation, if indeed calprotectin was the ERK-activating neutrophil factor. The mAb exclusively recognises the 27E10 epitope on the calprotectin molecule and not S100A8 or S100A9 individually. The results from this experiment were variable. In the vast majority of experiments, the calprotectin mAb had no effect and could not inhibit ERK activation by neutrophil conditioned medium (Figure 3.8), however some inhibition was occasionally observed (Figure 3.9) As S100A8 and A9 are highly abundant in neutrophils and large quantities are secreted following fMLP stimulation (Figure 3.10), it was probable that in most experiments the amount of antibody added was insufficient to bind all the secreted calprotectin.

3.4.2. Generation of S100A8 and S100A9 over-expressing cell lines

The treatment of endothelial cells with recombinant S100A8 and S100A9 was an alternative approach to investigate whether they are activators of endothelial ERK. Hence S100A8 and S100A9 were cloned and stably-expressed in HEK293 cells and the recombinant proteins purified to determine if they could activate ERK alone or in combination following calcium-induced dimer formation. Both S100A8 and A9 were cloned with HA and myc tags fused to the

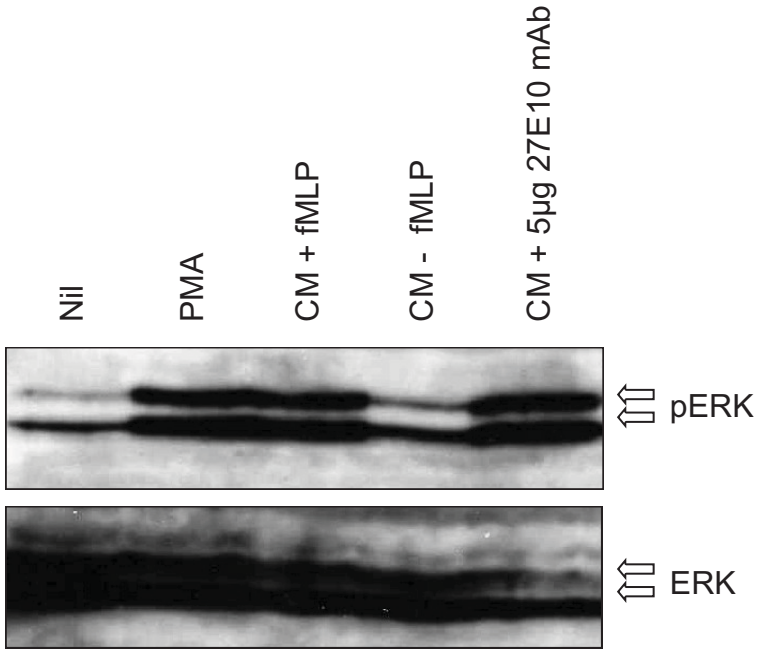


Figure 3.8: Calprotectin Ab does not inhibit ERK activation induced by fMLP-stimulated neutrophil conditioned medium.

Upper panel- Resting HUVEC monolayers were treated with PBS (Nil), 0.25µg/ml PMA, conditioned medium obtained from both unstimulated (CM-fMLP) and fMLP-stimulated neutrophils (CM+fMLP) and fMLP-stimulated conditioned medium with 5µg calprotectin mAb (CM + 5µg 27E10 mAb). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti-total ERK Ab after stripping

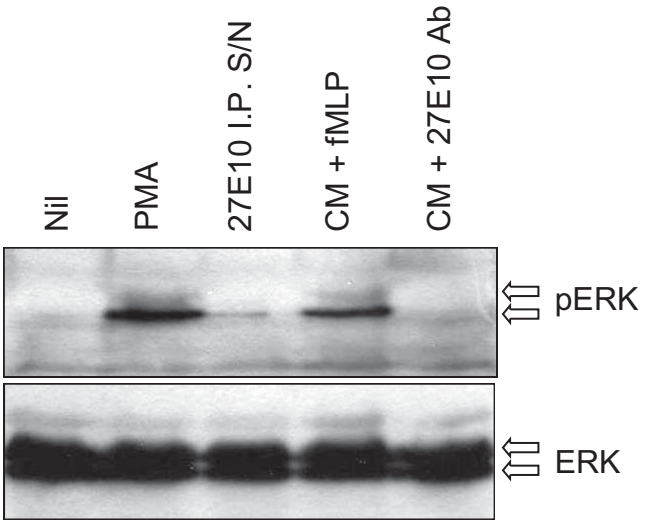


Figure 3.9: Calprotectin Ab can occasionally inhibit ERK activation induced by fMLP-stimulated neutrophil conditioned medium.

Upper panel- Resting HUVEC monolayers were treated with PBS (Nil), 0.25μg/ml PMA, supernatant from a calprotectin immunoprecipitation from neutrophil-conditioned medium (27E10 I.P. S/N), fMLP-stimulated neutrophil conditioned medium (CM+fMLP) and fMLP-stimulated conditioned medium with 5μg calprotectin mAb (CM + 27E10 mAb). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti-total ERK Ab after stripping

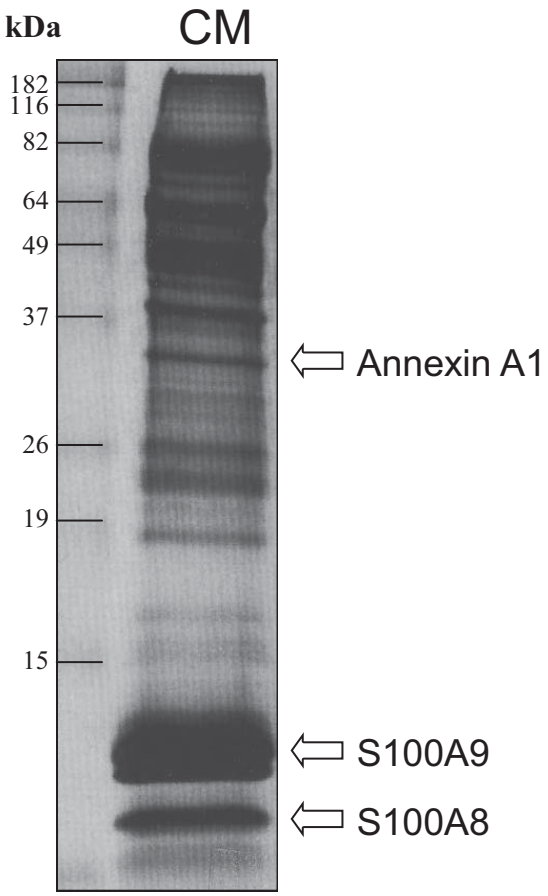


Figure 3.10: Visualisation of proteins present in fMLP-stimulated neutrophil conditioned medium.

Proteins secreted from 1 x 10^6 neutrophils stimulated with fMLP were separated on a 15% SDS-PAGE gel and visualised by staining with coomassie brilliant blue.

N-termini respectively, however neither protein could be detected by western blotting using in-house Abs directed to these tags (data not shown), therefore commercial calgranulin A (S100A8) and calgranulin B (S100A9) antibodies were used instead. Many clones expressed S100A9 (Figure 3.11), but only one clone expressing S100A8 was generated (Figure 3.12). The S100A8 clone subsequently lost expression after it was frozen down (Figure 3.13), consequently plans to test the effect of the S100A8/A9 complex formation on ERK activation in HUVECs could not be carried out.

3.4.3. S100A8 and S100A9 purified from neutrophils do not activate endothelial ERK

As neutrophils are an abundant source of S100A8 and A9, it was decided that the S100s would be purified from neutrophils rather than re-establishing a S100A8 over-expressing cell line. S100A8 and S100A9 were purified following a protocol loosely based on that described by van den Bos and colleagues [158]. Neutrophil lysate was subjected to a 55% ammonium sulphate precipitation to exploit the solubility of the S100 proteins at high ammonium sulphate concentrations. The resulting supernatant was dialysed and fractionated by anion exchange chromatography (Figure 3.14A and B). To test the effect of purified S100A8 and S100A9 on ERK activation, amounts of the purified protein equivalent to that present in unfractionated neutrophil conditioned medium used previously were added to HUVEC. As the neutrophil conditioned medium contained a mixture of proteins and the anion exchange column fractions had varying amounts of S100A8 and A9, the relative amounts of S100A8 and S100A9 between samples were estimated visually following resolution by SDS-PAGE and coomassie-staining the gel (Figure 3.15). On this gel, the amount of neutrophil conditioned medium was too low for the S100A8 band to be visible. This was not surprising as S100A8 was generally present at lower concentrations compared to S100A9 in both neutrophil conditioned medium and neutrophil lysate (see Figures 3.10 and 3.14B for examples). Therefore the amount of purified proteins added from each fraction was based on the amount of S100A9 present in each sample. Fractions 16, 17 and 18 were visually estimated to contain approximately 60%, 500% and 30% the amount of S100A9 observed in neutrophil conditioned medium, respectively. As 100µl conditioned medium (from 5 x 10^6 neutrophils) was used to elicit ERK activation in HUVEC and only 20µl was loaded on this gel, it was determined that 140µl fraction 16, 20µl fraction 17 and 170µl fraction 18 would be tested on HUVEC for ERK activation. To test for the effect that S100A8 and A9 heterodimerise to form

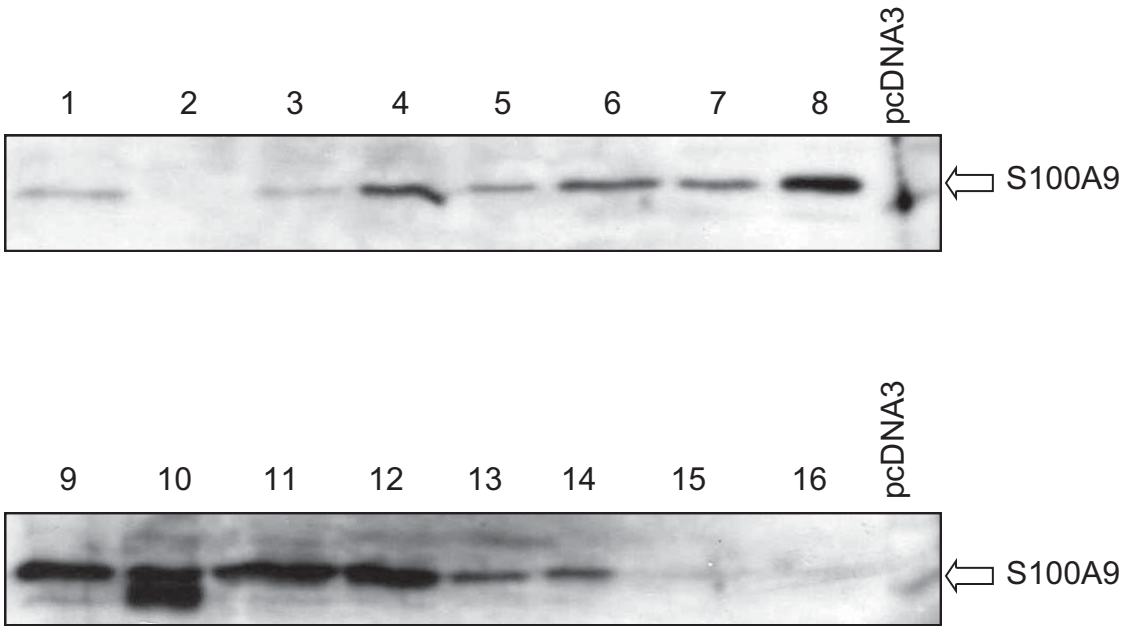


Figure 3.11: Analysis of S100A9 expression in G418-resistant HEK293 clones.

S100A9 was cloned into pcDNA3 and transfected into HEK293 cells, then grown in the presence of G418 to select for stable transformants. Isolated colonies were propagated individually and equal amounts of total protein from each clone were analysed for S100A9 expression by western blotting using an anti-calgranulin B (S100A9) Ab. Vector transfected lysate (pcDNA3) was included as a negative control.

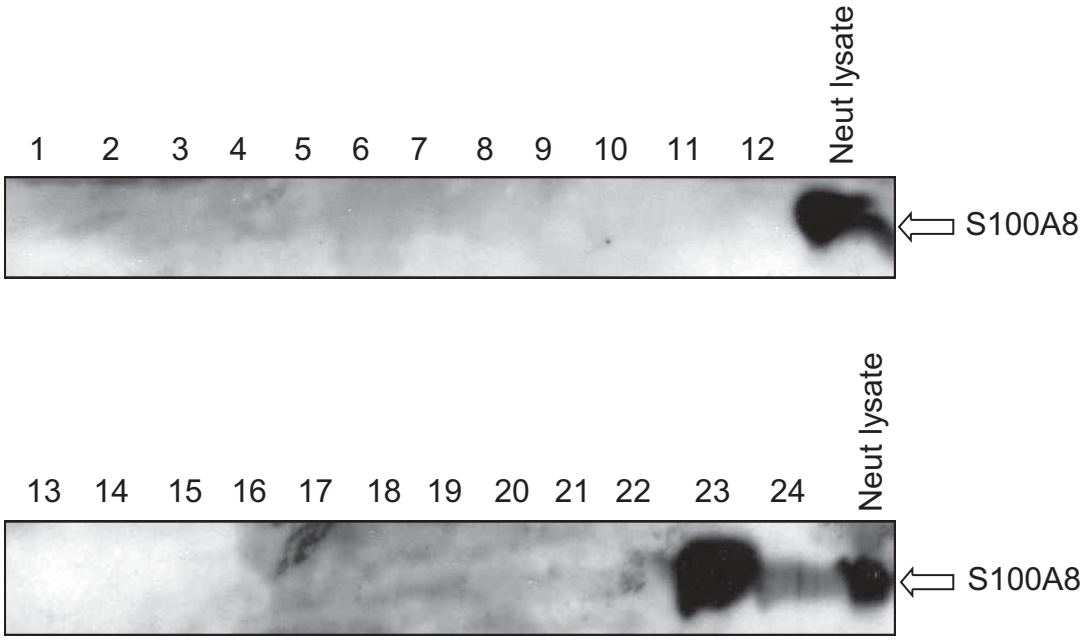


Figure 3.12: Analysis of S100A8 expression in G418-resistant HEK293 clones.

S100A8 was cloned into pcDNA3 and transfected into HEK293 cells, then grown in the presence of G418 to select for stable transformants. Isolated colonies were propagated individually and equal amounts of total protein from each clone were analysed for S100A8 expression by western blotting using an anti-calgranulin A (S100A8) Ab. Neutrophil lysate (Neut lysate) was included as a positive control.

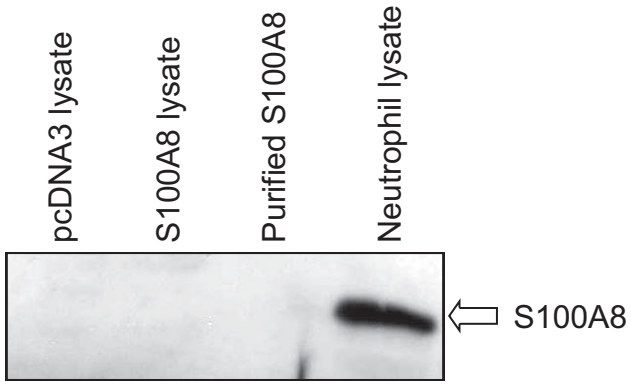


Figure 3.13: Western blot showing loss of S100A8 expression in stably transfected clone.

The S100A8 expressing clone was thawed after cryopreservation and found to have lost expression (S100A8 lysate). Neutrophil and pcDNA3 lysates were included as positive and negative controls, respectively. The S100A8 clone processed in the same way as neutrophils described in section 3.4.3 (purified S100A8), also failed to produce a detectable amount of S100A8 by western blotting with an Ab to calgranulin A (S100A8).

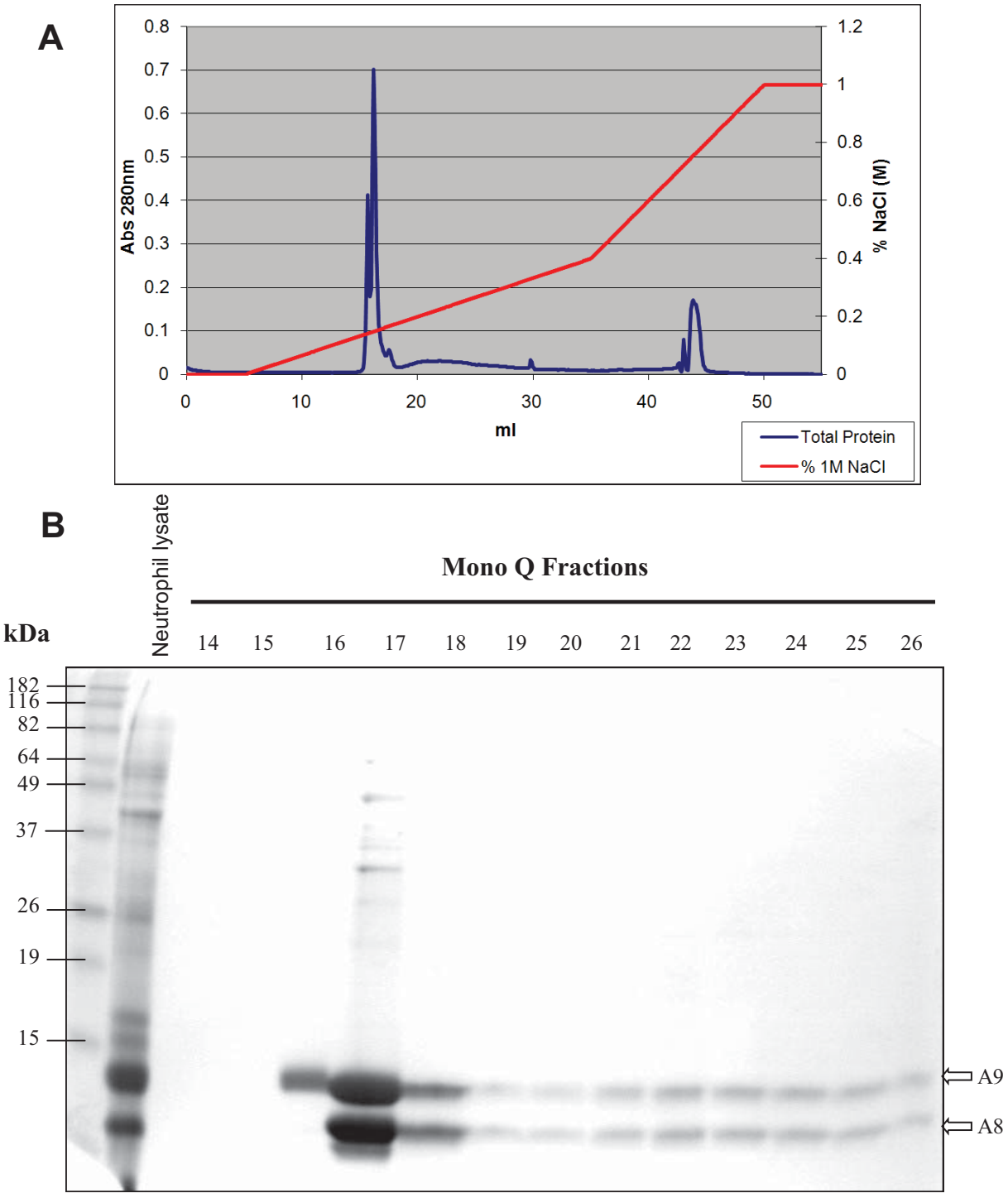


Figure 3.14: Purification of S100A8 and S100A9 from human neutrophils.

Following precipitation of contaminating proteins with 50% ammonium sulphate, the resulting supernatant was dialysed and fractionated by anion exchange chromatography.
A) Total protein elution profile from anion exchange chromatography of dialysed material.
B) Proteins present in the MonoQ fractions were resolved by 15% SDS-PAGE and visualised by staining with coomassie brilliant blue. S100A8 and S100A9 are indicated

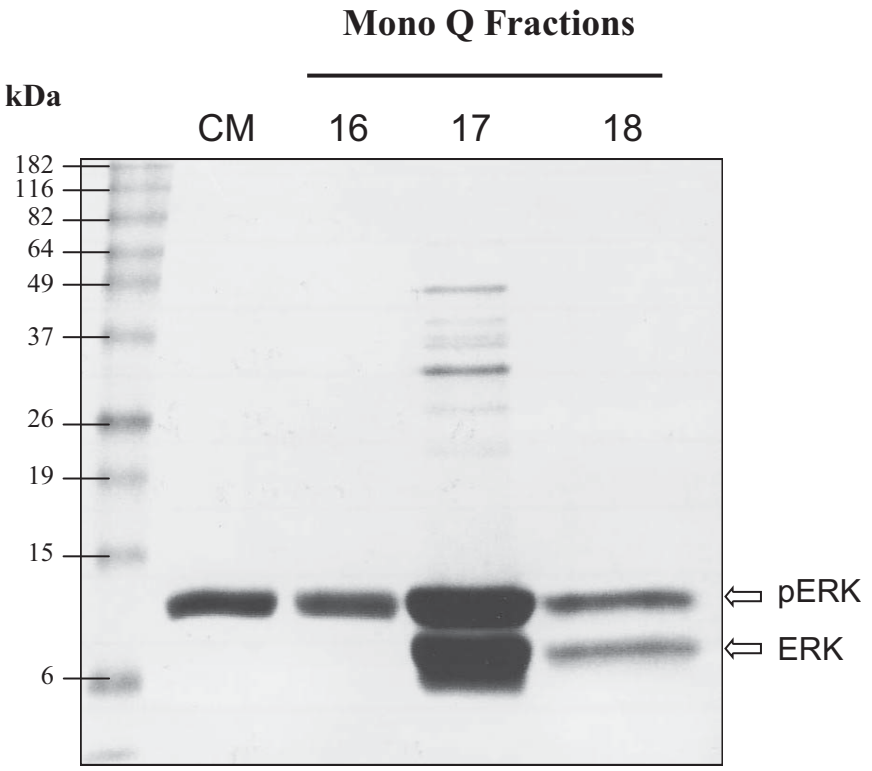


Figure 3.15: Comparison of purified S100A8 and S100A9 with levels in neutrophil conditioned medium.

20µl MonoQ fractions 16, 17 and 18 were compared to 20µl neutrophil conditioned medium (CM) on a 15% SDS-PAGE stained with coomassie brilliant blue.

calprotectin in the presence of calcium [165], each fraction was also incubated with calcium for 30 min before their addition to HUVEC. Following the incubation of each treatment on HUVEC monolayers, it was found that fraction 17 activated ERK, regardless of the presence of calcium (Figure 3.16). Fraction 16 was also able to give a small amount of ERK activation. Fraction 18 however had no effect, despite also containing both S100A8 and S100A9. Inexplicably, the calcium alone control spuriously activated ERK in this experiment, which was not observed in subsequent experiments (Figure 3.18). To investigate why fraction 17 could activate ERK whereas fraction 18 could not, both fractions (along with fraction 16) were analysed for annexin A1 content, a protein that was also a constituent of neutrophil conditioned medium whose role in activating endothelial ERK was being investigated concurrently with S100A8/A9 (see chapter 4). Western blot analysis of these fractions using an annexin A1 Ab established that fractions 16 and 17 contained greater amounts of annexin A1 compared to fraction 18, with fraction 17 containing the most (Figure 3.17). A band the approximate size of annexin A1 (37kDa) was also observed in fraction 17 following staining with coomassie blue (Figure 3.15). To verify these results, purified annexin A1 (outlined in section 4.5) and fraction 18, which contained very little annexin A1, were tested on HUVEC. It was found that only annexin A1 could activate endothelial ERK and fraction 18 could not, both of which were independent of calcium treatment (Figure 3.18). To eliminate the possibility of a synergistic effect of annexin A1 and S100A8/A9, a mixture containing half annexin A1 and half fraction 18 was also tested on HUVEC. There appeared to be no synergy between the proteins, and the small increase in phospho-ERK levels was probably due to annexin A1 (Figure 3.18). These results confirmed that S100A8 and A9 were not the factors in fMLP-stimulated neutrophil conditioned medium responsible for activating ERK. A more thorough investigation into the role of annexin A1 in endothelial ERK activation and neutrophil transmigration is detailed in chapter 4.

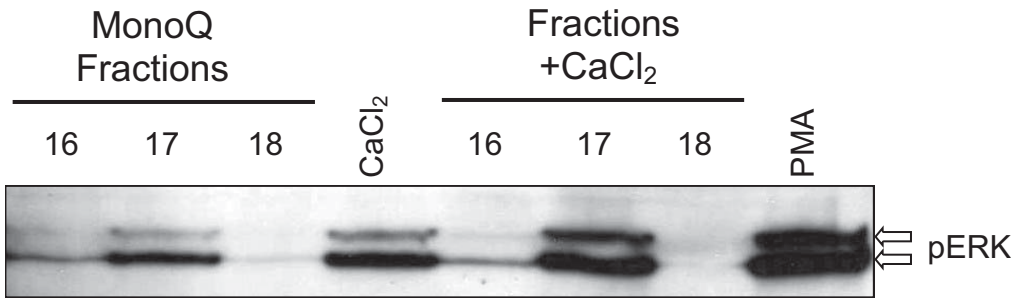


Figure 3.16: MonoQ fraction 17 from S100A8/A9 purification can activate endothelial ERK in HUVEC.

Resting HUVEC monolayers were treated with MonoQ fractions 16, 17 and 18, +/- 10mM $CaCl_2$. PMA and $CaCl_2$ controls were also included. HUVEC ERK activation was determined by western blotting lysates with an Ab to phosphorylated ERK.

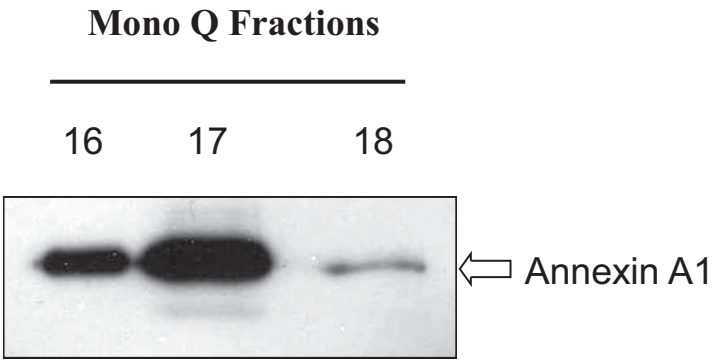


Figure 3.17: Annexin A1 content in purified S100A8/A9 MonoQ fractions.

Annexin A1 content in equal volumes of MonoQ fractions 16, 17 and 18 was analysed by western blotting using an Ab to human annexin A1.

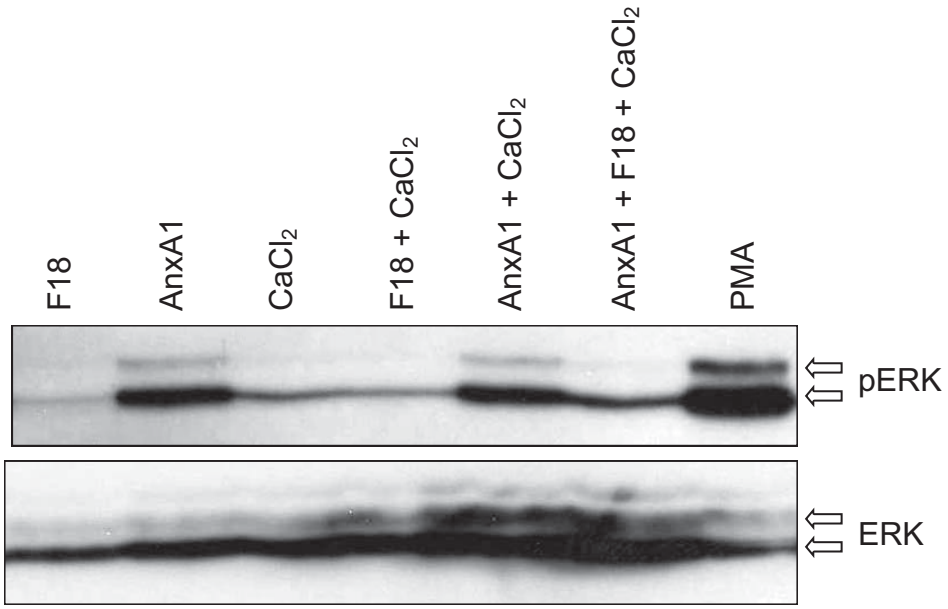


Figure 3.18: Purified annexin A1, but not purified S100A8 and S100A9, can activate ERK in HUVEC.

Upper panel- Resting HUVEC monolayers were treated with MonoQ fraction 18 (F18) and purified annexin A1, +/- 10mM $CaCl_2$. A sample with equal volumes of fraction 18 and annexin A1 + 10mM $CaCl_2$, equating to half that added in the sample alone treatments, was also tested. PMA and $CaCl_2$ controls were also included. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti-total ERK Ab after stripping, which highlights an uneven transfer of proteins to the PVDF membrane.

DISCUSSION

Activation of the ERK signalling pathway in endothelial cells has been shown to be crucial for neutrophil and eosinophil transmigration to occur, using both static and shear-flow *in vitro* models of transmigration [97, 99]. Previously our laboratory has shown that stimulated neutrophils secrete a soluble protein that is able to activate endothelial ERK [99]. In the present study, a strategy to identify the ERK-activating protein was formulated where soluble proteins released following neutrophil stimulation would be purified, with resulting candidate proteins to be analysed by mass spectrometry. The final purification scheme was relatively simple, after gel filtration chromatography of fMLP-stimulated neutrophil conditioned medium isolating the ERK-activating protein into just one fraction. The simplicity was largely due to the ERK-activating protein being a secreted protein, thus reducing the number of contaminating proteins encountered in comparison to the purification of cytosolic proteins. Other purification protocols were also evaluated, such as ammonium sulphate precipitation and anion/cation exchange chromatography (data not shown), however the purity of the active fraction from gel filtration chromatography was far superior.

Following mass spectrometry analysis of proteins present in the active fraction from gel filtration chromatography, MNEI, annexin A1, annexin A3, chloride intracellular channel protein, S100A8 and S100A9 were identified. A literature search of chloride intracellular channel protein highlighted that the protein did not have an obvious role in immune cell recruitment, aside from its intracellular function in chloride ion transport following neutrophil stimulation [166], as the name suggests. The presence of chloride intracellular channel protein in the active fraction was therefore thought to be a consequence of neutrophil degranulation rather than specific secretion following neutrophil stimulation and was not investigated further.

Neutrophil elastase is a serine protease with an established role in promoting neutrophil transmigration [167, 168] and it was therefore considered unlikely that its inhibitor, MNEI, could be a potential candidate for the pro-transmigration, ERK-activating protein. As expected, when purified recombinant MNEI was tested on HUVEC, no observable effect on phospho-ERK levels was detected.

Annexin A3 (also known as lipocortin III) was also investigated as a potential candidate for the ERK-activating neutrophil protein. Annexin A3 is a highly abundant protein in human neutrophils, comprising 1% of total cytosolic protein but is expressed at low levels or is undetectable in other cell types [169]. It has been shown to accumulate around the phagosomal region when neutrophils ingest opsonised yeast [170] and also promotes the aggregation of isolated specific granules from neutrophils *in vitro* in a calcium-dependent manner [169]. Evidence also suggests that annexin A3 can be secreted from neutrophils following stimulation [171]. This combined with the annexin A1 results detailed in chapter 4, led to the cloning of annexin A3 with a StrepII tag fused to the C-terminus, which was stably over-expressed in HEK293 cells (Appendix 2A). Unfortunately however, using conventional methods to purify annexin A3 by resins directed to the StrepII tag were unsuccessful (Appendix 2B). In light of the role of annexin A1 in neutrophil transmigration, further investigation into annexin A3 in this context is certainly warranted and is discussed in greater detail in chapter 6.

In contrast to the other candidates, the identification of S100A8 and S100A9 (also known as myeloid related proteins 8 and 14, respectively) appeared far more intriguing. A host of studies implicate S100A8 and S100A9 in a range of acute and chronic inflammatory disorders, including cystic fibrosis, inflammatory bowel diseases, rheumatoid arthritis, bronchitis and allograft rejection [172-176]. S100A8 and S100A9 are highly expressed in granulocytes, comprising 40-50% of total cytosolic protein [177], and myeloid cells staining positive for these proteins are the first immune cells to infiltrate inflammatory lesions [178, 179]. A close correlation between S100A8 and S100A9 serum levels and disease severity has led to the suggestion that both proteins could potentially be used as biomarkers for inflammation, as S100A8 and S100A9 can be detected in patients before the hallmarks of disease onset are evident [180, 181]. In the context of inflammation, evidence suggests that S100A8, S100A9 and the calprotectin heterodimer must be secreted for the proteins to exert an effect and has been shown to occur in both neutrophils [182] and monocytes [183] following their stimulation. S100A8 and S100A9 have also been detected extracellularly on vasculature where myeloid cells have transmigrated [184, 185] and have been shown to interact with several endothelial receptors [164, 172, 185], where blocking of one of these receptors was found to also inhibit neutrophil extravasation [164]. This data appeared to fit well with our data showing endothelial ERK activation by a soluble neutrophil protein being essential for neutrophil transmigration *in vitro*. To determine if calprotectin is a component of stimulated neutrophil conditioned medium involved in activating endothelial ERK, a mAb raised against an epitope specific to the calprotectin heterodimer, but not the S100A8 or

S100A9 subunits, was used. The heterodimer was investigated rather than the individual subunits, given the clear role for calprotectin in the immune response combined with the observation that calprotectin would be closer in size to proteins eluting in the ERK-activating fraction 10 resulting from gel filtration chromatography of neutrophil conditioned medium (Figure 3.5). When this mAb was added with neutrophil conditioned medium to HUVEC, it occasionally inhibited ERK activation in HUVEC (Figure 3.9). The inhibitory effect appeared dependent on the degree of ERK activation, in that when there was a weak endothelial response to neutrophil conditioned medium, the calprotectin mAb could inhibit ERK activation. However when a strong response was elicited by stimulated neutrophil conditioned medium (Figure 3.8), the mAb had no effect on phospho-ERK levels. This suggested that different amounts of calprotectin may be secreted by neutrophils between experiments and given the large amounts of S100A8 and S100A9 observed in neutrophil conditioned medium (Figure 3.10), the variability in inhibition by the calprotectin mAb could be due to insufficient quantities of the mAb being added to the blocking experiments. Therefore, rather than persisting with the calprotectin mAb to block ERK activation, it was decided that an alternative approach be used, i.e. to purify S100A8 and S100A9 and determine if each alone or in combination could activate endothelial ERK. Following the unsuccessful attempt at purifying recombinant S100A8 and S100A9, the proteins were ultimately purified from human neutrophils. In hindsight this was probably a more suitable direction to take, as all naturally occurring S100A8/A9 complexes secreted from neutrophils could be purified. After the addition of fraction 18, which contained both S100A8 and S100A9 (Figure 3.18), no effect on endothelial ERK was observed, even with the addition of calcium to promote the formation of S100A8/A9 complexes. Although tests were not carried out to verify that S100A8 and S100A9 purified from neutrophils retained activity, buffers and conditions described by van den Bos *et al* [158] were followed, which resulted in the purification of active proteins. Unlike fraction 18, fraction 17 could activate ERK but the effect was determined to be due to contaminating annexin A1 also present in the fraction, rather than S100A8/S100A9.

Although annexin A1 was identified as the ERK-activating factor in stimulated neutrophil conditioned medium in parallel studies (see chapter 4), it remains that the calprotectin mAb was also able to inhibit ERK activation on several occasions. It is unclear how the Ab produced this effect, however studies investigating S100-annexin complex formation may shed some light. There are 25 members in of S100 family of proteins and seven of those have been shown to interact with at least one member of the annexin family of proteins [186]. Annexin A1 has been shown to interact *via* the N-terminal domain with S100A11 [187] and with dicalcin, another

member of the S100 family, through the C-terminal core of the protein [188]. If the calprotectin mAb non-specifically recognised S100A11, dicalcin or an additional unidentified member of the S100 family that can bind annexin A1, the observed ERK inhibition by the mAb could have been an indirect effect caused by sequestration of annexin A1 away from the endothelium and thus prevented interaction. Alternatively, calprotectin may have been able to weakly bind to annexin A1, however immunoprecipitation experiments carried out to confirm a calprotectin-annexin A1 interaction were inconclusive (data not shown). Although a direct interaction has not been reported in the literature, results in Srikrishna *et al* [164] found that S100A8, S100A9 and annexin A1 assembly at carboxylated glycan endothelial receptors was involved in neutrophil transmigration, thus suggesting an interaction between these proteins.

Other preliminary experiments were also carried out to investigate whether additional types of white blood cells were capable of producing an ERK-activating factor similar to stimulated neutrophils. The mononuclear cell layer (buffy coat) resulting after density-gradient separation of human peripheral blood was isolated and stimulated with fMLP. The resulting conditioned medium was found to activate ERK in HUVEC in the same manner as fMLP-stimulated neutrophil conditioned medium (Figure 3.2), indicating that a soluble protein was also being released from these cells. Although lymphocytes, monocytes and platelets are all present in the mononuclear cell layer, monocytes were most likely the cell type responsible for producing the ERK-activating factor after fMLP stimulation, as they express all three formyl peptide receptors (FPR) [189], whereas lymphocytes do not express FPR [190]. Purified eosinophils were also stimulated with fMLP to determine if a soluble ERK-activating protein was secreted, as eosinophils are known to respond to fMLP stimulation and release a range of pro-inflammatory mediators [191]. The conditioned medium from fMLP-stimulated eosinophils did not induce ERK activation in HUVEC, which could indicate a number of possibilities: that eosinophils cannot produce an ERK-activating factor, that they can not release a soluble form of the ERK-activating factor or that fMLP is not an appropriate inducer. This experiment was attempted only once however and should be repeated to be confident of these findings, especially considering the results from a study showing ERK-dependent eosinophil transmigration across HUVEC monolayers under shear-flow [97], although in this study ERK activation was suggested to be the result of endothelial VCAM-1 and/or E-selectin engagement. Western blot analysis to detect annexin A1 in the stimulated conditioned medium from eosinophils and mononuclear cells was not carried out, as annexin A1 induced ERK activation had not been confirmed at that stage. This experiment needs to be performed with the inclusion of additional relevant proinflammatory

mediators, before drawing any conclusions relating to ERK activation by soluble annexin A1 and subsequent extravasation of white blood cells other than neutrophils.

SUMMARY

The key purpose of experiments performed in this chapter was to screen proteins present within conditioned medium from fMLP-stimulated neutrophil to identify the one responsible for activating ERK in endothelial cells, which is required for neutrophil transendothelial migration. Mass spectrometry analysis detected MNEI, annexin A1, annexin A3, chloride intracellular channel protein, S100A8 and S100A9 all within the active fraction of partially purified neutrophil conditioned medium. Given their function in inflammation and leukocyte extravasation, S100A8 and S100A9 initially appeared to be the likely candidates; however S100A8 and S100A9 purified from neutrophils failed to have any effect on endothelial phospho-ERK levels. Following the systematic testing of other potential targets, annexin A1 was found to be the ERK-activating protein in neutrophil conditioned medium. The role of annexin A1 in ERK activation and neutrophil transmigration is addressed further in chapter 4, with the effects of annexin A1 on the endothelium detailed in chapter 5.

Chapter 4

ANNEXIN A1 ACTIVATES ENDOTHELIAL ERK TO PROMOTE NEUTROPHIL TRANSENDOTHELIAL MIGRATION

INTRODUCTION

Studies carried out using mass spectrometry to identify the protein in the active fraction of fMLP-stimulated neutrophil conditioned medium (detailed in chapter 3) revealed that annexin A1 was a component of this fraction. However, other studies [192] have suggested that annexin A1 is an anti-inflammatory agent rather than pro-inflammatory as expected of the ERK-activating factor. Nevertheless, its potential role as the ERK-activating factor was investigated further (as will be expanded upon herein) because annexin A1 Abs could effectively block ERK-activation induced by neutrophil conditioned medium and recombinant annexin A1 induced ERK activation in HUVEC.

Annexin A1, also known as lipocortin 1, is a 37 kDa member of the annexin superfamily of calcium and phospholipid-binding proteins. Thirteen members of the annexin family have been described to date in humans, with a wide distribution of expression across different species and tissues, however localisation varies considerably [193]. The annexins are structurally characterised by two principle domains: the N-terminal domain and a C-terminal protein core that is highly conserved and is responsible for binding to negatively charged phospholipids in a calcium-dependent manner. Conversely, the N-terminal domain can vary in length and is the most divergent region of the otherwise highly conserved members of the annexin family. The unique N-terminus of each annexin protein is thought to convey the functional diversity and biological specificity required for the activity of each protein. Contained within this domain are a number of phosphorylation and protease recognition sites that regulate activity and localisation [194, 195].

Annexin A1 is a multi-faceted protein with a number of functions ranging from membrane fusion, inflammation, apoptosis, cell signalling and regulation of cell growth and differentiation [196]. Annexin A1 is particularly abundant in human neutrophils and comprises between 2-4% of total cytosolic protein. The majority of annexin A1 detected in inflamed tissues has been shown to be a consequence of neutrophil infiltration [197, 198]. Annexin A1 is predominantly cytosolic and localised in gelatinase granules of resting neutrophils, however 50-70% is rapidly mobilised to the plasma membrane upon the activation and adhesion to endothelial cells, where it is then proteolytically cleaved [199-201]. Consequently, the predominant form annexin A1 remaining within the neutrophil is N-terminally cleaved following extravasation. N-terminally truncated

annexin A1 is also secreted from neutrophils following adhesion to endothelial monolayers [202] and has been detected on the surface and within endothelial cells in close proximity to the site of neutrophil adhesion and extravasation [192, 200], however the mechanism by which truncated annexin A1 is taken up by endothelial cells is unknown. As observed with other annexins, annexin A1 possesses sites within the N-terminal domain that target the protein for glycosylation, phosphorylation and limited proteolytic processing [203], where such post-translational modifications can result in a number of effects. Annexin A1 contains phosphorylation sites for kinases including protein kinases A and C [204], TRPM7 channel kinase [205], hepatocyte growth factor receptor [206] and epidermal growth factor receptor [207]. Phosphorylation of annexin A1 can alter its sub-cellular localisation, where dexamethasone and IL-6 have been shown to induce serine phosphorylation leading to the translocation of annexin A1 to the cell surface [208, 209]. Annexin A1 phosphorylation appears to facilitate binding to the plasma membrane as phosphorylation decreases the concentration of Ca^{2+} required for phospholipid binding [210]. Proteolytic cleavage of annexin A1 has also been found to increase its affinity for Ca^{2+} and also reduce the Ca^{2+} requirement for membrane binding [194]. Once located at the plasma membrane, there is evidence that phospholipid-bound annexin A1 is more susceptible to proteolysis [194].

Arguably, the best-characterised feature of annexin A1 and N-terminal peptide derivatives is the profound anti-inflammatory effect exhibited on leukocyte activation and extravasation [211, 212]. Annexin A1 was originally described as a glucocorticoid-inducible protein with the ability to inhibit phospholipase A_2 activity and therefore the generation of prostaglandins [213]. Both the full length protein and a peptide derived from the N-terminus, Ac2-26, were shown soon after to display anti-inflammatory properties in a number of acute models of inflammation [214, 215]. Following on from these early findings, a host of studies utilising neutralising antibodies and exogenously administered protein have described further anti-inflammatory actions for annexin A1 and Ac2-26 *in vitro* and *in vivo* [211, 216], many of which primarily affect neutrophil function and trafficking. Perhaps the most compelling data from this set of studies was the generation of the annexin A1 knockout mouse, which demonstrates an irregular inflammatory response [217]. Annexin A1-null mice display a heightened response to exogenous stimuli in models of acute and chronic inflammation [218]. These inflammatory events take longer to resolve compared to wild-type mice and are resistant to the anti-inflammatory effects of glucocorticoids [219, 220]. Other features quantified included an upregulation of other annexins,

increased spontaneous migratory behaviour of leukocytes and altered leukocyte adhesion molecule expression (reviewed in [217]).

Evidence is emerging that annexin A1 may also play an important regulatory role in tumour development. The detection of annexin A1 in clinical cancer tissues has clearly shown altered expression, however whilst expression is reduced in some cancers, it has been observed at elevated levels in others [221]. Given the variation of annexin A1, this may indicate that expression is linked to factors such as cancer progression, tumour type or differentiation status, however its precise function still remains to be identified.

Through the use of blocking antibodies and recombinant annexin A1, the experiments performed in this chapter confirmed that annexin A1 was the ERK-activating factor present in fMLP-stimulated neutrophil conditioned medium. Unexpectedly, it was determined that the active form of annexin A1 was not full length protein, but a truncated product potentially generated following neutrophil calpain I cleavage.

RESULTS

4.1. Annexin A1 Ab inhibits endothelial ERK-activation by fMLP-stimulated neutrophil conditioned medium

As outlined in chapter 3, annexin A1 was identified as a component of fMLP-stimulated neutrophil conditioned medium by mass spectrometry (Figure 3.6, band 2). To determine if annexin A1 was the ERK-activating factor, an annexin A1 mAb was added with the neutrophil conditioned medium to endothelial cells and its ability to neutralise the ERK-activating effect was examined. The level of activated ERK in HUVEC induced by neutrophil conditioned medium was markedly reduced in the presence of the annexin A1 mAb when compared to that of HUVEC treated with conditioned medium alone (Figure 4.1). In contrast, a control irrelevant, isotype matched mAb to keyhole limpet hemocyanin (KLH) had no effect on ERK activation induced by neutrophil conditioned medium (Figure 4.1). This suggested that annexin A1 could be the ERK-activating factor in neutrophil conditioned medium. An annexin A1 immuno-depletion experiment was also performed, where the protein A sepharose-Ab-annexin A1 complex was removed from the conditioned medium. When annexin A1 immunodepleted neutrophil conditioned medium was tested on resting HUVEC, its potency in activating ERK was significantly reduced when compared to conditioned medium alone (Figure 4.1). This further supported the role of annexin A1 as the ERK-activating protein in neutrophil conditioned medium. To ensure the ERK-inhibiting effect was not peculiar to the annexin A1 monoclonal Ab, an annexin A1 polyclonal Ab was also tested and showed identical results (Figure 4.2), although an irrelevant rabbit IgG control should have also been tested to validate this result.

4.2. Annexin A1 Ab inhibits endothelial ERK-activation by IL8-stimulated neutrophil conditioned medium

A wide range of pro-inflammatory mediators involved in the inflammatory response can stimulate neutrophil degranulation. Therefore to determine if the release of annexin A1 is specific to fMLP or if it is pertinent other neutrophil activators, neutrophils were incubated with the pro-inflammatory cytokine IL-8 and the resulting conditioned medium was tested on resting HUVEC monolayers for endothelial ERK activation. IL-8 stimulated neutrophil conditioned medium

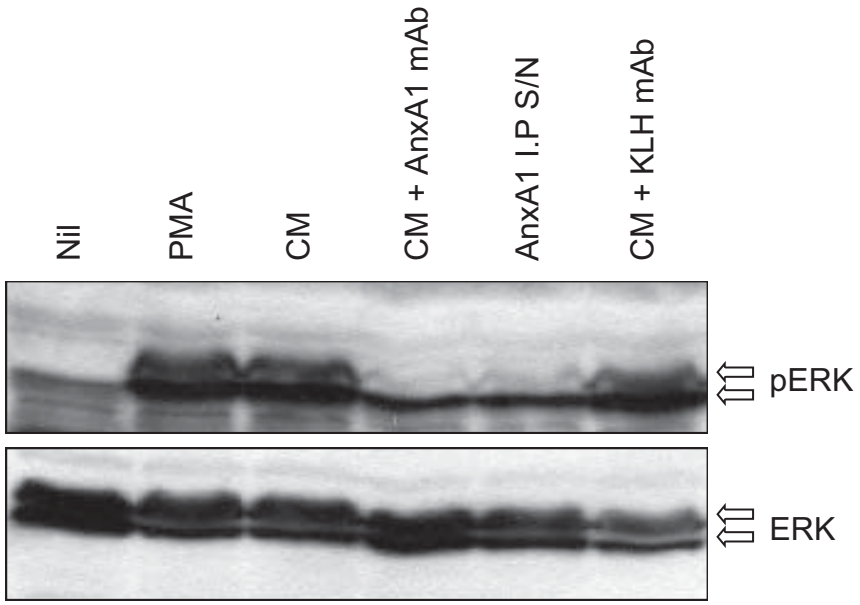


Figure 4.1: Annexin A1 mAb inhibits ERK-activation in HUVECs induced by fMLP-stimulated neutrophil conditioned medium.

Upper panel- Resting HUVEC monolayers were treated with PBS (Nil), 0.25μg/ml PMA as a positive control, neutrophil conditioned medium (CM), neutrophil conditioned medium with 5μg annexin A1 mAb (CM + AnxA1 mAb), supernatant from an annexin 1 immunodepleted neutrophil-conditioned medium (AnxA1 I.P S/N), neutrophil conditioned medium with 5μg irrelevant anti-KLH mAb (CM + KLH Ab), for 10 min. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti– total ERK Ab after stripping.

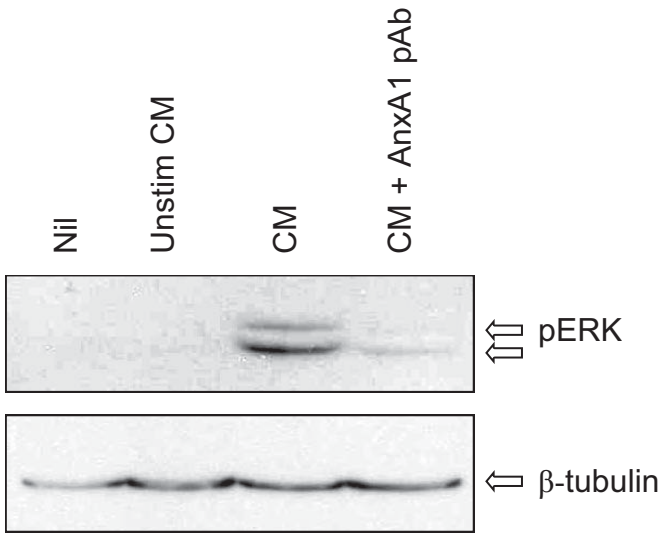


Figure 4.2: Annexin A1 pAb inhibits ERK activation in HUVECs induced by fMLP-stimulated neutrophil conditioned medium.

Upper panel- Resting HUVEC monolayers were treated with PBS (Nil), unstimulated neutrophil conditioned medium (Unstim CM), fMLP-stimulated neutrophil conditioned medium (CM), and neutrophil conditioned medium + 5μg annexin A1 pAb (CM + AnxA1 pAb). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with β-tubulin Ab.

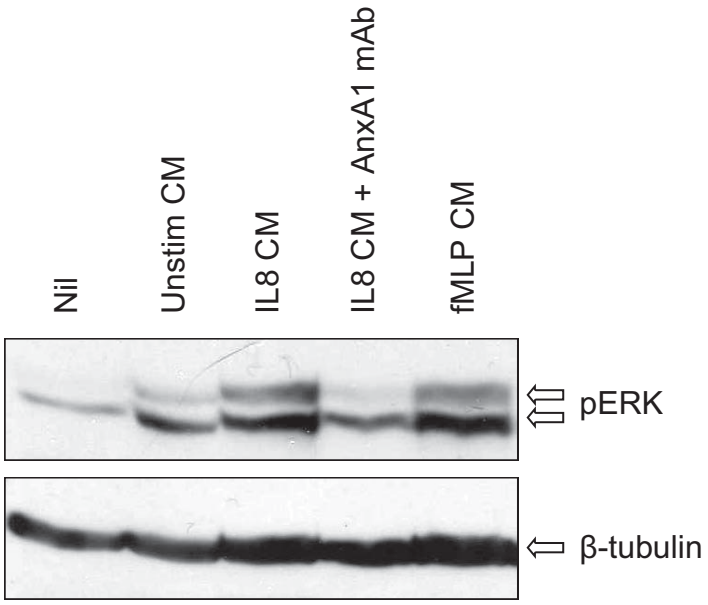


Figure 4.3: Annexin A1 mAb inhibits ERK activation in HUVECs induced by IL-8-stimulated neutrophil conditioned medium.

Upper panel- Resting HUVEC monolayers were treated with PBS (Nil), unstimulated neutrophil conditioned medium (unstimulated CM), IL-8-stimulated neutrophil conditioned medium (IL8 CM), IL-8 neutrophil conditioned medium + 5μg annexin A1 mAb (IL8 CM + AnxA1 Ab), and fMLP-stimulated neutrophil-conditioned medium (fMLP CM). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with β-tubulin Ab.

could activate endothelial ERK (Figure 4.3). Furthermore, the addition of annexin A1 mAb to IL-8-stimulated conditioned medium effectively reduced ERK activation to a degree similar to that induced by conditioned medium from unstimulated neutrophils (Figure 4.3). This indicated that neutrophil stimulation and subsequent release of the ERK-activating factor was not limited to stimulation by the formyl peptide receptors and could also be released in response to IL-8 receptor binding.

4.3. Annexin A1 Ab inhibits neutrophil transendothelial migration *in vitro*

We have previously shown that endothelial ERK activation is an essential requirement for neutrophil transmigration to occur [99]. Therefore the effect of the annexin A1 Ab on neutrophil transmigration was investigated. This was conducted using an *in vitro* model of neutrophil migration across a confluent monolayer of cultured HUVEC, as outlined in materials and methods (Figure 2.4). Transmigration was induced by the addition of an exogenous chemoattractant gradient (fMLP) or by activating the endothelial cells with TNF-α, a cytokine that is released during the pro-inflammatory response and activates endothelial and immune cells *in vivo*. The addition of annexin A1 Ab to the assay significantly impaired neutrophil transmigration across HUVEC monolayers in response to an fMLP gradient (Figure 4.4, reduced by ~ 3.5 fold) and across TNF-α-activated HUVEC (Figure 4.5, reduced by ~ 3 fold) compared to the control isotype-matched KLH Ab. Basal levels of transmigration can be seen in the first column of Figures 4.4 and 4.5, where neither a stimulus nor Ab was added to the assay. This data suggested that blocking neutrophil induced ERK activation in endothelial cells with the annexin A1 mAb could effectively inhibit neutrophil transendothelial migration *in vitro*.

4.4. Cloning and expression of human annexin A1 in human embryonic kidney cells

Inhibition of endothelial ERK activation induced by neutrophil conditioned medium as well as neutrophil transmigration by the annexin A1 Ab suggested annexin A1 was critical for activating endothelial ERK that is essential for transmigration. It was therefore investigated whether purified recombinant annexin A1 could activate endothelial ERK. Untagged human annexin A1 was cloned from human granulocyte mRNA and inserted into the pcDNA3 mammalian expression vector, where expression is driven by the CMV promoter (Figure 2.3). The entire

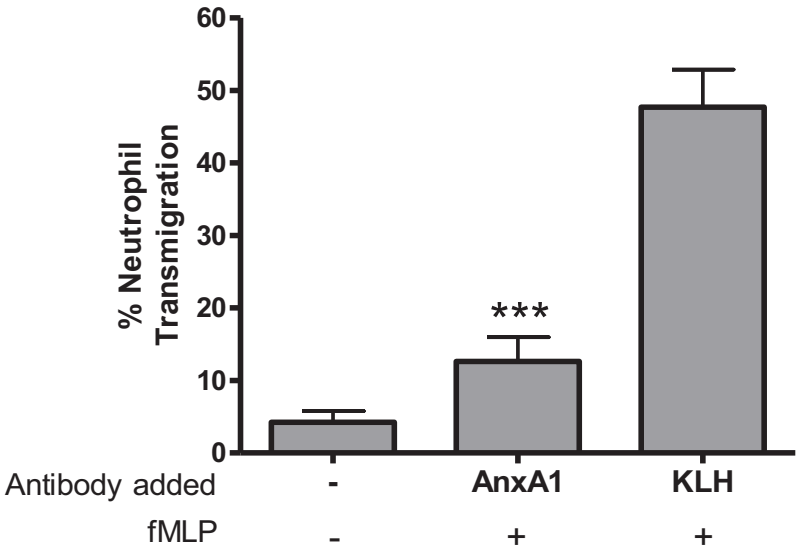


Figure 4.4: Annexin A1 mAb inhibits neutrophil transendothelial migration induced by fMLP *in vitro*.

HUVEC monolayers were established on Transwells as described. 5 x 10^5 neutrophils were added to the top chamber with 5μg annexin A1 mAb (AnxA1), 5μg irrelevant KLH mAb (KLH) or without antibody (-). Transmigration was measured across unstimulated endothelium, with and without a 10nm fMLP gradient, and expressed as a percentage of the total amount of neutrophils added. The experiment was conducted 3 times in triplicate. *** $p<0.0001$ (unpaired t-test comparing AnxA1 and KLH columns). Bars indicate +/- SEM.

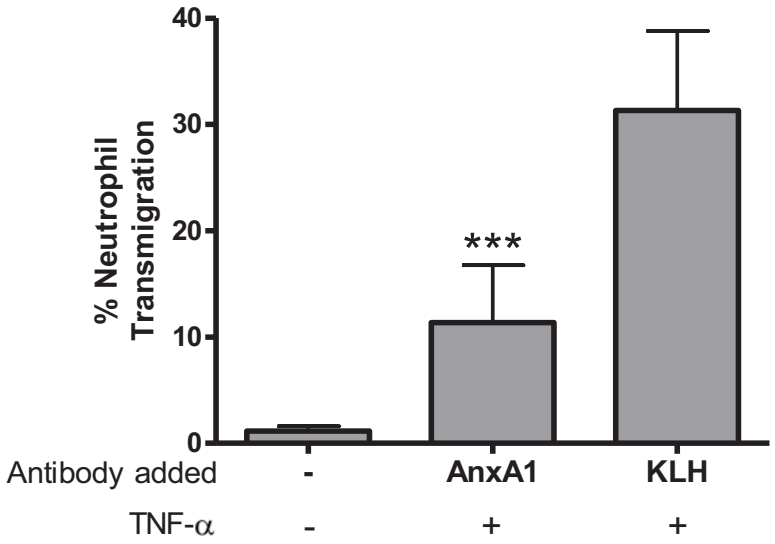


Figure 4.5: Annexin A1 mAb inhibits neutrophil transendothelial migration across TNF-α activated HUVEC *in vitro*.

HUVEC monolayers were established on Transwells as described and were either activated with TNF-α (4ng/ml) for 4h before conducting the assay or were left untreated. 5 x 10^5 neutrophils were added to the top chamber with 5μg annexin A1 mAb (AnxA1), 5μg irrelevant KLH mAb (KLH) or without antibody (-). Transmigration was measured and expressed as a percentage of the total amount of neutrophils added. The experiment was conducted 3 times in triplicate. *** $p<0.001$ (unpaired t-test comparing AnxA1 and KLH columns). Bars indicate +/- SEM.

cDNA was sequenced and determined to be mutation free. The annexin A1 plasmid was transfected into HEK293 cells and clonal cell lines stably expressing annexin A1 were generated following selection with geneticin (G418). Annexin A1 expression levels in the HEK293 clones was analysed by western blotting (Figure 4.6). The high level of annexin A1 expression in clone 1 made it the clone of choice for purification of recombinant annexin A1.

4.5. Purification of recombinant annexin A1

Annexin A1 was purified from clone 1 using an established method employing differential calcium precipitation and anion exchange chromatography, previously outlined by Rothhut and colleagues [159]. Following calcium precipitation of cell lysates, the pellet was resuspended in buffer containing EGTA and a final ultra-centrifugation step performed. The supernatant containing annexin A1 was then injected onto a Mono Q anion exchange column and proteins were eluted by applying a salt gradient. Total protein eluted from the Mono Q column can be seen in Figure 4.7A, and the fractions containing annexin A1 present in these fractions were analysed by western blotting (Figures 4.7B). The proteins that passed through the column and did not bind to it were TCA precipitated and analysed by western blotting, to reveal that only a small amount of annexin A1 did not bind (Figure 4.8). It was therefore concluded that the vast majority of the protein was binding to the column and was not being lost in the flow-through. Examination of particulate material remaining after resolubilisation with EGTA for annexin A1 by western blotting (Figure 4.8) showed that a large amount was retained in the pellet and had not been resolubilised completely. Steps were taken subsequently to reduce the amount of annexin A1 remaining in the pellet, such as increasing the EGTA concentration, increasing the EGTA resolubilisation time and sonicating the pellet, but were without success (data not shown). Analysis of annexin A1 in the Mono Q fractions showed that the majority of the protein eluted rapidly from the column and was present in fractions 1 and 2 (Figure 4.8) and had rapidly eluted from the column at a salt concentration very much lower than the reported concentration of 0.23M NaCl [159]. More annexin A1 further eluted off the column in fractions 9 and 10, corresponding to approximately 0.06M – 0.08M NaCl, which is still significantly lower than 0.23M NaCl. The difference in the amount of NaCl needed to elute annexin A1 from the column was probably due to the condition of the Mono Q column, as it had been extensively used and had undergone repeated chemical cleaning. Fractions collected after fraction 16 were also examined, but no other significant annexin A1 peaks were detectable by western blotting (data

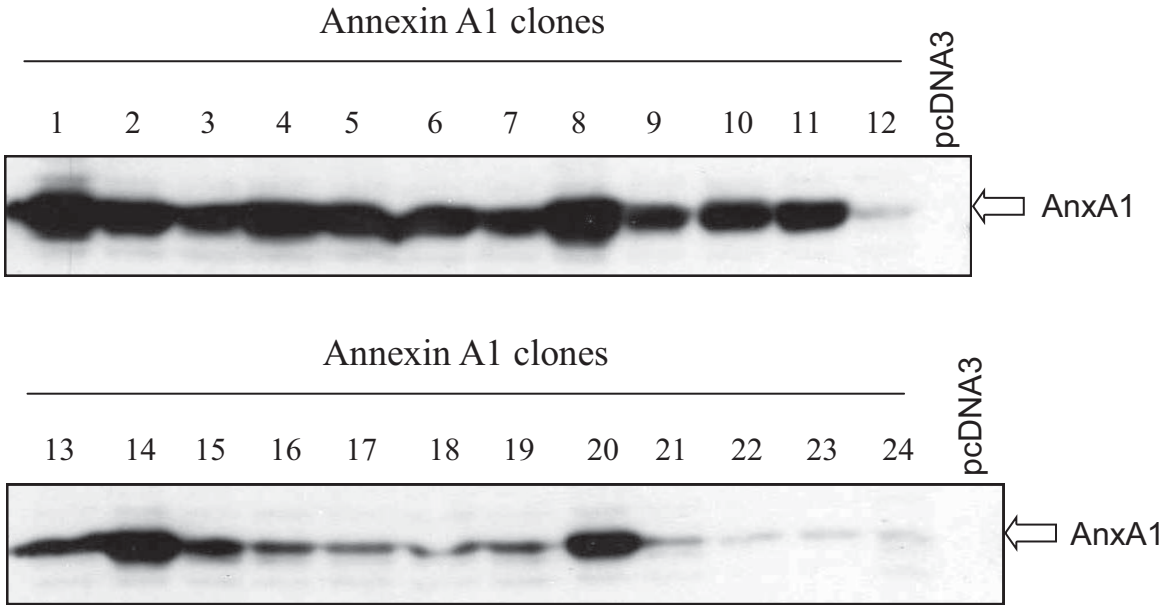


Figure 4.6: Analysis of annexin A1 expression in G418-resistant HEK293 clones.

Annexin A1 was cloned into pcDNA3 and transfected into HEK293 cells, then grown in the presence of G418 to select for stable transformants. Isolated colonies were propagated individually and equal amounts of total protein from each clone were analysed for annexin A1 expression by western blotting using an anti-annexin A1 mAb. Vector transfected lysate (pcDNA3) was included as a negative control.

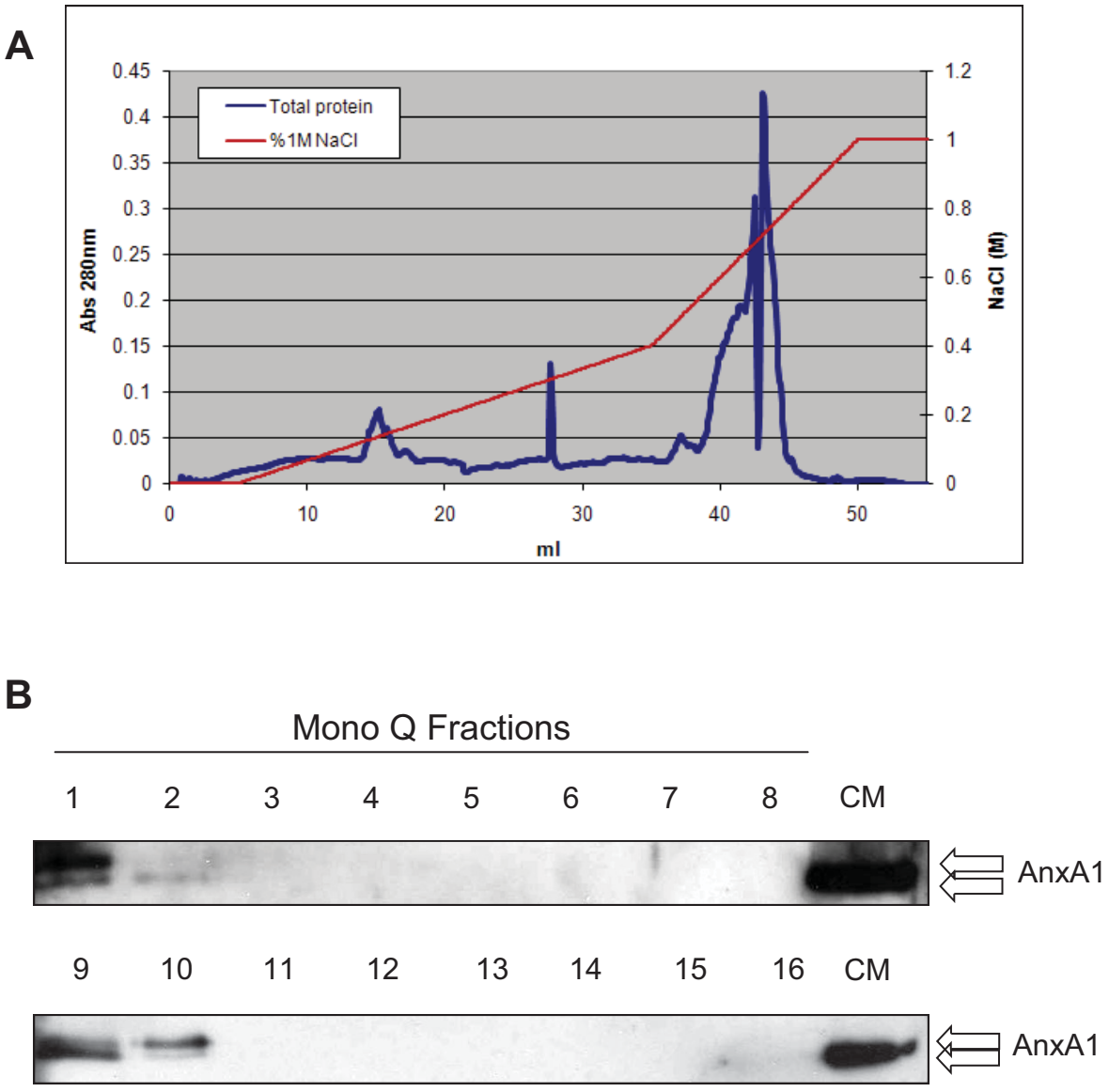


Figure 4.7: Analysis of annexin A1 levels in Mono Q fractions.

A) Elution profile of total protein from Mono Q anion exchange chromatography. **B**) 30µl of fractions 1-16 were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed with an annexin A1 mAb. fMLP-stimulated neutrophil conditioned medium (CM) was also run as a positive control.

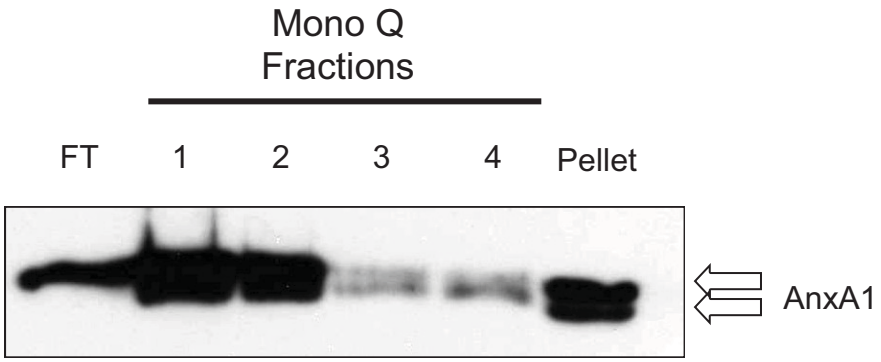


Figure 4.8: Analysis of annexin A1 levels in Mono Q fractions.

Mono Q fractions and the entire flow-through (FT) were TCA precipitated and run on a 10% SDS-PAGE gel. One-fiftieth of the pellet remaining after EGTA resolubilisation(Pellet) was also run on the gel. Annexin A1 content was analysed by western blotting with an annexin A1 mAb.

not shown). Since a significant proportion of annexin A1 eluted in fraction 1, this would be the fraction of choice for subsequent analyses. Hence, the level of contaminating proteins present in this fraction were analysed by coomassie blue staining (Figure 4.9A) and silver staining (Figure 4.9B) to determine if the annexin A1 was sufficiently pure to be used. The contaminating proteins were found to be negligible, with only two minor bands being detected by silver staining. Therefore the annexin A1-containing fraction 1 was deemed to be suitable for use in subsequent experiments.

4.6. Purified recombinant annexin A1 activates endothelial ERK

Purified annexin A1 from fraction 1 was incubated on resting HUVEC monolayers. Western blotting analysis showed a marked increase in ERK-activation in cells treated with 1.25 µg/ml annexin A1, compared the vehicle control where there was no effect (Figure 4.10). Increasing the amount of annexin A1 added to 2µg/ml further increased the degree of ERK-activation (Figure 4.10). The finding that recombinant annexin A1 could activate endothelial ERK reinforced our earlier conclusion from studies using the blocking Abs to annexin A1; that the ERK-activating factor present in fMLP-stimulated neutrophil conditioned medium was annexin A1. Further characterisation of annexin A1 and its role in neutrophil transmigration were therefore carried out.

4.7. Truncated annexin A1 activates endothelial ERK

During the purification of recombinant annexin A1, both full-length and a 33 kDa truncated form of annexin A1 routinely co-fractionated, with their ratio often varying between preparations (see Figures 4.7B, 4.9A and 4.9B for examples). At times, the two forms eluted separately in adjacent fractions from the Mono Q column (see fractions 9 and 10, Figure 4.7B). Equal volumes of fractions 9 and 10, containing 5µg of total protein, were tested on HUVEC for their ability to activate ERK. It was found that fraction 9, containing predominantly truncated annexin A1, activated ERK to a greater extent than fraction 10, which contained predominantly full length annexin A1 (Figure 4.11), suggesting that the truncated form of annexin A1 is much more active than full length annexin A1. Other earlier results were also reviewed in light of this finding and it was found that many of the annexin A1 bands observed were not the full length 37kDa form, but

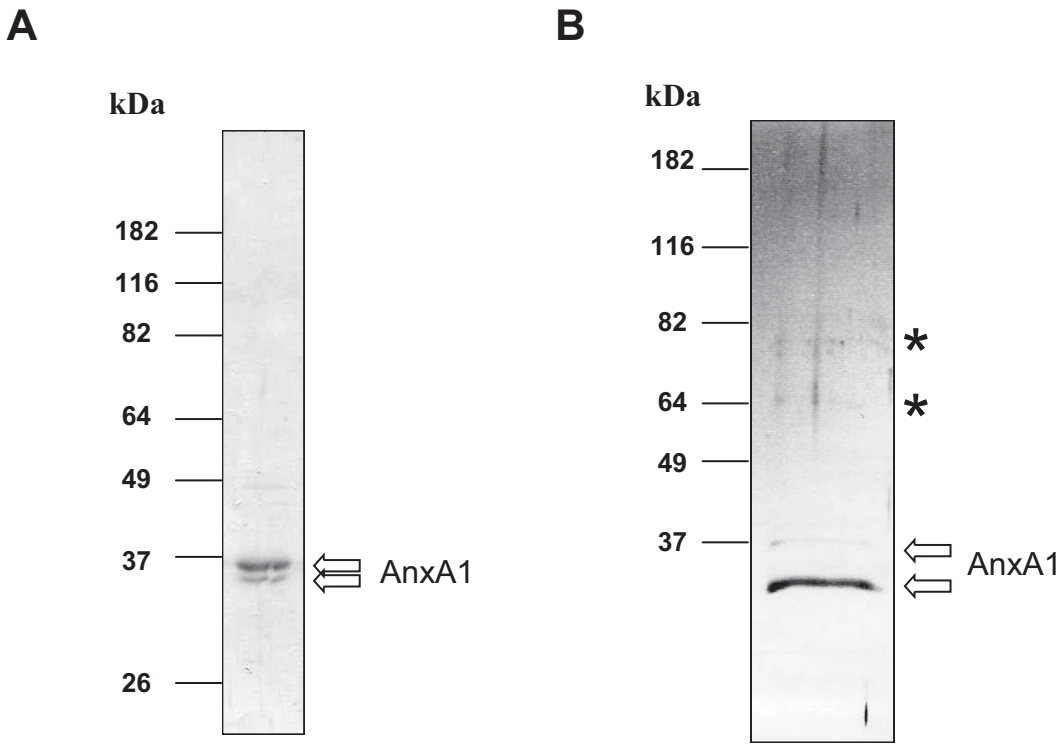


Figure 4.9: Analysis of Mono Q Fraction 1.

A) The entire fraction 1 was TCA precipitated, the proteins resolved on a 10% SDS-PAGE gel and transferred to PVDF membrane. The PVDF was stained with coomassie blue to detect total protein.
B) Proteins present in 40µl of fraction 1 were resolved on a 10% SDS-PAGE gel, which was then silver stained to detect total protein. Stars denote contaminating protein bands.

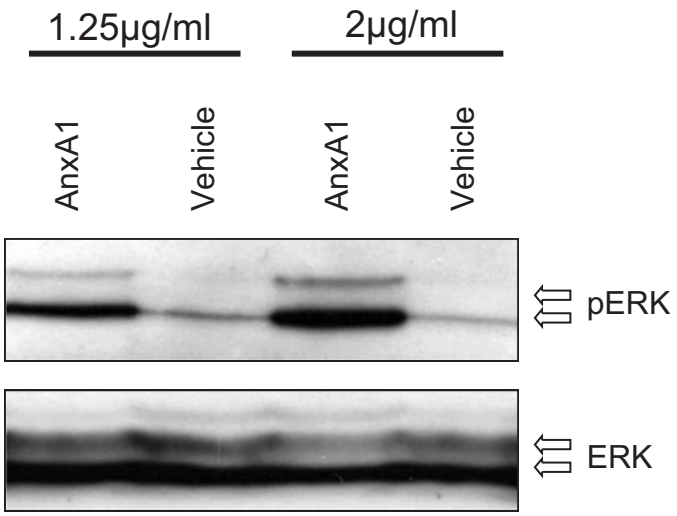


Figure 4.10: Recombinant annexin A1 activates endothelial ERK.

Upper panel- Resting HUVEC monolayers were treated with 1.25µg/ml or 2 µg/ml recombinant annexin A1 and the corresponding volumes of vehicle control (20mM ethanolamine). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with anti– total ERK after stripping.

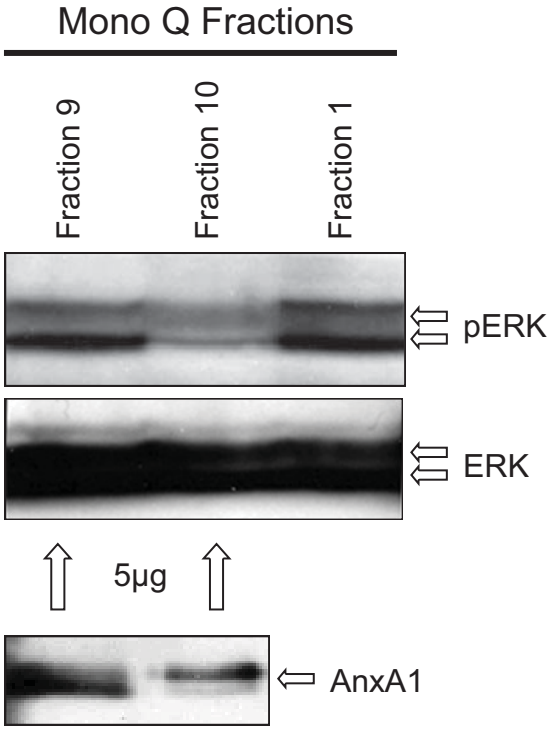


Figure 4.11: Truncated annexin A1 activates endothelial ERK.

Upper panel- 5µg of Mono Q fractions 9 and 10 (lower panel) were incubated on resting HUVEC monolayers for 10 min, then phosphorylated ERK was detected by western blotting. A positive control (fraction 1) containing both forms of the protein was also included. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Middle panel- The membrane probed with anti-total ERK Ab after stripping.

rather a smaller form of the protein (see Figure 3.6, a SDS-PAGE gel of fractionated neutrophil conditioned medium used for mass spectrometry where annexin A1 was initially identified). This suggested the smaller molecular weight form of annexin A1 present in the protein preparation was the form responsible for activating endothelial ERK, instead of the full length protein.

4.8. N-terminal sequencing of annexin A1 present in fMLP-stimulated neutrophil conditioned medium

The results in 4.7 suggested that a truncated form of annexin A1, potentially due to proteolytic cleavage, was responsible for activating endothelial ERK. The annexin A1 present in fMLP-stimulated neutrophil conditioned medium was subjected to N-terminal sequencing (performed by Chris Cursaro, Adelaide Proteomics Centre) with the purpose of identifying potential cleavage sites and consequently the protease responsible for cleaving annexin A1. However, after a number of attempts using different extraction techniques to obtain annexin A1 from neutrophil conditioned medium (extracting the annexin A1 from both SDS-PAGE gel and PVDF membrane), the N-terminus could not be detected for the protein (data not shown). Such a result could be because the protein is N-terminally blocked by post-translational modifications and therefore suggests the protein has not been cleaved by a protease, or simply that the N-terminal sequencing reaction was unsuccessful.

4.9. Calpain I proteolysis of annexin A1 yields an ERK-activating product

Cathepsin D, calpain I and plasmin have previously been shown to cleave annexin A1 in the N-terminal region after Trp-12, Lys-26 and Lys-29 respectively [222], therefore all three were tested. Full length, purified recombinant annexin A1 (40µg total protein) was cleaved with human calpain I and the resulting products were assayed on HUVEC (Figure 4.12A). Under these conditions, calpain treatment for 20 minutes appeared to result in the greatest degree of ERK activation, which persisted for 40 minutes but returned to vehicle-treated control levels by 60 minutes (Figure 4.12A). Full length annexin A1 alone could not activate ERK (Figure 4.12A). This suggests that a calpain cleavage product of annexin A1 activates ERK but that this cleavage product may have a limited lifetime. The annexin A1 present in each of these samples (25µg total protein, ~2/3 of that assayed on HUVEC) was analysed by western blotting (Figure 4.12B),

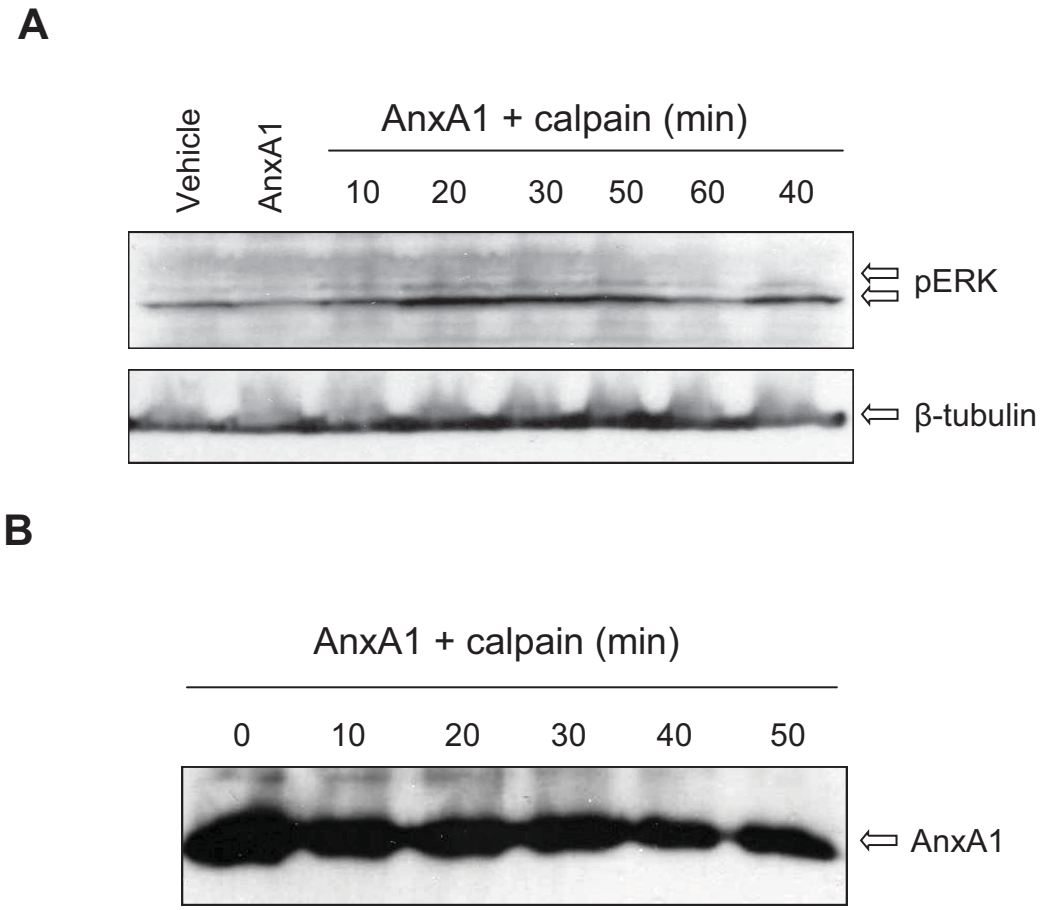


Figure 4.12: Calpain-cleaved recombinant annexin A1 activates endothelial ERK.

A) ***Upper panel***– 40μg partially purified annexin A1 (total protein concentration) was proteolysed by human calpain I for the indicated time periods, then incubated on resting HUVEC monolayers for 10 min. 40μg uncleaved annexin A1 (AnxA1) and vehicle control (vehicle) were also tested. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK. ***Lower panel***– The membrane reprobed with β-tubulin mAb.

B) 25μg of calpain-cleaved annexin 1 from each time point was analysed by western blotting with an annexin A1 mAb. The 60' calpain cleavage timepoint is absent.

however a cleavage product could not be detected, although a loss of full length protein was observed, presumably due to its low concentration as a consequence of its short half-life. In an experiment using the same conditions but with the inclusion of additional controls, it was again shown that full length annexin A1 could not activate ERK in HUVEC, whereas annexin A1 cleaved with calpain I could (Figure 4.13). The addition of 1μM calpain I inhibitor during annexin A1 proteolysis effectively inhibited ERK activation induced by the annexin A1/calpain I mixture (Figure 4.13), most likely by preventing the formation of the annexin A1 cleavage product, although again, the cleavage product could not be detected by western blotting (Figure 4.13). Calpain incubated with the vehicle control could not induce ERK activation above that observed in the annexin A1 alone sample (Figure 4.13). Annexin A1 plus inhibitor and vehicle alone controls are absent from this blot and should have been included. These results suggested that an annexin A1 cleavage product and not the full length form of the protein, was activating endothelial ERK. The inability to detect the active cleavage product along with the observation that prolonged incubation with calpain I led to a loss of activity suggested that the active cleavage product was transient. fMLP-stimulated neutrophil conditioned medium was therefore treated with calpain I to determine if the ERK-activating product was being processed by calpain I at an additional site, as the only annexin A1 released by neutrophils is proteolytically cleaved in the secretion process. The level of annexin A1 in conditioned medium increased following the treatment of neutrophils with fMLP compared to unstimulated neutrophils, however annexin A1 in the same fMLP-stimulated conditioned medium was undetectable after incubation with calpain I (Figure 4.14). This indicated that calpain I did indeed cleave at an additional recognition sequence located within annexin A1. This was the most likely reason that a product was not observable when recombinant annexin A1 was cleaved with exogenous calpain I and that the ERK-activating annexin A1 was a transient product in these experiments.

4.10. Calpain inhibitor prevents secretion of active annexin A1 from neutrophils

To demonstrate that calpain I also plays a role in generating active annexin A1 from stimulated neutrophils, a calpain inhibitor was included either during the fMLP-stimulation of neutrophils or the endothelium prior to the addition of neutrophil conditioned medium (generated without calpain inhibitor). Although the unstimulated conditioned medium and conditioned medium lanes have electrophoresed oddly, it was clear that conditioned medium obtained from neutrophils

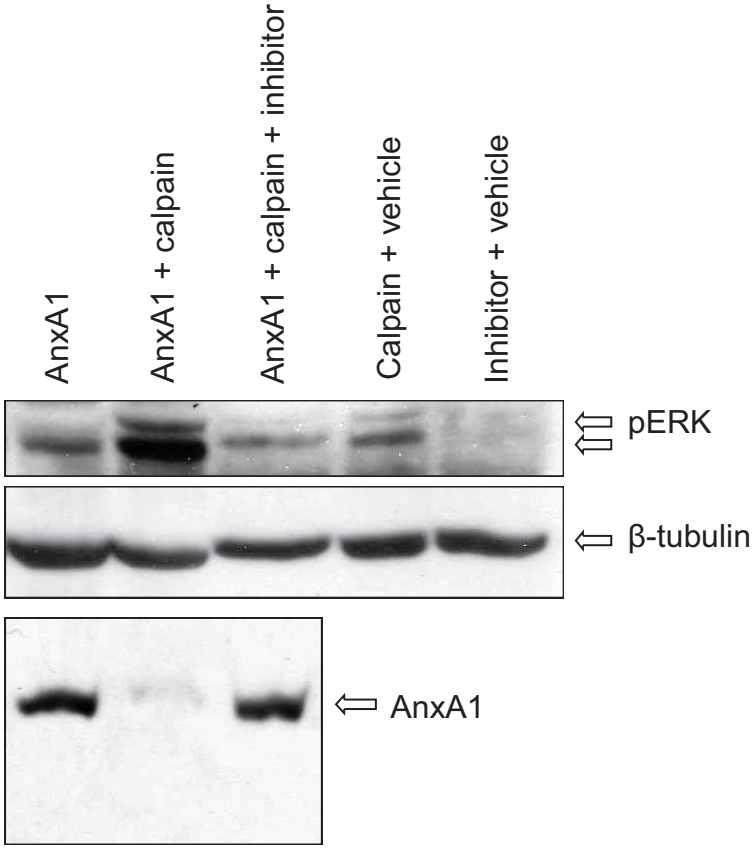


Figure 4.13: Calpain-cleaved recombinant annexin A1 activates endothelial ERK.

Upper panel- Resting HUVEC monolayers were treated with 20μg partially purified annexin A1 (AnxA1), 20μg calpain-cleaved purified annexin A1 (AnxA1 + calpain), 20μg purified annexin A1 with calpain inhibitor included during calpain treatment (AnxA1 + calpain + inhibitor), vehicle control with calpain (Calpain + vehicle), vehicle control with inhibitor (Inhibitor + vehicle), for 10 min. HUVEC ERK activation was determined by western blotting lysates with an Ab to phosphorylated ERK.
Middle panel- The membrane reprobed with β-tubulin mAb.
Lower panel– Samples tested on HUVEC were analysed for annexin A1 content by western blotting with an Ab to annexin A1.

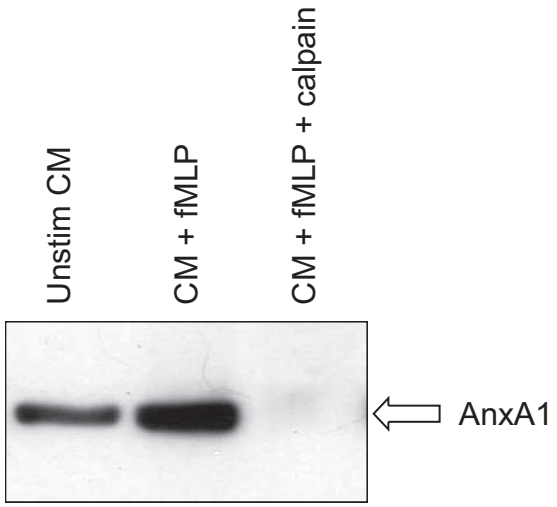


Figure 4.14: Annexin A1 content in unstimulated, fMLP-stimulated and calpain-treated fMLP-stimulated neutrophil conditioned medium.

Conditioned medium from vehicle-treated neutrophil conditioned medium, fMLP-stimulated neutrophil conditioned medium and calpain-treated fMLP-stimulated neutrophil conditioned medium were analysed for annexin A1 content by western blotting with an Ab to annexin A1.

stimulated in the presence of the calpain inhibitor could not activate ERK to the same extent as when the inhibitor was added to HUVEC but not neutrophils during the stimulation (Figure 4.15). This highlights that the effect of the inhibitor was due to the inhibition of neutrophil calpain I, not endothelial calpain I.

4.11. Analysis of peptides from mass spectrometry

Following the observation that calpain cleavage was essential for generating the endothelial ERK-activating factor and the lack of success with obtaining an N-terminal sequence, mass spectrometry data of the active annexin A1 found in neutrophil-conditioned medium was returned to in an attempt to locate the calpain cleavage site. Tryptic digestion of full-length annexin A1 would yield the peptides shown in Figure 4.16, however there was no evidence of peptides 1 (residues 1-9) and 2 (residues 10-26) being present in neutrophil conditioned medium. Peptide 3 (residues 27-29) of which the C-terminal end sits adjacent to the calpain-cleavage site, was also not detected, possibly due to its size and was probably not retained on the reverse-phase column. Peptide 4 (residues 30-53) was strongly detected, as were many of the following C-terminal peptides. This data suggested that the previously reported calpain cleavage site between Lys-26 and Ser-27 could potentially be the site recognised by calpain to generate 33kDa annexin A1 present in fMLP-stimulated neutrophil conditioned medium. A peptide with an N-terminus beginning at Ser-37, which follows Val-36 in the intact protein, was also found in the mass spectrometry data (Figure 4.16). Tryptic digestion could not have generated this peptide, as trypsin specifically cleaves after lysine or arginine residues, which therefore suggests that this peptide was generated by an additional protease, most likely neutrophil elastase (see Discussion).

4.12. Annexin A1 N-terminal peptide, Ac2-26, does not activate endothelial ERK

In numerous studies investigating annexin A1 function both *in vitro* and *in vivo*, the N-terminal annexin A1 peptide Ac2-26 has been used as it mimics the activity of full length annexin A1 [211, 223]. This peptide was synthesised and different concentrations were tested on HUVEC, but could not activate ERK in the same manner as neutrophil-conditioned medium containing truncated annexin A1 (Figures 4.17 and 4.18). To determine if Ac2-26 had a potential inhibitory effect on endothelial ERK, the annexin A1 N-terminal peptide was added with neutrophil conditioned medium to HUVECs, but did not affect ERK activation (Figure 4.18). This

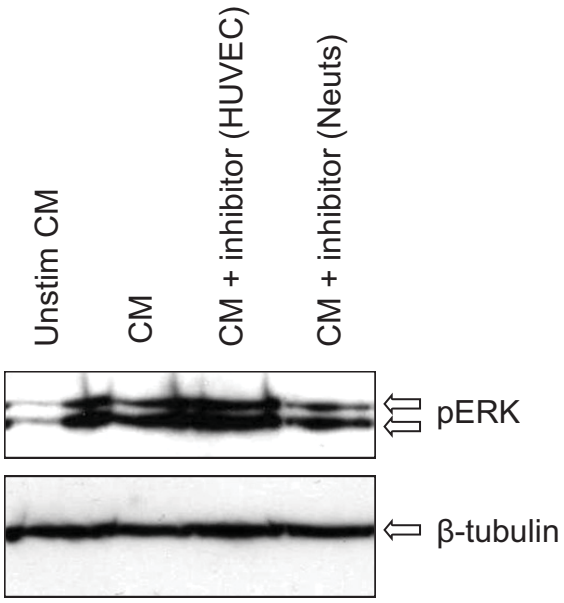


Figure 4.15: Calpain inhibitor prevents neutrophil secretion of an ERK-activating product.

Upper panel- Resting HUVEC monolayers were treated with unstimulated neutrophil conditioned medium (Unstimulated CM), fMLP-stimulated neutrophil conditioned medium (CM) and conditioned medium from neutrophils that were pre-incubated with calpain inhibitor XII for 10 min before fMLP stimulation (CM + inhibitor (neuts)). HUVEC were also treated with the calpain inhibitor for 10 min, then with fMLP-stimulated neutrophil conditioned medium (CM + inhibitor (HUVEC)). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane reprobed with a β-tubulin mAb.

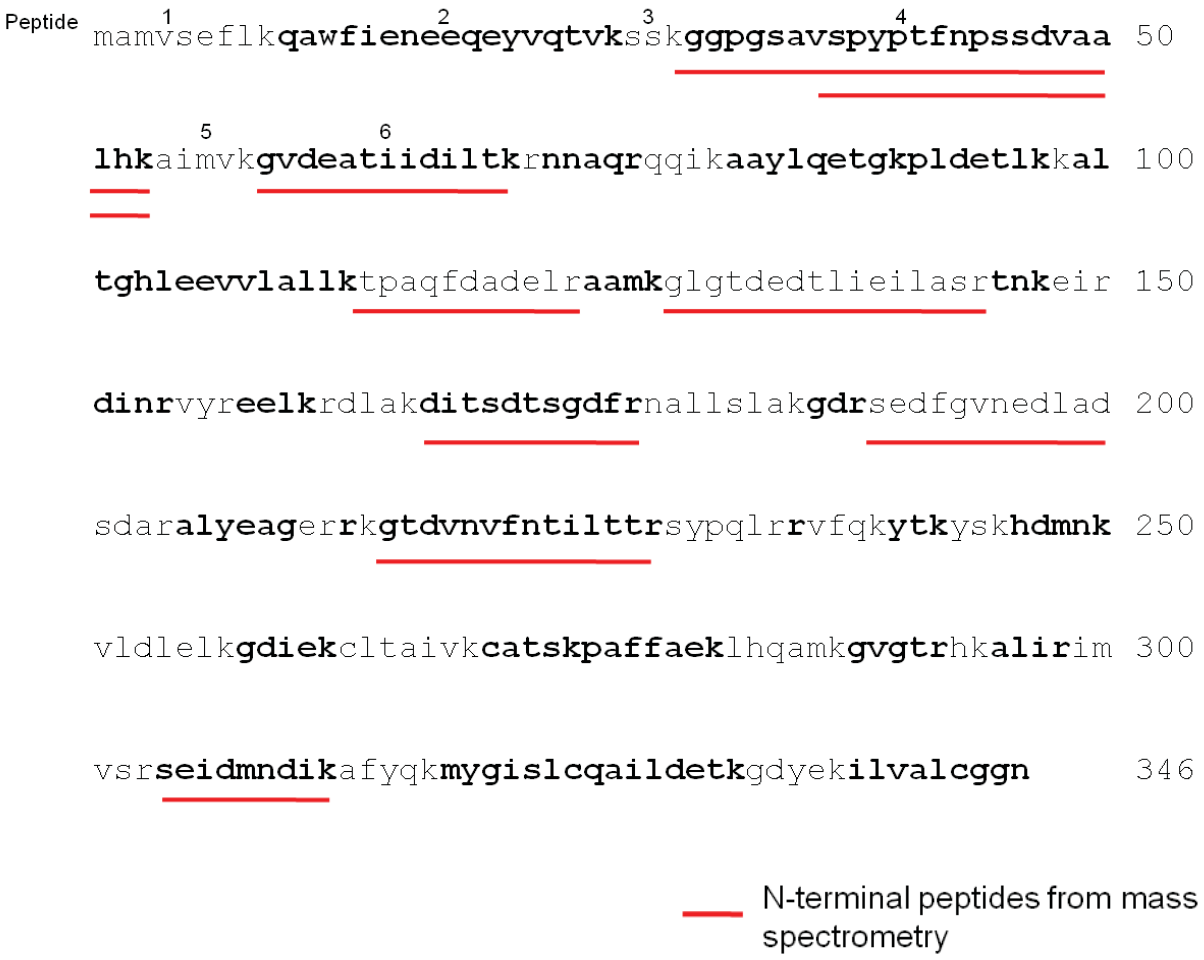


Figure 4.16: Annexin A1 peptides identified by mass spectrometry.

Mass spectrometry data returned for annexin A1-containing band 2, Figure 3.6. Alternating bold sequences indicate theoretical peptides that would be generated following tryptic digestion of full length annexin A1. Peptides underlined in red were detected during mass spectrometry analysis.

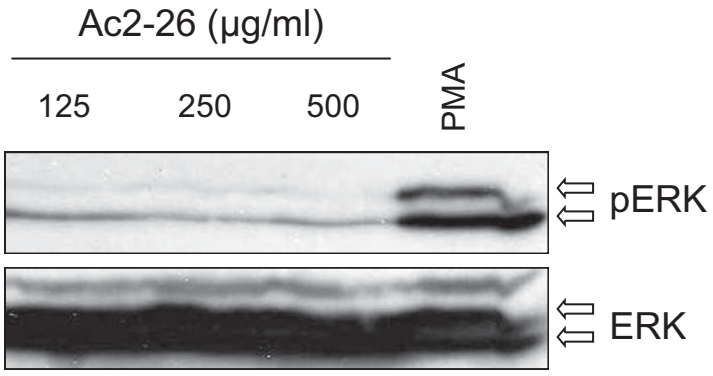


Figure 4.17: Annexin A1 N-terminal peptide, Ac2-26, does not activate endothelial ERK.

Upper panel- Resting HUVEC monolayers were treated with 125, 250 and 500µg/ml Ac2-26. HUVEC were also treated with PMA as a positive control. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Lower panel- The membrane probed with anti-total ERK after stripping.

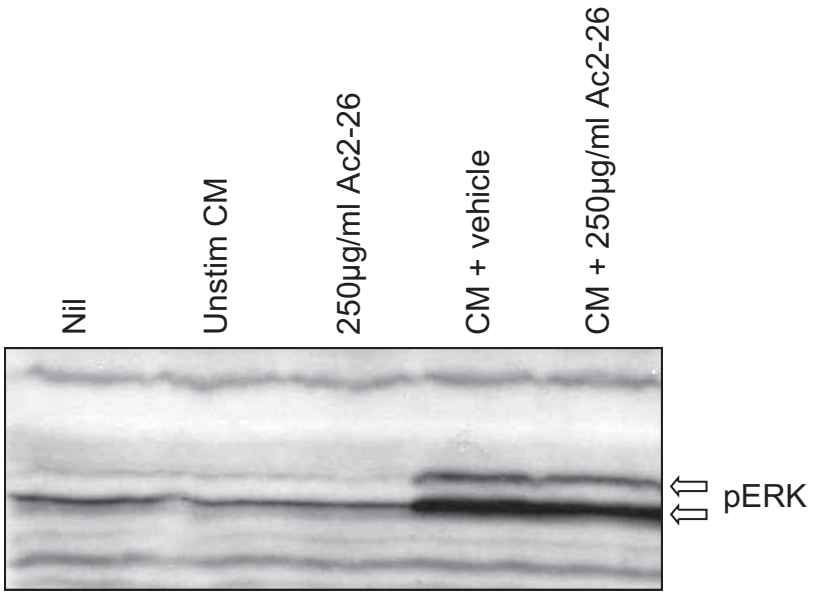


Figure 4.18: Annexin A1 N-terminal peptide, Ac2-26, does not activate endothelial ERK alone and cannot inhibit ERK activation induced by fMLP-stimulated neutrophil conditioned medium.

Resting HUVEC monolayers were treated with PBS (Nil), unstimulated neutrophil conditioned medium (Unstim CM), 250µg/ml Ac2-26, fMLP-stimulated neutrophil conditioned medium with Ac2-26 vehicle (CM + vehicle) and fMLP-stimulated neutrophil conditioned medium with 250µg/ml Ac2-26 (CM + 250µg/ml Ac2-26). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.
Equal amounts of total protein were loaded in each lane but the blot was not reprobed to verify this, however the intensity of non-specific bands indicate even loading.

confirmed that the N-terminal portion of annexin A1, proximal to the calpain cleavage site, did not possess the capacity to activate ERK like the C-terminal calpain cleavage product, nor could it inhibit ERK activation induced by annexin A1 present in neutrophil conditioned medium.

DISCUSSION

This chapter identified the pro-inflammatory, ERK-activating protein of interest present in fMLP-stimulated neutrophil conditioned medium as annexin A1. Both annexin A1 monoclonal and polyclonal Abs had the ability to inhibit ERK-activation induced by neutrophil conditioned medium (Figures 4.1 and 4.2), presumably by binding to annexin A1 and preventing it from interacting with its endothelial receptor. In addition, when annexin A1 was removed from conditioned medium by immunoprecipitation, the depleted conditioned medium could no longer activate endothelial ERK, which further confirmed that annexin A1 was the soluble ERK-activating protein. The Ab behaved similarly in an *in vitro* model of neutrophil transmigration, where it significantly inhibited transmigration in response to fMLP and across activated endothelial monolayers (Figures 4.4 and 4.5). Cumulatively, these results suggested that following the secretion of annexin A1 from stimulated neutrophils, it functions in a pro-inflammatory manner by activating endothelial ERK to promote transmigration.

In direct contrast, the current literature pertaining to annexin A1 describes it as an anti-inflammatory protein with a well-established role in mediating the effects of glucocorticoids [224]. Exogenously administered annexin A1 and peptides derived from the N-terminal region display strong anti-inflammatory effects both *in vitro* [225] and *in vivo* in several models of acute inflammation [215, 226-229], through an inhibitory effect on neutrophil recruitment [211, 230] and interference with neutrophil adhesion to the endothelium [216, 231]. In light of the data in these studies, the initial results observed showing an inhibitory effect of the annexin A1 mAb on endothelial ERK activation and neutrophil transmigration were regarded with caution. To demonstrate unequivocally that annexin A1 could activate endothelial ERK, the activity of exogenously administered annexin A1 on HUVEC needed to be evaluated. It was therefore resolved that annexin A1 would be over-expressed and purified using established protocols to address this issue. As human annexin A1 is glycosylated *in vivo* [232], its over-expression was performed in a mammalian cell line rather than in bacteria, so this and other potentially relevant post-translational modifications would be present on the recombinant protein. Expression in mammalian cells also circumvented potential issues of LPS contamination of purified protein, as HUVEC are particularly reactive to LPS and could become artificially activated if LPS was present in the final protein preparation [233, 234]. Initial experiments showed that the addition of

purified annexin A1 could increase phospho-ERK levels in HUVEC (Figure 3.18), however further investigation found that this was not due to full length annexin A1. Recombinant annexin A1 purified from mammalian HEK293 cells was generally comprised of two forms present in the active fraction: the 37kDa full length protein and a smaller molecular weight form of approximately 33kDa. The presence of this smaller molecular weight form was not surprising as it has been widely observed in the literature, but investigations into its biological function have been limited to calcium requirements for membrane binding [194, 235]. Following the separation of the two forms, it was found that full length annexin A1 had no effect on endothelial ERK, whereas the smaller molecular weight form alone was sufficient to induce ERK-activation (Figure 4.11). A question still remained however, regarding the origin of the truncated protein. As annexin A1 can be glycosylated *in vivo*, the two annexin A1 bands observed could reflect differences in glycosylation of the protein, thus providing an explanation as to why annexin A1 species of different molecular weights were observed. It was also possible that the truncated form of annexin A1 was the result of proteolytic activity. It is well established that annexin A1 possesses signals within the N-terminal domain that target the region for post-translational modification [236-238] and proteolysis [239, 240]. This occurs in many cell types, including neutrophils where phosphorylation, proteolysis and then secretion of annexin A1 results after activation [200, 202]. This suggested that that the smaller molecular weight form of annexin A1 observed could potentially be N-terminally truncated due to proteolysis.

To identify the protease responsible for cleaving annexin A1 into its ERK-activating form, N-terminal sequencing of the annexin A1 cleavage product was attempted, however a sequence could not be obtained. Truncated annexin A1 was prepared for N-terminal sequencing in two ways. Purified recombinant annexin A1 from HEK293s was initially used (the preparation shown in Figure 4.9B, which contained a higher proportion of cleaved annexin A1), despite full length protein being present. Full length annexin A1 is N-terminally blocked due to an acetylation modification [241] and will therefore not generate a signal to interfere with data obtained from N-terminal sequencing truncated annexin A1. As a signal could not be obtained using this method, fMLP-stimulated neutrophil conditioned medium was fractionated using gel filtration chromatography, then the ERK-activating fraction separated by SDS-PAGE and transferred to PVDF membrane. The annexin A1 band was excised, extracted from the membrane and subjected to N-terminal sequencing, however this method also failed to yield a sequence. The effectiveness of the N-terminal sequencing reaction was questioned, as both reactions also failed to identify the species of annexin A1 cleaved between Val-36 and Ser-37 by neutrophil elastase

[242], which was known to be present in the annexin A1 band from the original mass spectrometry data where annexin A1 was initially identified as a possible target (Figure 4.16). It was therefore concluded that the problem probably resided in the N-terminal sequencing rather than the protein preparation itself. Due to the lack of availability of an alternative sequencing service, another method was required to identify the N-terminus of ERK-activating annexin A1.

A number of proteases have been shown to cleave annexin A1 in the N-terminal domain: cathepsin D [222], calpain I [222], plasmin [222], neutrophil elastase [242, 243], proteinase 3 [244] and an unidentified matrix metalloprotease [245]. As the ERK-activating fractions contained a form of annexin A1 of approximately 33kDa (Figure 4.7B), cleavage sites located within the first 30 amino acids were investigated, with a particular focus on cathepsin D, calpain I and plasmin. Calpain I, which has a recognition site after residue 26 in annexin A1, was first tested to ascertain whether it could generate an annexin A1 cleavage product capable of activating ERK in endothelial cells. The reasoning behind this choice was the widely accepted fact that an increase in cytoplasmic calcium is required for signal transduction events involved in the neutrophil response to inflammatory stimuli [246, 247] and calpain I is a calcium-dependent neutral protease present in neutrophils that is activated following fMLP stimulation [248]. This is in contrast to cathepsin D and plasmin, which do not require calcium for proteolytic activity. Additionally it has been shown that annexin A1 cleavage in activated neutrophils strictly requires calcium [249]. It was found that the incubation of HUVEC with full-length recombinant annexin A1 cleaved with calpain could increase phosphorylated ERK levels (Figures 4.12 and 4.13). ERK activation induced by annexin A1 cleaved by calpain for 60 minutes appeared diminished compared to the other time points, probably due to inactivation of calpain I. The aspartic protease cathepsin D and the serine protease plasmin are also documented as being able to cleave annexin A1 in the N-terminal domain [222]. These were also tested and were not able to generate annexin A1 cleavage products capable of activating ERK in endothelial cells (Appendix 3). Furthermore, the calpain inhibitor was able to completely inhibit the generation of the active form of annexin A1, suggesting that other proteases were not involved in this process. This suggested that the calpain cleavage of annexin A1 in neutrophils is a specific processing event required to confer the ability to activate endothelial ERK.

ERK activation induced by the annexin A1/calpain mixture was presumably due to the formation of an annexin A1 cleavage product; however the presence of a processed form of annexin A1 in the reaction mixture could not be verified by western blotting (Figures 4.12B and 4.13). This was

intriguing, as two lines of evidence indicated that that calpain I converted inactive full length annexin A1 into an active form, namely; the ability of exogenous calpain I to generate ERK-activating annexin A1 and the loss of secretion of active annexin A1 from neutrophils when pre-treated with a calpain inhibitor prior to fMLP stimulation (Figure 4.15). Together this suggested that a calpain cleavage product in the neutrophil conditioned medium was responsible for the ERK activation observed. As the recombinant annexin A1 data convincingly showed that it could activate endothelial ERK, the problem had to lie in the detection of the annexin A1 cleavage product. When annexin A1 was cleaved endogenously, either in neutrophils or HEK293 cells, the cleavage product could be observed by both western blotting and by coomassie staining if the preparation was large enough (Figures 4.7 and 4.9). However when the protein was cleaved *in vitro* with exogenously applied calpain I, the annexin A1 was probably processed to a point where no full-length protein was remaining, due to the addition of excessive amounts of protease. There is the possibility that an additional calpain cleavage site is located elsewhere on the protein, so that following an initial calpain I cleavage event to produce a transient ERK-activating product, annexin A1 is then cleaved again and results in the epitope recognised by the antibody being removed. Indeed if annexin A1 is behaving in a similar manner to other chemokines, such as TNF-α and IL-8 [160], only minute amounts are required to achieve an effect and would not be detectable by western blotting. As an exact calpain I recognition sequence is not known [250], annexin A1 could not be screened for additional calpain I cleavage sites. To test this hypothesis however, fMLP-stimulated neutrophil conditioned medium was cleaved with calpain I, which resulted in the loss of the annexin A1 band recognised by the antibody following western blotting (Figure 4.14). This suggested that an additional calpain cleavage site is located within the annexin A1 sequence, which may be responsible for the difficulty in detecting the ERK-activating calpain cleavage product generated *in vitro*. To support this theory, annexin A1 treated with calpain I should have been analysed by silver staining to identify all cleavage products.

In an effort to verify the N-terminus of ERK-activating annexin A1, mass spectrometry data that originally identified annexin A1 in neutrophil conditioned medium was reanalysed (Figure 4.16). Aligning annexin A1 peptides from the mass spectrometry analysis to the full annexin A1 sequence highlighted a number of features. Significantly, no N-terminal peptides corresponding to the first 29 amino acids were returned. This was not surprising for peptide 3, which would not be retained on the reverse phase column due its small size. However peptides 1 and 2 (notably, calpain I cleaves immediately after the last amino acid of peptide 2) did not display characteristics that would prevent them from being ionised. A number of peptides at the C-

terminus were also not returned for reason pertaining to the properties of these peptides, with the exception of one. The C-terminal peptide ILVACGGN would only be singly charged during the analysis and was therefore not subjected to automated fragmentation to give internal sequence information, as a peptide cannot be confidently identified from a singly charged ion. Other peptides from the C-terminus, AFYQK and GDYEK would also not have been detected in the analysis due to their size. Therefore the only remaining C-terminal peptide that was unaccounted for was MYGISLCQAILDETK, for which there appears to be no explanation. An additional peptide containing an N-terminus of Ser-37 was also identified (Figure 4.16), which could not have been generated through tryptic digestion and indicates processing by another protease. Interestingly, a truncated form of annexin A1 with Ser-37 has also been identified in bronchoalveolar lavage fluid from cystic fibrosis patients [242], which was attributed to neutrophil elastase activity. However because another peptide was returned aligning to the sequence preceding Ser-37, it was improbable that this was the N-terminus of ERK-activating annexin A1. Whilst calpain cleavage from the C-terminus cannot be ruled out, proteolysis after residue 26 appears most likely given the extrusion of the N-terminal domain of annexin A1 following calcium binding [251] and that the two N-terminal peptides preceding the calpain recognition site were not returned in the analysis.

Ac2-26 is an acetylated annexin A1 peptide that has been shown to mimic the anti-inflammatory activity of the full length protein [215], thus it has been used extensively to investigate the effects of annexin A1 *in vitro* and *in vivo* [223, 225, 252, 253]. Conveniently, the Ac2-26 peptide also corresponds to the N-terminal portion of annexin up to the calpain cleavage site, where calpain cleavage occurs between residues 26 and 27. This provided the perfect means to test whether the N-terminal portion of annexin A1 possessed the capacity to activate endothelial ERK in a similar manner to calpain-cleaved annexin A1. Like full length annexin A1, Ac2-26 was unable to activate ERK in HUVEC, even when an excess of peptide was added (Figure 4.17). Therefore despite reports showing that Ac2-26 possesses comparable anti-inflammatory activity to full length annexin A1, the peptide failed to mimic the pro-inflammatory properties of calpain-truncated annexin A1. This presents an interesting situation however, where annexin A1 appears to mediate both pro- and anti-inflammatory signals in the context of neutrophil extravasation. The area responsible for the anti-inflammatory activity displayed by annexin N-terminal peptides has been narrowed down to a stretch of residues ranging from 19-26 [254]. Cleavage with calpain I would remove this sequence from annexin A1, therefore abolishing the anti-inflammatory effect from the remaining C-terminus of the protein. To determine if the N- and C-terminal portions of

annexin A1 were competing for the same endothelial receptor to give opposing effects, Ac2-26 was incubated on HUVEC with neutrophil conditioned medium to assess whether the peptide could inhibit ERK activation. Ac2-26 had no inhibitory effect, suggesting that the two annexin A1 entities were signalling through different endothelial receptors, which was interesting given the findings described in section 5.2. This set of experiments highlighted that calpain cleavage of annexin A1 was a specific processing event required to generate an ERK-activating product and that the source of the activity resided in the C-terminal core of the protein.

It has been shown that glucocorticoid treatment results in annexin A1 upregulation in many cells. To determine if glucocorticoids could stimulate the secretion of annexin A1 from neutrophils, neutrophils were stimulated with dexamethasone and the resulting conditioned medium was assayed on HUVEC. The effect of dexamethasone-stimulated neutrophil conditioned medium on ERK activity could not be effectively analysed however, as it was found that dexamethasone alone could inhibit phospho-ERK levels in HUVEC (Appendix 4). The inhibitory effect of dexamethasone was particularly obvious in this line of HUVEC, as it displayed an elevated level of basal ERK phosphorylation which dexamethasone could effectively reduce. The mechanism behind this inhibition was unclear, especially since a recent study has shown the exact opposite in that dexamethasone treatment increased phospho-ERK levels in HUVEC, although this was following a 60 minute incubation period and therefore may be involved in the activation of alternate signalling cascades [255].

An interesting difference observed between this study and published data is that immunoneutralisation of annexin A1 with annexin A1 Abs has been shown to exacerbate inflammation and reverse the protective anti-inflammatory effects afforded by glucocorticoids [212, 224, 229, 230]. However in our static model of neutrophil transmigration the annexin A1 Ab essentially inhibited all transmigration across HUVEC. It is possible that the Ab was inhibiting transmigration in two ways: 1) by binding to truncated annexin A1 released from neutrophils to prevent ERK-activation in HUVEC and 2) by binding to annexin A1 present on the neutrophil surface to prevent transmigration by posing as a steric hindrance. The effectiveness of annexin A1 Abs in modulating neutrophil extravasation would presumably hinge on the epitope that is recognised by the Ab, whether it is specific to the full length protein, truncated annexin A1 or the N-terminal domain. Many of the studies showing the exacerbation of inflammation following passive immunisation with annexin A1 Abs used antiserum to the N-terminal peptide only [229, 230], which would not recognise the pro-inflammatory truncated form of annexin A1.

These experiments would serve to remove full length annexin A1 and leave the pro-inflammatory truncated form still functioning, which explains the increased inflammatory activity, particularly if full length annexin A1 plays a role in resolving inflammatory events. This is important to keep in mind when reviewing data from experiments where annexin A1 expression has been investigated under inflammatory conditions, as the Abs used to detect annexin A1 may only recognise certain forms of the protein and not others.

SUMMARY

Annexin A1 was identified as a component of stimulated neutrophil conditioned medium that could activate endothelial ERK. To confirm if this was the protein responsible, annexin A1 Abs were added to neutrophil conditioned medium to determine if they could immunoneutralise annexin A1 and abolish the ERK-activating effect. The Abs inhibited ERK-activation induced by neutrophil conditioned medium and additionally inhibited neutrophil transmigration towards a chemoattractant and across activated endothelial monolayers in an *in vitro* model of neutrophil transmigration. Although this provided compelling evidence that the soluble neutrophil protein was annexin A1, the vast majority of literature reporting the involvement of annexin A1 in inflammation describes it as being anti-inflammatory. The hypothesis that annexin A1 was the endothelial ERK-activating protein was further confirmed when recombinant annexin A1 was produced and was found to increase phospho-ERK levels in HUVEC. Intriguingly, the purification of recombinant annexin A1 resulted in two forms being produced; full length annexin A1 and a smaller molecular weight form of approximately 33 kDa. This shorter annexin A1 product has been described as biologically inactive in the literature, however when its activity was compared to full length annexin A1, the smaller form was responsible for inducing ERK-activation and the full length protein had no effect. As a number of protease recognition sites are located in the N-terminal domain of annexin A1, this was pursued as the likely mode of annexin A1 truncation. Calpain I, a calcium dependent protease that is activated upon neutrophil stimulation with chemoattractant and has also been shown to cleave annexin A1 after Lys-26, was able to modify full length inactive recombinant annexin A1 into an unidentified product that possessed the capacity to activate endothelial ERK. A calpain I inhibitor was also found to inhibit neutrophils from secreting an ERK-activating protein following stimulation with chemoattractant, further suggesting that calpain I is involved in the release of pro-inflammatory annexin A1 from neutrophils.

This chapter describes a previously unrecognised, pro-inflammatory function for annexin A1 and suggests that a truncated form, most likely generated following calpain I cleavage, is responsible for the pro-inflammatory activity observed. Additional experiments need to be performed to confirm this hypothesis.

Chapter 5

EFFECTS OF ANNEXIN A1 ON THE ENDOTHELIUM.

INTRODUCTION

Experiments carried out up to this point were focussed on characterising annexin A1 as the soluble neutrophil factor and the features involved in its generation. The direct effects of endothelial ERK activation induced by annexin A1 and how this modulated neutrophil transendothelial migration remained unclear however, so experiments were directed towards addressing this aspect.

Until this point, the identity of the endothelial receptor for truncated annexin A1 had also remained elusive. The only known receptors for annexin A1 and Ac2-26 are the formyl peptide receptors (FPRs), a class of 7 transmembrane domain G-protein-coupled receptors [189, 223, 254]. To date, the FPR family consists of three proteins in humans: FPR, FPRL-1 (formyl peptide receptor like 1) and FPRL-2 (formyl peptide receptor like 2). FPR, the first described member of the family, has the ability to bind *N*-formylated peptides with high affinity and is the chemotactic receptor involved in neutrophil response to fMLP [256]. As the only known sources of *N*-formylated peptides are of bacterial or mitochondrial origin, this suggested a role for the receptor in host leukocyte recruitment in response to bacterial infection. FPRL-1 and FPRL-2 were later identified as related receptors through a low-stringency hybridisation screen using FPR cDNA, where FPRL-1 possesses 69% identity to FPR [257-259] and FPRL-2 shares 56% and 83% identity with FPR and FPRL-1 respectively [260, 261]. FPR and FPRL-1, but not FPRL-2, are expressed on neutrophils and endothelial cells [262]. It has been established that full length annexin A1 and Ac 2-26 are ligands for FPRL-1, and while Ac2-26 has been shown to bind FPR, the ability of full length annexin A1 to bind FPR is a matter of speculation [223, 252]. Interestingly annexin 1 has been implicated in binding to a carboxylated glycan receptor present on bovine lung, which appeared to be part of a complex with the pro-inflammatory proteins S100A8 and S100A9 [164]. However bovine annexin A1 bound to the carboxylated glycan receptor was found to be a truncated form devoid of the first 12 amino acids when aligned to human annexin A1 and annexin A1 derived from other species. As neutrophil transendothelial migration is a highly complex process involving many molecules, narrowing down the effect of endothelial ERK activation to a particular protein or process without knowing the endothelial receptor through which it was being activated would be difficult. It was anticipated that the identification of the endothelial receptor for truncated annexin A1 would assist in identifying the

downstream consequences of ERK activation, or at least limit the number of possibilities required to be investigated.

Although able to significantly attenuate the degree of neutrophil transmigration, endothelial ERK-inhibition has previously been shown to have no effect on β_2-integrin-mediated neutrophil adhesion to HUVEC monolayers [99]. This suggests that endothelial ERK activation is not necessary for the initial adhesion of neutrophils to endothelial cells, but may be important for the subsequent steps in transendothelial migration, such as ICAM-1 clustering for the formation of transmigratory cups or the actual diapedesis step. Following neutrophil adhesion, endothelial transmigratory cup structures enriched in ICAM-1 and VCAM-1 have been shown to form around transmigrating neutrophils and are engaged during both paracellular and transcellular transmigration [81, 83]. To determine if annexin A1 Abs could affect transmigratory cup formation during transcellular and paracellular neutrophil transmigration, an assay was devised that monitored the endothelial adhesion molecule ICAM-1 during this process.

This chapter provides evidence that truncated annexin A1, like the full length protein and Ac2-26, signals through the FPR family to activate ERK in HUVEC. Furthermore, it was found that neutrophil adhesion to HUVEC monolayers and subsequent transcellular migration was impaired following treatment with annexin A1 blocking Abs. This highlighted a novel role for annexin A1-induced ERK-activation in transmigratory cup formation during neutrophil extravasation.

RESULTS

5.1. fMLP-stimulated neutrophil conditioned medium does not increase endothelial monolayer permeability

It has been previously proposed that the isometric tension generated following endothelial cell activation by stimulated neutrophils leads to increased paracellular permeability, therefore facilitating leukocyte extravasation through the endothelial monolayer [148, 263]. To investigate the effect of fMLP-stimulated neutrophil conditioned medium on endothelial permeability, conditioned medium was added to confluent HUVEC monolayers and the permeability to FITC-conjugated dextran was measured after a 1 hour incubation period. It was found that there was no significant difference in the monolayer permeability between conditioned medium treated and vehicle alone treated cells (Figure 5.1). In contrast, thrombin, a known inducer of endothelial permeability [264], was used as a positive control and produced an elevated rate of FITC-dextran flux through the HUVEC monolayer. The ERK inhibitor U0126, which has been shown to block neutrophil transmigration by inhibiting endothelial ERK-activation [99], slightly decreased permeability when added alone but had no significant effect in the presence of neutrophil conditioned medium. Therefore neutrophil conditioned medium did not appear to alter the paracellular permeability of the endothelial monolayer.

5.2. Formyl peptide receptor antagonist inhibits ERK activation by neutrophil-conditioned medium

Annexin A1 has been shown to be a ligand of formyl peptide receptor like-1 (FPRL-1) [252]. Therefore a non-selective FPR antagonist, Boc2 (Boc-Phe-Leu-Phe-Leu-Phe), was employed to determine the effect of blocking this receptor family on endothelial ERK activation by neutrophil-conditioned medium. Treatment of HUVEC with Boc2 resulted in a dose-dependent inhibition of ERK activation induced by fMLP-stimulated neutrophil-conditioned medium, with 20μM antagonist markedly reducing ERK activation (Figure 5.2A). The vehicle alone could not inhibit ERK activation in the same manner, nor could Boc2 non-specifically inhibit ERK activation induced by PMA (Figure 5.2B). This data confirmed that truncated annexin A1

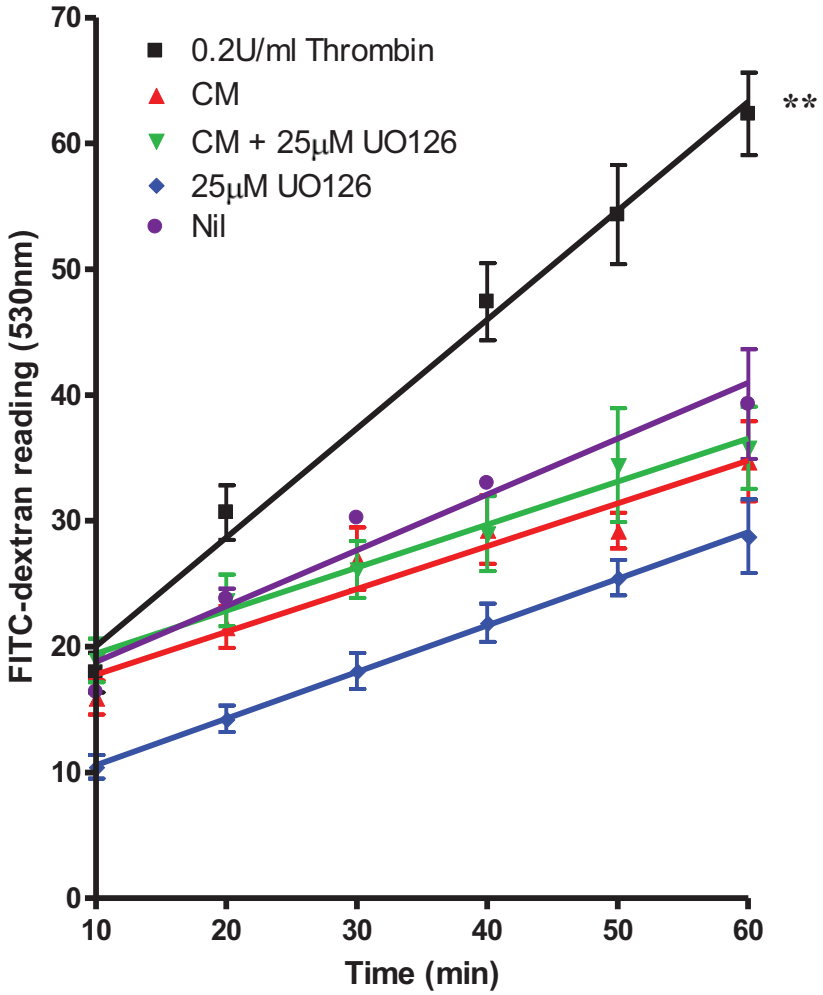


Figure 5.1: fMLP-stimulated neutrophil conditioned medium does not alter HUVEC monolayer permeability to FITC-conjugated dextran.

HUVEC monolayers grown in Transwells were treated with the indicated samples mixed with FITC-conjugated dextran in the apical chamber for 1h. FITC-conjugated dextran present in the basolateral chamber was sampled at 10 minute intervals for the duration of the experiment. Figure shows one representative experiment performed of a total of three, each in triplicate. Error bars indicate SEM. Lines of best fit were generated by linear regression analysis. ** $p<0.01$, one-way ANOVA, followed by Tukey's multiple comparison post test, comparing the Thrombin slope to each of the treatments. The slopes from other treatment groups were not significantly different.

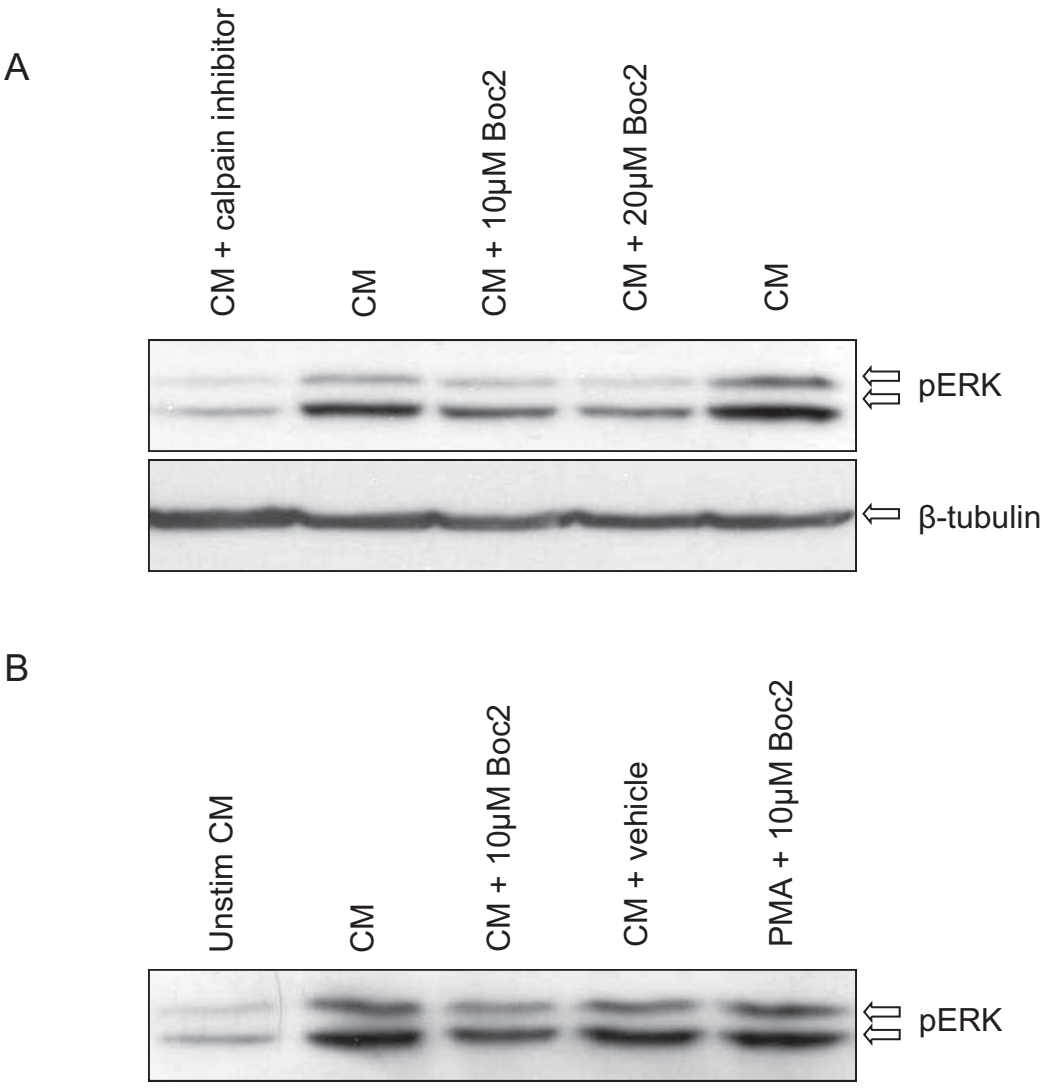


Figure 5.2: Pre-treatment of HUVEC with Boc2 inhibits ERK activation induced by fMLP-stimulated neutrophil conditioned medium.

A) ***Upper panel-*** Resting HUVEC monolayers were pre-treated with either 10 or 20 μg/ml Boc2 for 10 min as indicated. HUVEC were then treated with fMLP-stimulated neutrophil conditioned medium with calpain inhibitor (CM + calpain inhibitor) or fMLP-stimulated neutrophil conditioned medium (CM) for 10 min. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK. ***Lower panel-*** The membrane reprobed with β-tubulin Ab.
B) Resting HUVEC monolayers were pre-treated with 10 μg/ml Boc2 or a equivalent volume of 50% methanol vehicle control for 10 min as indicated. HUVEC were then treated with unstimulated neutrophil conditioned medium (Unstim CM), fMLP-stimulated neutrophil conditioned medium (CM) or PMA (0.25μg/ml) for 10 min.

released from fMLP-stimulated neutrophils was signalling through the FPR family on the endothelium to activate ERK and permit neutrophil transmigration.

5.3. Annexin A1 N-terminal peptide, Ac2-26, cannot inhibit neutrophil transendothelial migration *in vitro*

A number of reports have shown that annexin A1 and the N-terminal peptide, Ac2-26, exert their anti-inflammatory effects on neutrophils by signalling through FPRL-1 [252]. Given the results in section 5.2, the effect of the peptide on transmigration was evaluated by adding a titration of Ac2-26 to the *in vitro* model of neutrophil transmigration, which was driven by a 10nM fMLP chemoattractant gradient. The inclusion of the peptide did not significantly inhibit the level of neutrophil transmigration at any of the concentrations tested (Figure 5.3). The duration of the transmigration assay was reduced from 1 hour to 15 or 30 min (Figure 5.4), but Ac2-26 again did not affect neutrophil transmigration at these shorter time points. These results indicated that Ac2-26 could not significantly inhibit neutrophil transmigration in this experimental system.

5.4. Exogenous annexin A1 protein can reduce neutrophil transendothelial migration *in vitro*

Annexin A1 protein was also added with neutrophils to the transmigration assay to determine if transmigration could be increased by the addition of ERK-activating truncated annexin A1. Annexin A1 preparations amounting to 10 and 20 µg total protein were added with neutrophils to the apical chamber of the Transwells and 10nM fMLP was added to the basolateral chambers to establish a chemoattractant gradient to ensure neutrophil activation. After compiling the data from six experiments conducted in triplicate, the overall effect of adding exogenous annexin A1 to the assay was to decrease transmigration compared to the vehicle control (Figure 5.5). This indicated that the annexin A1 protein preparation that was added could interfere with neutrophil transmigration. This is in direct contrast to the results described in section 5.3 where the annexin A1 N-terminal peptide had no effect on neutrophil transmigration. The annexin A1 preparations used contained both full length and truncated forms of the protein, where Figure 4.9 shows one such preparation that was used. Given that truncated annexin A1 is the predominant form

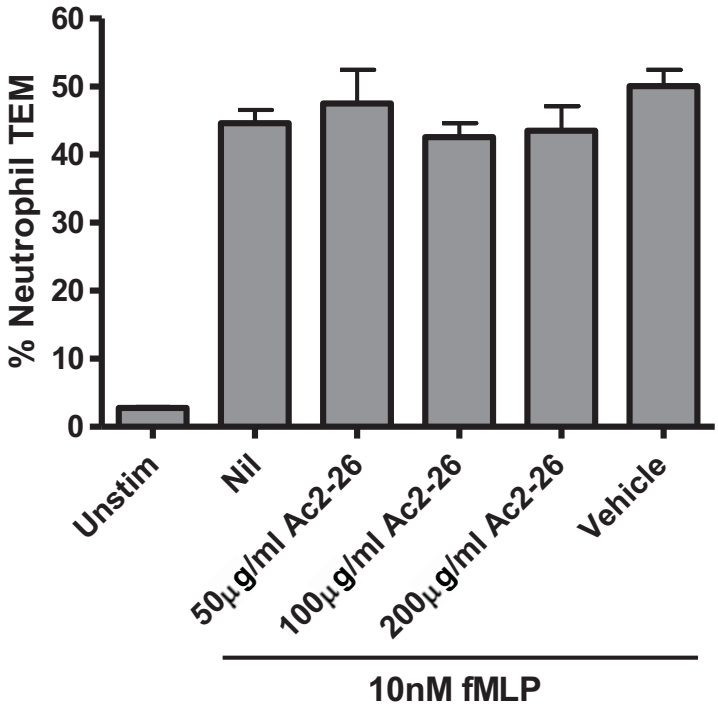


Figure 5.3: Annexin A1 N-terminal peptide, Ac2-26, does not affect neutrophil transendothelial migration.

HUVEC monolayers were established on Transwells as described and were either activated with TNF-α (4ng/ml) for 4h before conducting the assay or were untreated. 10nM fMLP was added to the basolateral chambers of the indicated samples. 5 x 10^5 neutrophils were added to the apical chamber with indicated concentrations of Ac2-26 or vehicle corresponding to amount added for 200μg/ml Ac2-26. Transmigration of neutrophils into the basolateral chamber of the Transwell was measured and expressed as a percentage of the total amount of neutrophils added. Experiment was performed twice in triplicate. Error bars indicate SEM.

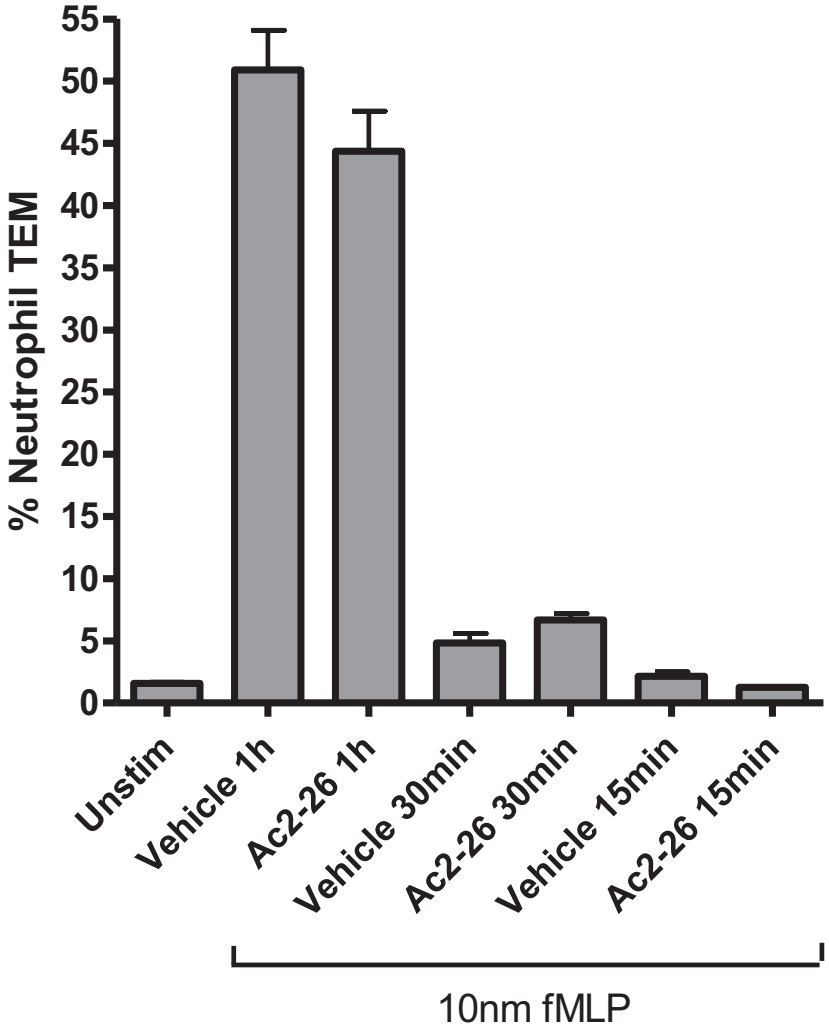


Figure 5.4: Annexin A1 N-terminal peptide, Ac2-26, does not affect neutrophil transendothelial migration.

HUVEC monolayers were established on Transwells as described and were activated with TNF-α (4ng/ml) for 4h before conducting the assay or were left untreated. 10nM fMLP was added to the basolateral chambers of the indicated samples. 5 x 10^5 neutrophils were added to the apical chamber with 150µg/ml Ac2-26 or equal volume of vehicle. Transmigration was allowed to proceed for the indicated time. Neutrophil transmigration into the basolateral chamber of the Transwell was measured and expressed as a percentage of the total amount of neutrophils added. Experiment was performed once in triplicate. Error bars indicate SEM.

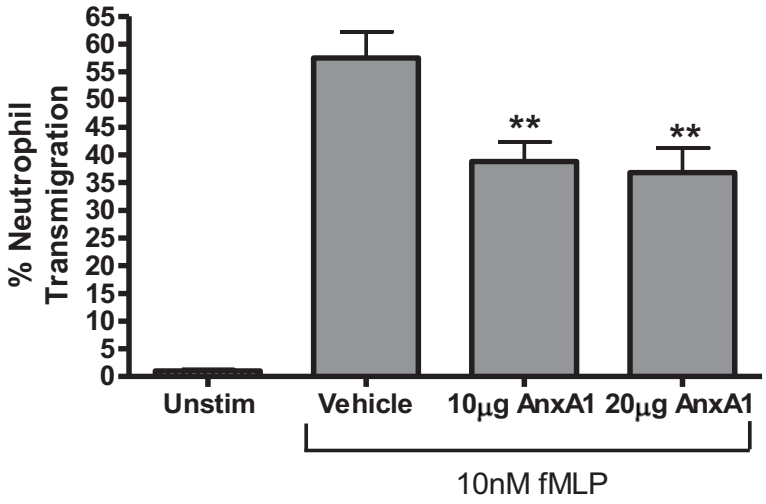


Figure 5.5: Effect of exogenously administered annexin A1 on neutrophil transendothelial migration *in-vitro*.

HUVEC monolayers were established on Transwells as described and were either activated with TNF-α (4ng/ml) for 4h before conducting the assay or were untreated. 10nM fMLP was added to the basolateral chambers of the indicated samples. 5 x 10^5 neutrophils were added to the apical chamber with the indicated concentrations of annexin A1 or equimolar vehicle corresponding to the amount added for 20μg/ml annexin A1. Neutrophil transmigration into the basolateral chamber of the Transwell was measured and expressed as a percentage of the total amount of neutrophils added. n= 6 for 10μg AnxA1 and n=4 for 20μg AnxA1, with each experiment conducted in triplicate. ** $p< 0.01$, one-way ANOVA with Bonferroni's Multiple Comparison Post-test (annexin A1 treatment groups compared to vehicle treatment). Error bars indicate SEM.

secreted by activated neutrophils, it is likely that the full length annexin A1 in the preparation is the form that is interfering with neutrophil transmigration.

5.5. Annexin A1 mAb interferes with neutrophil transcellular migration

As it has been previously shown that endothelial ERK activation was not required for β_2-integrin-mediated neutrophil adhesion to HUVEC monolayers [99], this suggested that molecules downstream of the adhesion phase were involved in ERK-mediated neutrophil transmigration. As the endothelial adhesion molecules ICAM-1 and VCAM-1 are components of transmigratory cups that have been shown to form around transmigrating neutrophils [81, 83], an assay was developed to visualise transmigratory cups during neutrophil transmigration by monitoring ICAM-1. This assay was used to measure the effect of the annexin A1 Ab on transmigratory cup formation.

5.5.1 Assay development

The assay was based on experiments performed by Carman *et al* [81], where these experiments primarily examined monocyte transmigration. Briefly, HUVEC monolayers grown on glass slides were activated with TNF-α to induce ICAM-1 and VCAM-1 upregulation. Neutrophils were stimulated with fMLP to activate adhesion molecules required for neutrophil-endothelial interactions. Stimulated neutrophils were added to activated HUVEC monolayers for a 2 min period, then non-adherent neutrophils were washed away. The HUVEC were then fixed and probed with in-house ICAM-1 or VCAM-1 mAbs [154] and an Alexa488-conjugated anti-mouse secondary Ab.

5.5.1.1 TNF-α stimulation

To ensure ICAM-1 and VCAM-1 were being sufficiently upregulated on the surface of HUVEC monolayers following TNF-α stimulation, a 6h time course was carried out (Figure 5.6). As shown in Figure 5.6, 4-6h stimulation was suitable as this showed an adequate level of ICAM-1 and VCAM-1 staining on the cell surface.

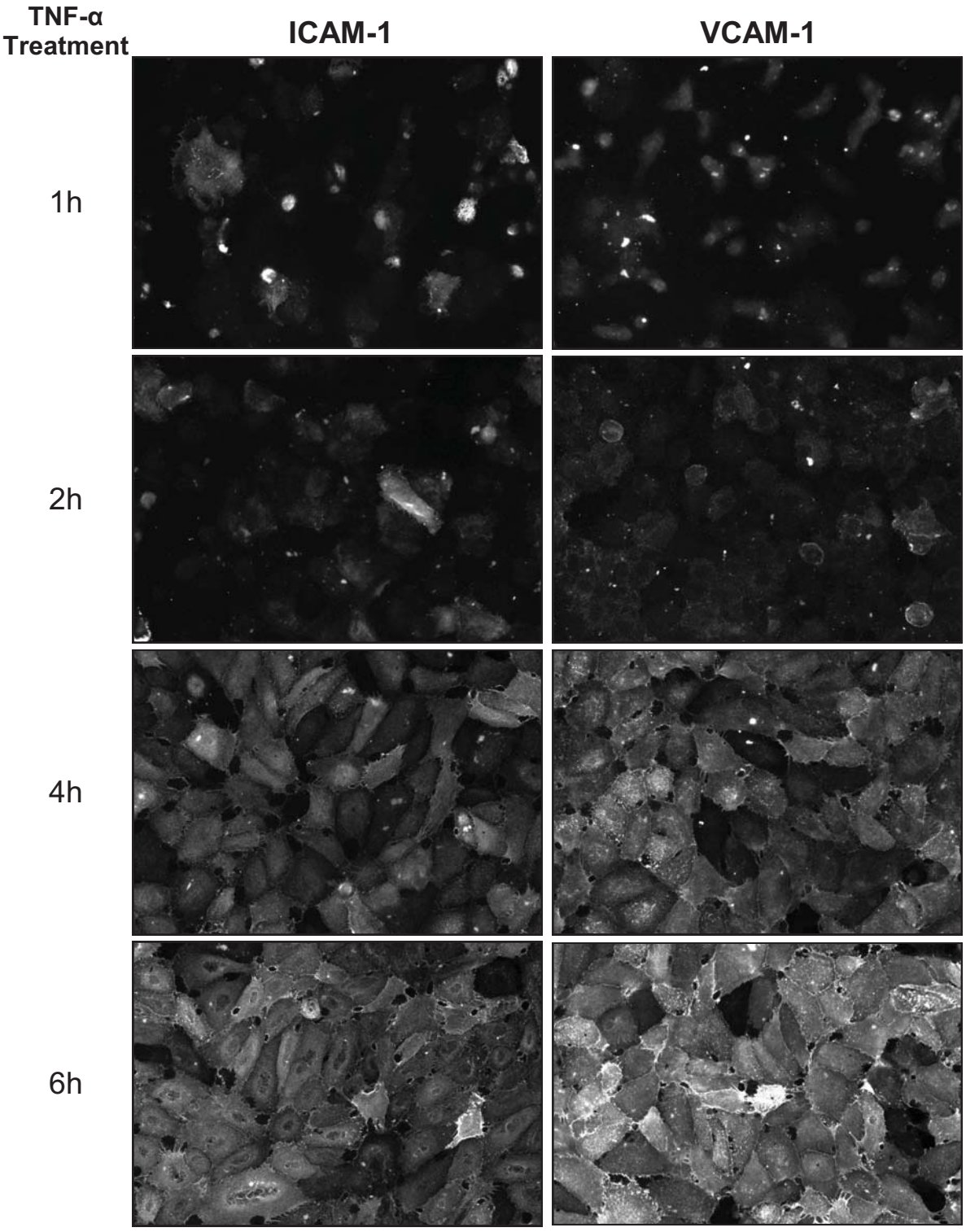


Figure 5.6: TNF-α induced ICAM-1 and VCAM-1 upregulation on HUVEC.

HUVEC monolayers were stimulated with TNF-α for 2, 4 or 6 hours, then washed, fixed and stained for either ICAM-1 or VCAM-1 and visualised by indirect immunofluorescence using Alexa488-conjugated anti-mouse secondary Ab. Magnification x200.

5.5.1.2 Neutrophil incubation with HUVEC monolayers

Following HUVEC activation with TNF-α, neutrophils were stimulated with 100nM fMLP for 2 min before being added to the HUVEC. In pilot experiments, stimulated neutrophils were incubated with HUVEC for a period of 30 min, as published results investigating monocyte transmigration had used incubation times varying between 10 min to 1 hour [81]. However it was found that the majority of neutrophils had migrated through the HUVEC monolayer by 30 min and were located between the HUVEC monolayer and the glass slide (data not shown). The remaining neutrophils present on the apical surface of the monolayer after 30 min did not display discernable ICAM-1 staining in the form of a transmigratory cup (data not shown). To determine an optimal neutrophil incubation time, stimulated neutrophils were added to activated HUVEC monolayers and monitored using real-time light microscopy. It was observed that a considerable portion of transmigration occurred 2-3 min after the addition of neutrophils to HUVEC, following a short crawling phase of approximately 15-30 seconds. To arrest neutrophil progression at a point where transmigratory cups were in the early stages of forming around the neutrophil (so ICAM-1 staining would be readily observable) neutrophils were incubated with HUVEC for 2 min, non-adherent neutrophils washed away, then the monolayer and attached neutrophils were immediately fixed. Slides were stained with ICAM-1 to visualise transmigratory cups, along with DAPI to emphasise areas of neutrophil adhesion to the monolayer. Following staining with an ICAM-1 mAb, rings of staining around the periphery of the neutrophils were detected and ICAM-1 enriched projections extended from the HUVEC to surround and encapsulate the transmigrating neutrophil (Figures 5.7, 5.8 and 5.9). DAPI was also used as an indicator of neutrophil progression, as the intensity of DAPI staining decreased as neutrophils penetrated further into the monolayer (Figure 5.8). The assay conditions were therefore considered suitable to further examine transmigratory cup formation during neutrophil transendothelial migration.

5.5.1.3 HUVEC monolayer integrity

At the commencement of each experiment, the integrity of HUVEC monolayers were examined and validated by light microscopy before use to ensure cell-cell contacts were well-formed and that the monolayers were confluent. HUVEC monolayer integrity was additionally confirmed following staining with $AgNO_3$ to visualise the HUVEC borders (Figure 5.10), where it was also

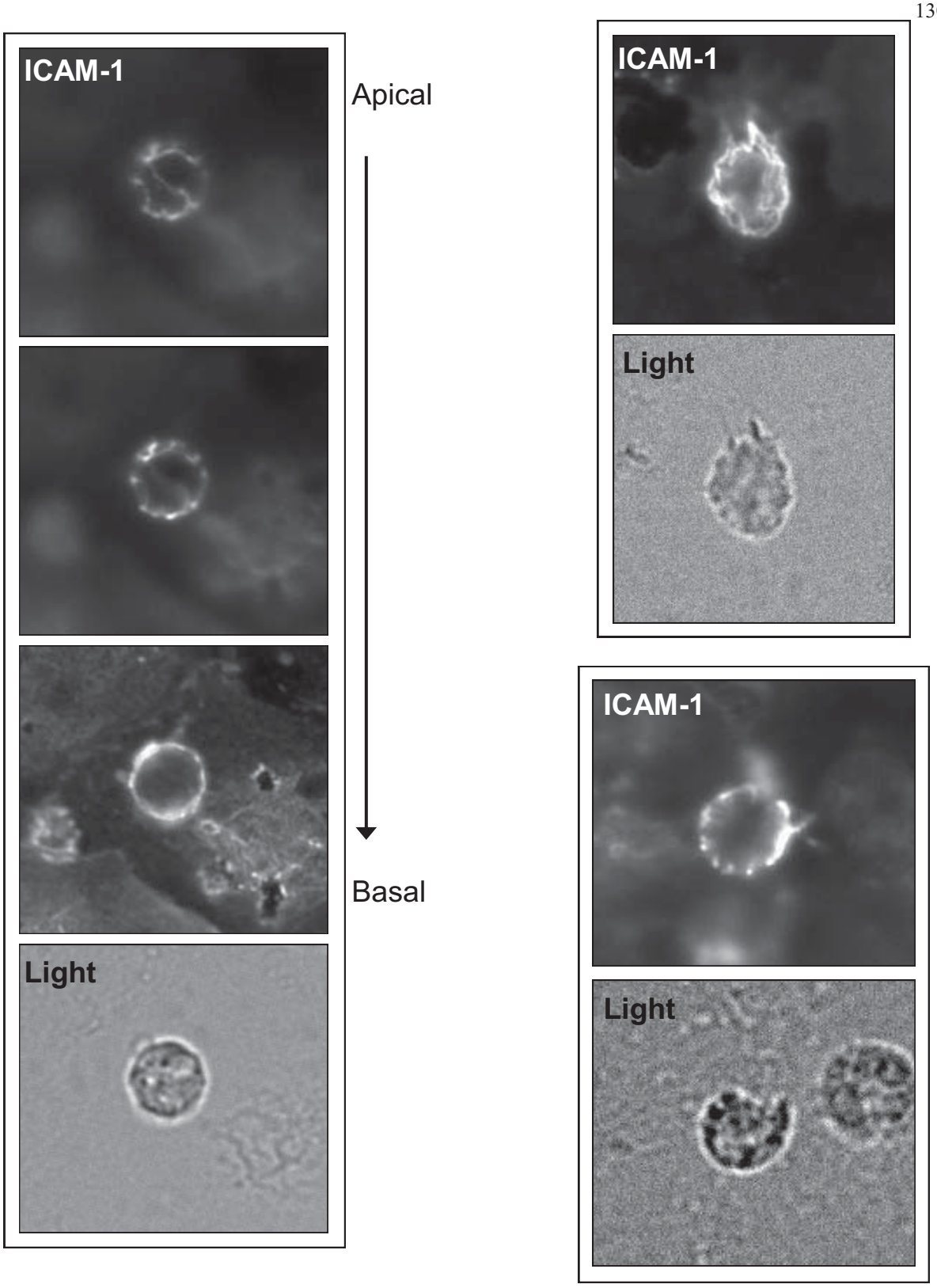


Figure 5.7: Visualisation of transmigratory cup formation around migrating neutrophils.

HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min. Non-adherent neutrophils were then washed away and the monolayers fixed. Transmigratory cups were visualised by staining for ICAM-1 and a corresponding bright field image was also taken to visualise the neutrophils. Magnification x 600.

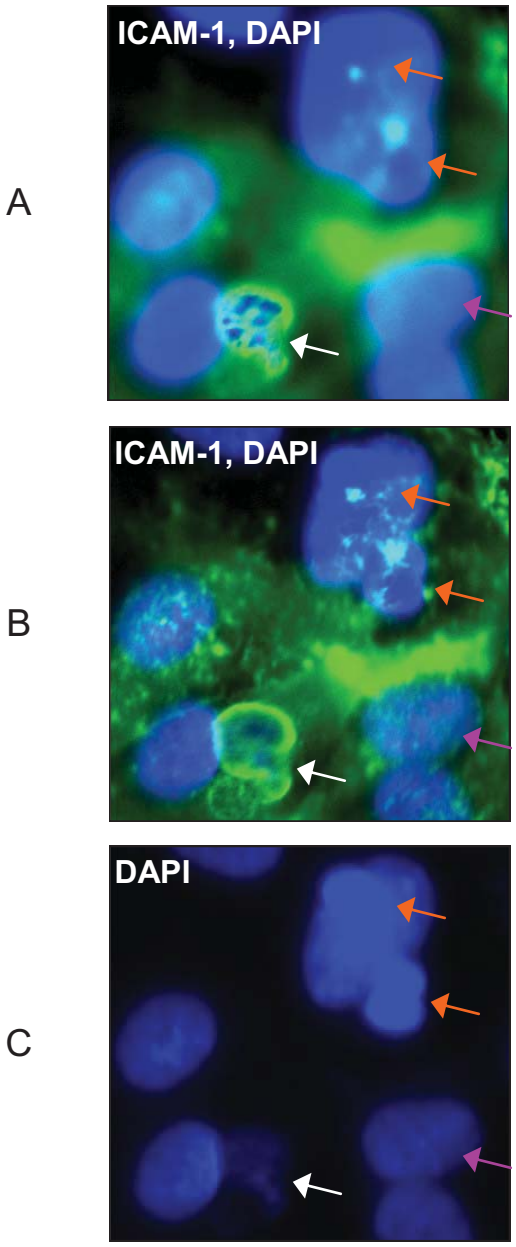


Figure 5.8: Visualisation of transmigratory cup formation around transmigrating neutrophils.

HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min. Non-adherent neutrophils were then washed away and the monolayers fixed. Transmigratory cups were visualised by staining for ICAM-1 (green) and both HUVEC and neutrophil cell nuclei were stained with DAPI (blue). A and B show a composite ICAM-1/DAPI image, with C showing DAPI staining alone. Image A is higher on the focal plane than B to highlight the neutrophil being encapsulated by endothelial ICAM-1-enriched projections (white arrow). The white arrow indicates a transmigrating neutrophil, orange arrows indicate neutrophils adherent to the apical surface of the HUVEC monolayer and the pink arrow shows an example of a HUVEC nucleus. Magnification x 600.

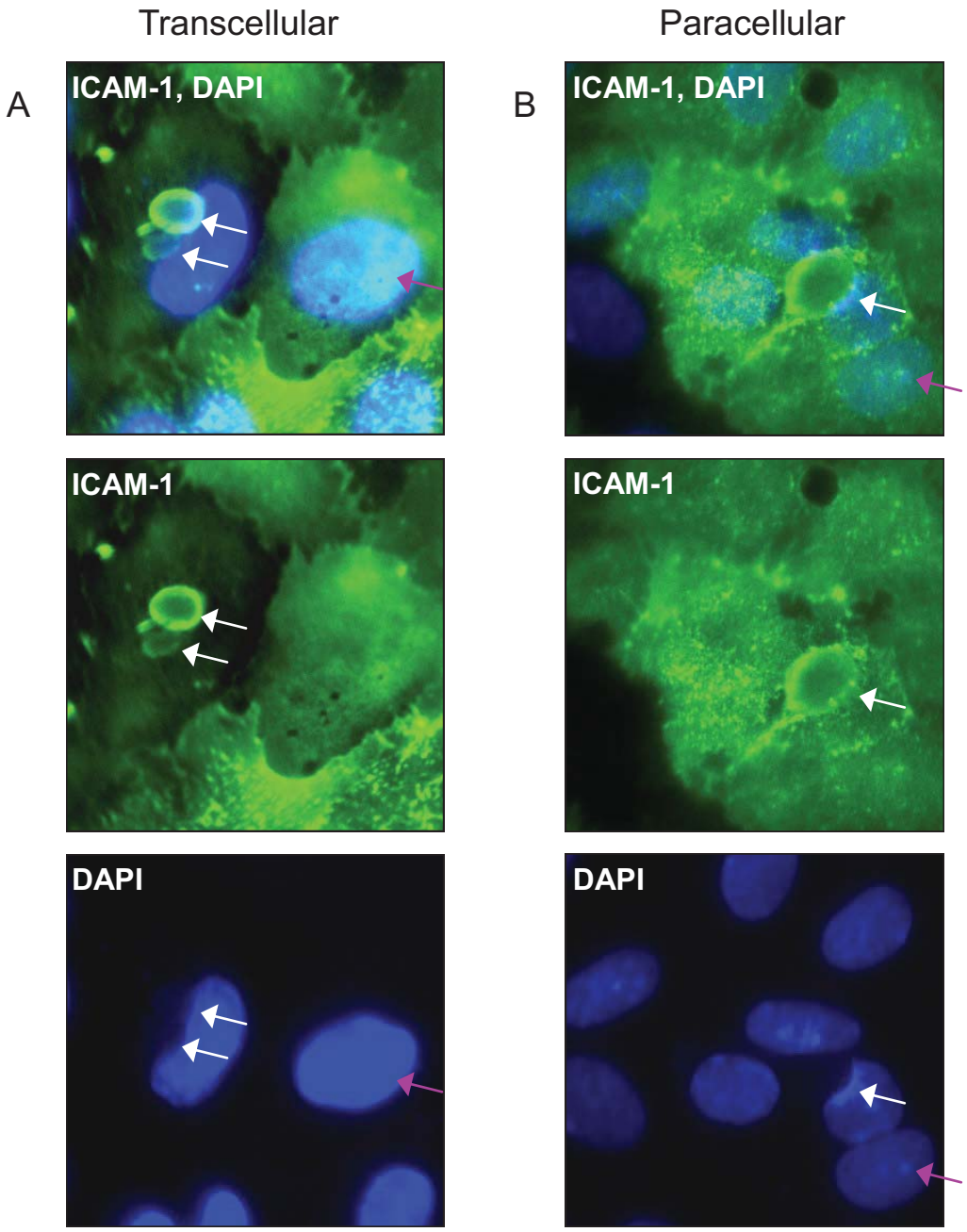


Figure 5.9: Visualisation of transmigratory cup formation around neutrophils transmigrating transcellularly and paracellularly.

HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min. Non-adherent neutrophils were then washed away and the monolayers fixed. Transmigratory cups were visualised by staining for ICAM-1 (green) and both HUVEC and neutrophil cell nuclei were stained with DAPI (blue). Image set A shows transcellular migration and image set B shows paracellular migration. White arrows indicate transmigrating neutrophils and pink arrows indicate HUVEC nuclei. Magnification x 600.

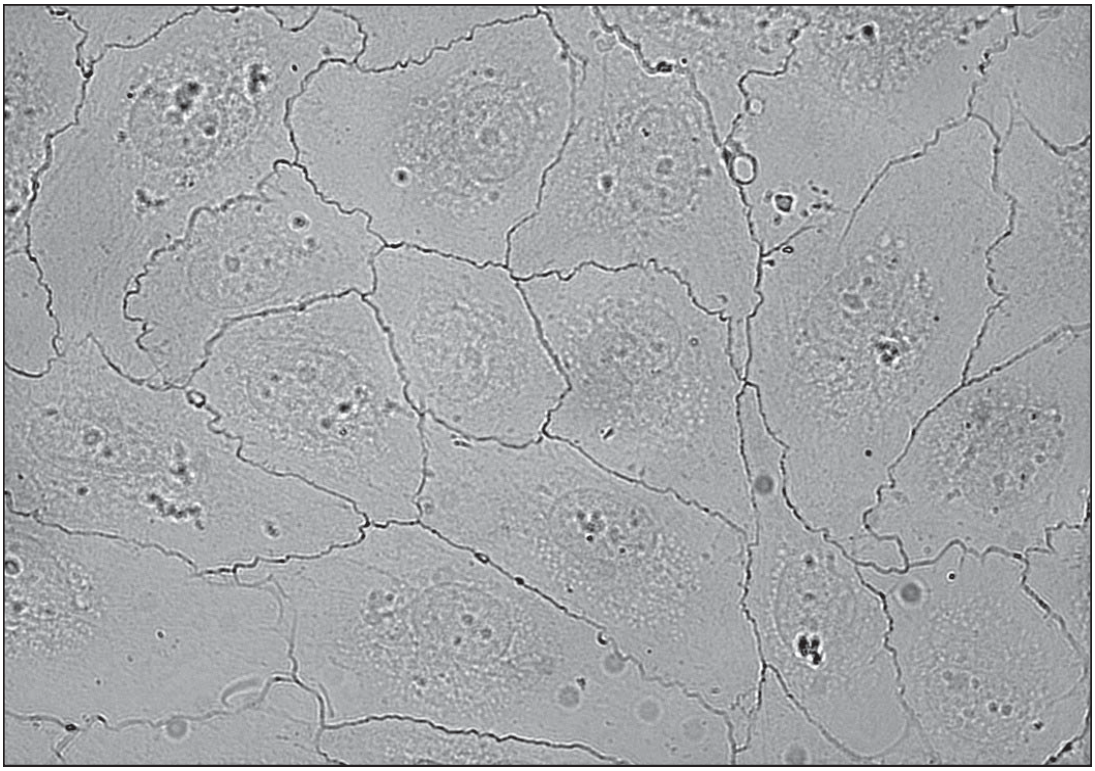


Figure 5.10: Silver-stained HUVEC monolayer.

HUVEC were seeded at high density onto fibronectin-coated glass slides and grown overnight. Monolayers were washed and stained with 1% $AgNO_3$, washed and fixed with 4% paraformaldehyde. Magnification x600.

found that the addition of neutrophils did not adversely affect the monolayer (Figure 5.11). Small ‘holes’ were occasionally observed at intercellular junctions following staining for ICAM-1 and VCAM-1 (see Figure 5.6 for an example), although $AgNO_3$ staining showed this was not an accurate reflection of the state of the monolayer during the experiment and was most likely the result of the immunohistochemistry process. Unexpectedly, neutrophil transmigration could also be observed following staining with $AgNO_3$, where light diffraction at the neutrophil plasma membrane was noticeably reduced in transmigrated neutrophils compared to those adherent to the apical surface of the HUVEC monolayer (Figure 5.11).

5.5.2 Neutrophil adhesion to endothelial monolayers and ICAM-1 staining in transmigratory cups is reduced in the presence of annexin A1 mAb

Given the inhibitory effect of the annexin A1 Ab on endothelial ERK activation and neutrophil transmigration, the Ab was tested using the assay developed to analyse its effects on transmigratory cup formation. A decrease in the total number of neutrophils adhering to HUVEC in the presence of the annexin A1 Ab was found, compared to Nil treatment (Figure 5.12). Despite the difference observed in total numbers of adherent neutrophils, >90% of these exhibited ICAM-1 staining around the periphery of the cell, independent of the treatment or method of transmigration employed by the neutrophil (transcellular or paracellular) (Figure 5.13). It was apparent however, that the overall intensity of ICAM-1 staining in the transmigratory cups of annexin A1 Ab treated samples was reduced compared to that of the Nil and KLH mAb treated samples. Figures 5.14 and 5.15, showing Nil and annexin A1 Ab treatments from the same experiment respectively, are representative images of multiple experiments and highlight the difference in intensity of transmigratory cup ICAM-1 staining between the different treatments. The intensity of ICAM-1 staining in all transmigratory cups was quantified by averaging multiple grey value measurements taken from each and normalising this value to surface ICAM-1 staining on HUVEC. Multiple readings from each transmigratory cup were required as the ICAM-1 staining would often be irregular, depending on the topography of the endothelial cells and the neutrophil, combined with the occurrence of lateral neutrophil locomotion, which could also affect endothelial ICAM-1 clustering surrounding the neutrophil. It was found that the average intensity of ICAM-1 staining in the transmigratory cups for Nil and KLH mAb-treated samples were higher than those from the annexin A1 mAb treatment (Figure 5.16, mean denoted by red line), despite there being no significant difference in surface ICAM-1 staining between the

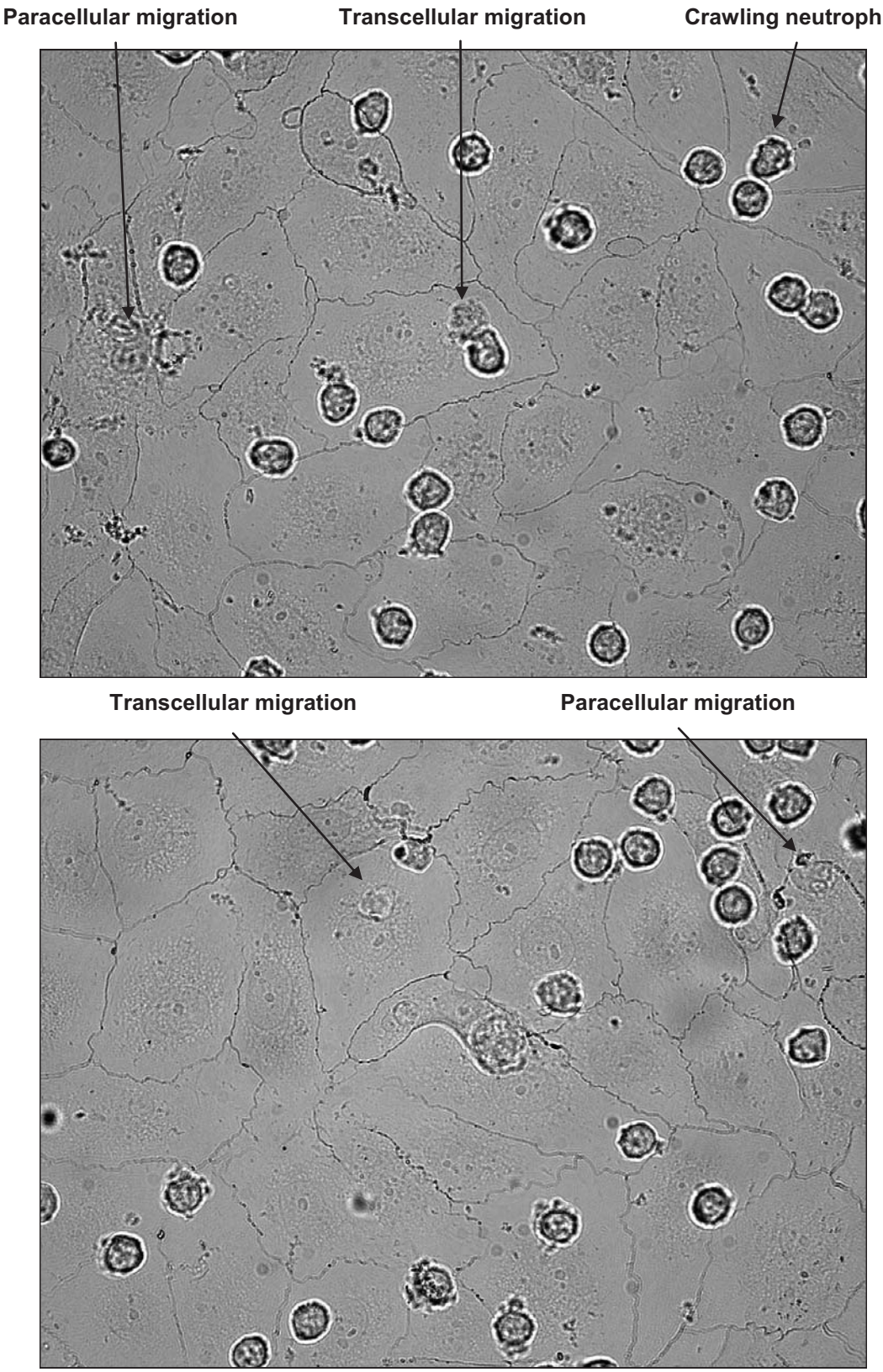


Figure 5.11: Silver-stained HUVEC monolayers with adherent neutrophils.

HUVEC were seeded at high density onto fibronectin-coated slides and grown overnight. HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min. Non-adherent neutrophils were washed away, then the monolayers were stained with 1% $AgNO_3$ and fixed. Magnification x600.

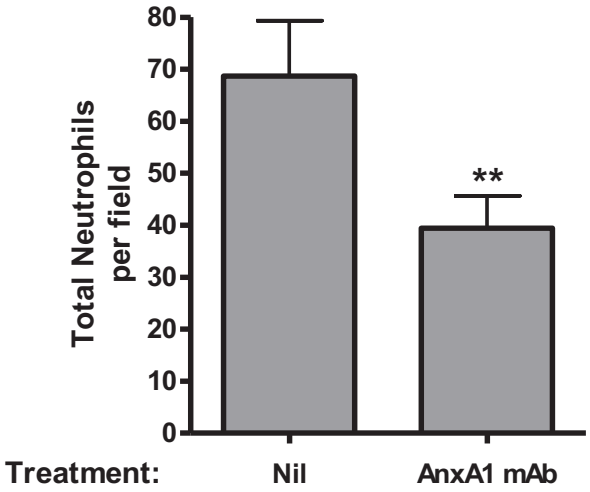


Figure 5.12: Annexin A1 mAb inhibits neutrophil adhesion to activated HUVEC monolayers.

HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min. Non-adherent neutrophils were washed away and the monolayers fixed. The total amount of adherent neutrophils from 30 representative fields of view (x 200 magnification) was determined from 3 individual experiments.
** $p<0.005$, unpaired t test. Error bars indicate SEM.

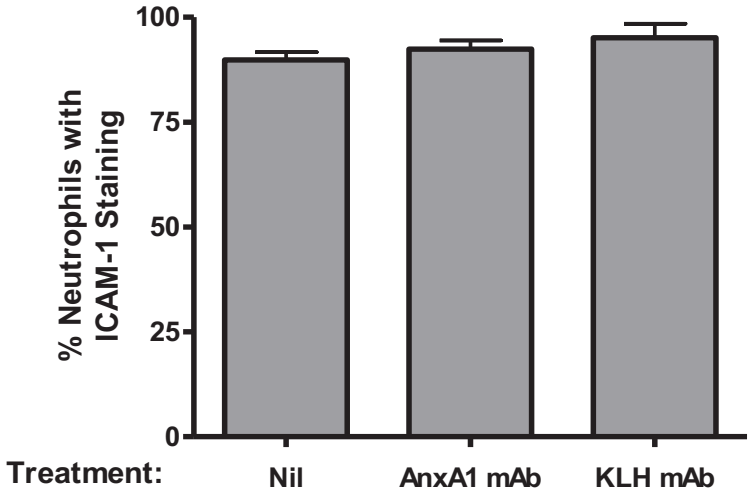


Figure 5.13: ICAM-1 is present in >90% of transmigratory cups surrounding adherent neutrophils.

Following adhesion to activated HUVEC monolayers, the percentage of neutrophils displaying ICAM-1 staining around the periphery was determined. 811 neutrophils contained within 59 representative fields of view were counted and the percentage of neutrophils with ICAM-1 transmigratory cup staining was calculated for Nil, AnxA1 mAb and KLH mAb treatments. Data was compiled from three individual experiments, where each experiment was performed in duplicate using 2 lines of HUVEC obtained from different women. Error bars indicate SEM.

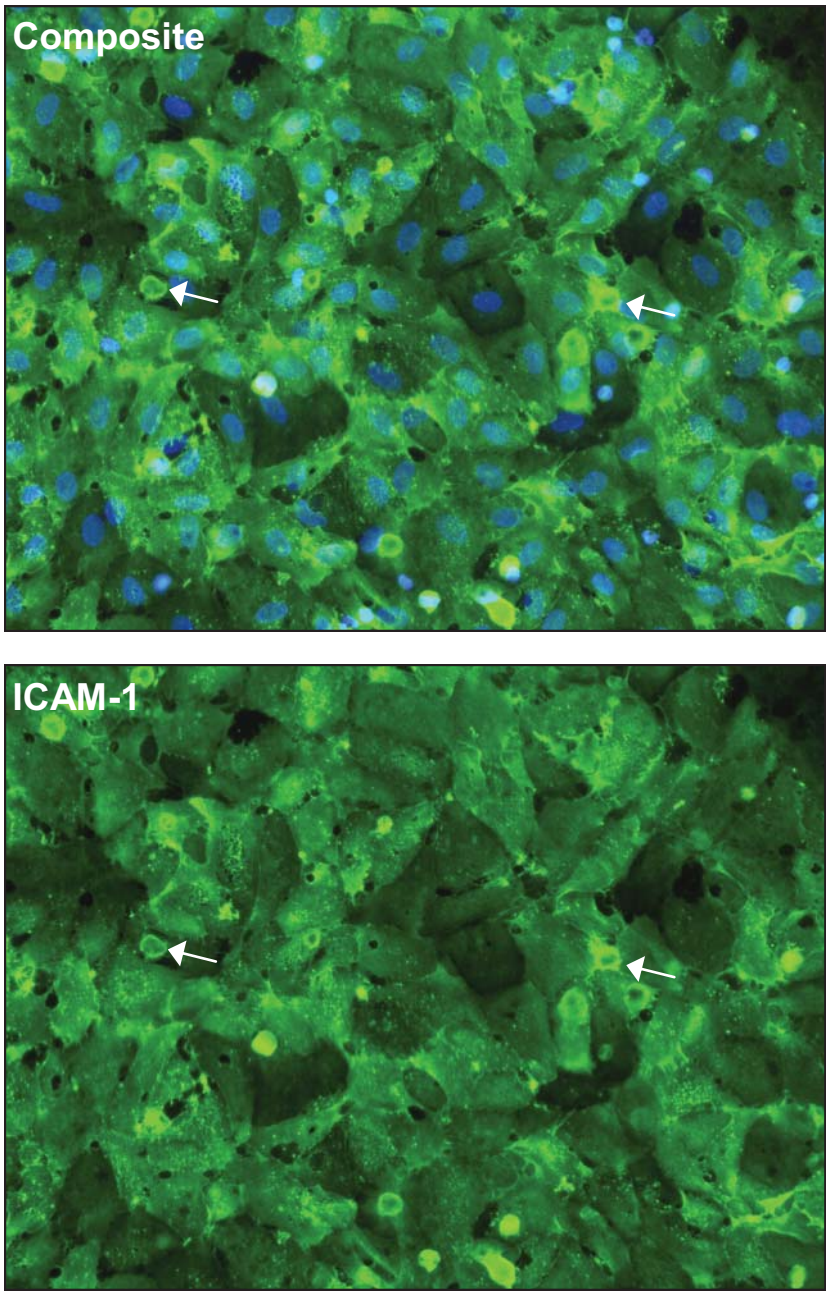


Figure 5.14: Nil treatment.

HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min. Non-adherent neutrophils were then washed away and monolayers were fixed. Transmigratory cups were visualised by staining for ICAM-1 (green) and both HUVEC and neutrophil cell nuclei were stained with DAPI (blue). White arrows indicate examples of transmigratory cups. Magnification x 200.

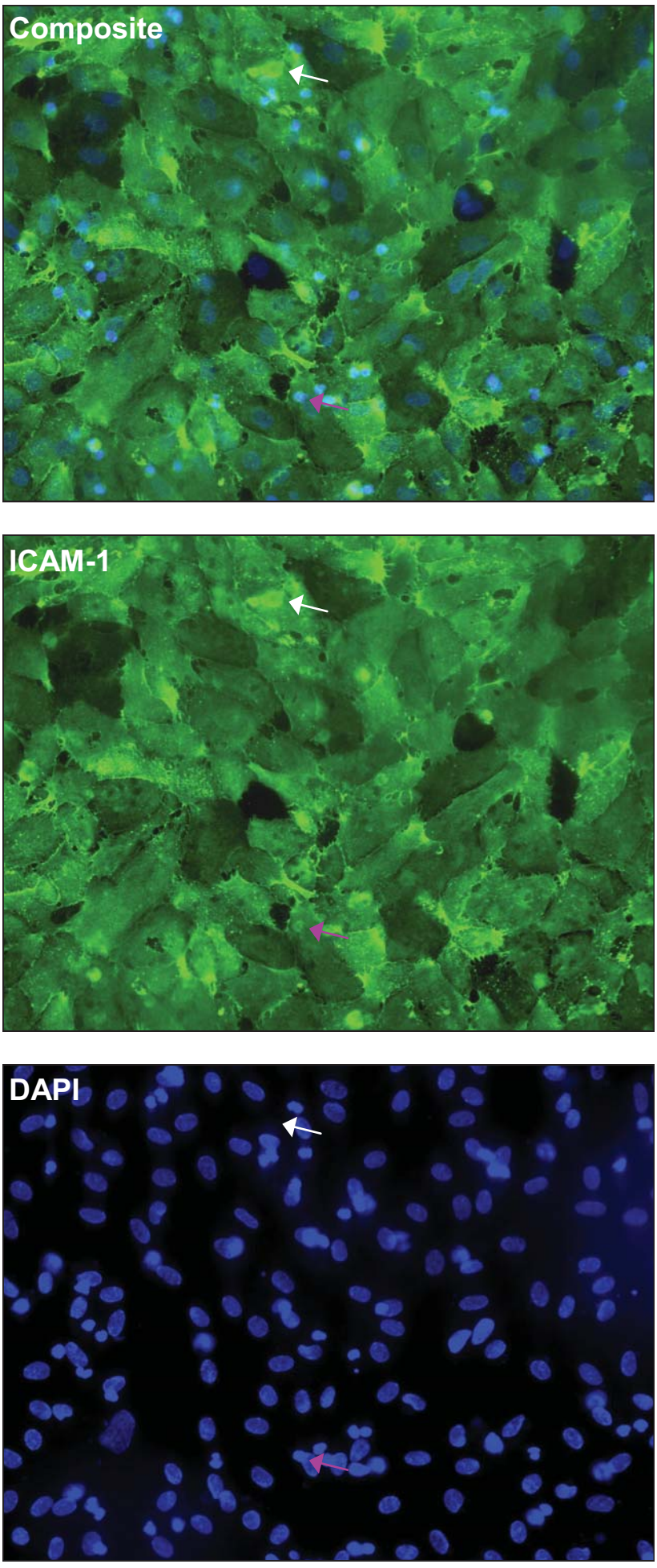


Figure 5.15: AnxA1 Ab treatment.

HUVEC monolayers were stimulated with TNF-α for 4h, then fMLP-stimulated neutrophils were added for 2 min with the annexin A1 Ab. Non-adherent neutrophils were then washed away and monolayers were fixed. Transmigratory cups were visualised by staining for ICAM-1 (green) and both HUVEC and neutrophil cell nuclei were stained with DAPI (blue). White arrow indicates an example of a transmigratory cup with strong staining, pink arrow indicates an example of a transmigratory cup with weaker intensity.
Magnification x 200

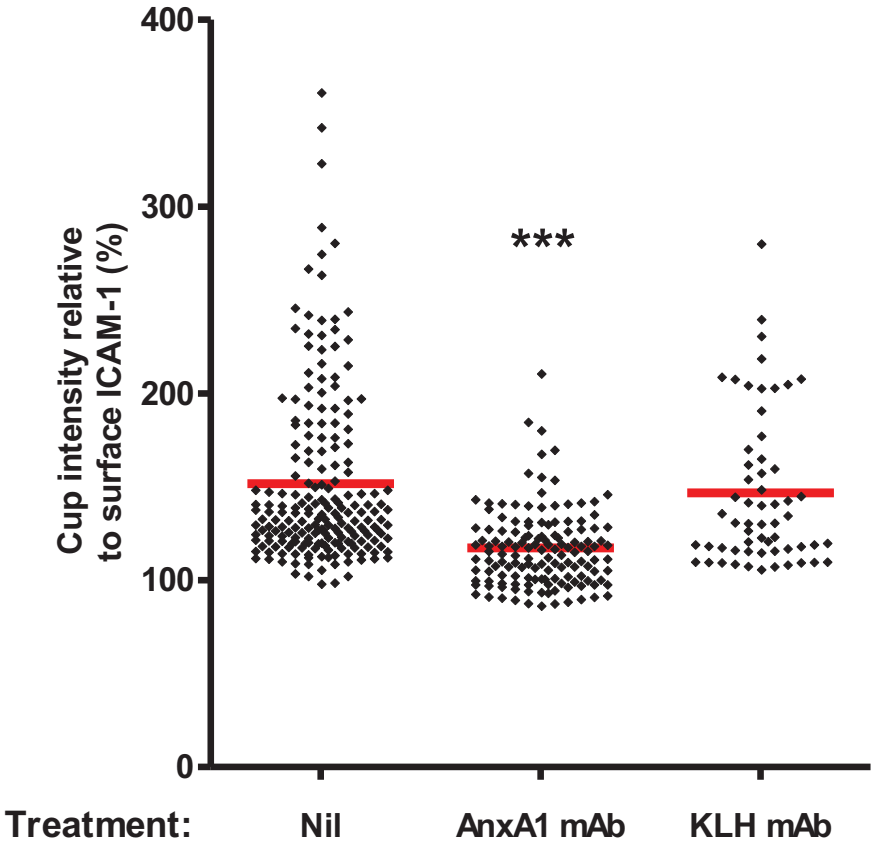


Figure 5.16: Annexin A1 Ab reduces the intensity of ICAM-1 staining in transmigratory cups surrounding adherent neutrophils.

Four measurements of ICAM-1 intensity in each transmigrator cup were taken from transmigrating neutrophils displaying ICAM-1 staining around the periphery. These measurements were averaged and expressed as a percentage relative to averaged HUVEC surface ICAM-1 staining. Red bars denote mean ICAM-1 staining in transmigratory cups for each treatment. 400 transmigratory cups in total from 59 representative fields of view were analysed from Nil, AnxA1 mAb and KLH mAb treatments from three individual experiments. Each experiment was performed in duplicate using 2 lines of HUVEC obtained from different women. *** $p<0.0001$, unpaired t test comparing Nil and AnxA1 mAb treatments.

treatments (Figure 5.17). Reduced levels of neutrophil adhesion and ICAM-1 staining within transmigratory cups of annexin A1 Ab treated samples suggested that endothelial ERK activation induced by annexin A1 was involved in the clustering of ICAM-1 around the neutrophil to form the transmigratory cup and engage the neutrophils to the endothelium.

5.5.3 The inhibitory effect of annexin A1 Ab is greater on transcellular than paracellular transendothelial migration

As transmigrating neutrophils using both transcellular and paracellular mechanisms were observed in this assay, the ratio of each mode of transmigration was calculated from neutrophils surrounded by endothelial ICAM-1 projections. In the absence of the annexin A1 Ab, it was found that the majority of transmigration initiated was *via* the transcellular pathway in this *in vitro* system (68% ± 2.5% transcellular, Nil, Figure 5.18), which was not significantly different to treatment with an irrelevant isotype-matched Ab (data not shown). However following treatment with the annexin A1 Ab, the bulk of transmigrating neutrophils were doing so in a paracellular manner (39% ± 2.3% transcellular, AnxA1 mAb, Figure 5.18). When the ratios of paracellular and transcellular migration were plotted as a percentage of total adherent neutrophils for each treatment (Figure 5.18), it became apparent that paracellular migration was unaffected by the annexin A1 Ab and its effects of transendothelial migration was predominantly on the transcellular route.

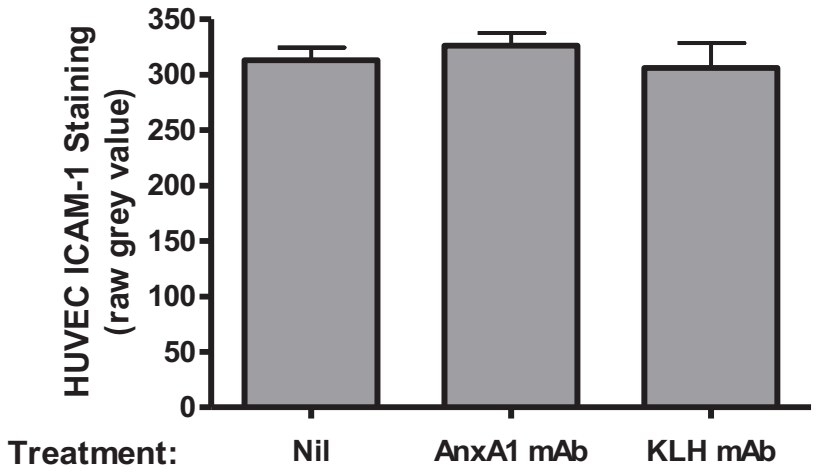


Figure 5.17: Annexin A1 Ab has no effect on total HUVEC surface ICAM-1 staining.

Four measurements of HUVEC surface ICAM-1 staining were taken and averaged from 59 representative fields of view where transmigratory cup intensity was quantified. The data from Nil, AnxA1 mAb and KLH mAb treatments from three individual experiments were compiled. Each experiment was performed in duplicate using 2 lines of HUVEC obtained from different women. Error bars indicate SEM.

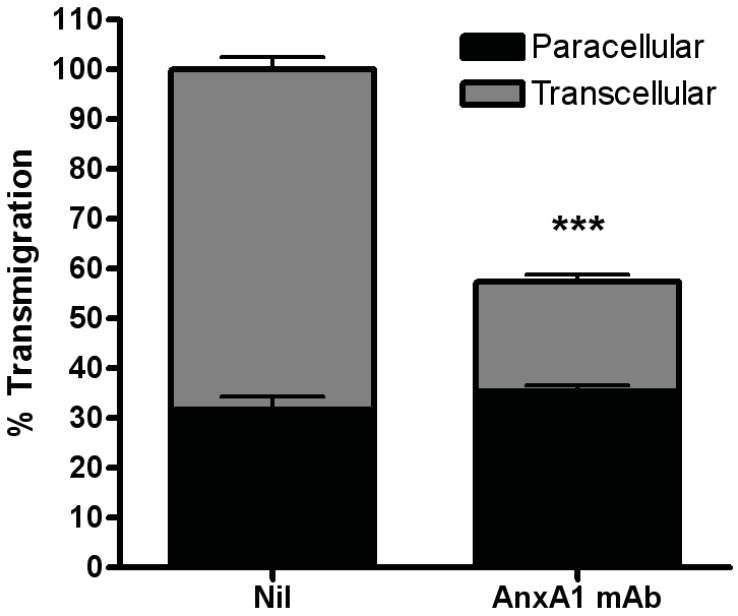


Figure 5.18: Annexin A1 Ab reduces the level of transcellular transmigration.

Following adhesion to activated HUVEC monolayers, the mode of transmigration of 400 neutrophils displaying ICAM-1 staining was examined and the percentage of neutrophils transmigrating via the transcellular or paracellular route was calculated. Paracellular and transcellular migration was plotted as a percentage of total adherent neutrophils for each treatment (as determined in Figure 5.12). The data from Nil and AnxA1 Ab treatments are from three individual experiments. Each experiment was performed in duplicate using 2 lines of HUVEC obtained from different women. Error bars indicate SEM.
*** $p<0.0001$, unpaired t-test comparing transcellular transmigration between Nil and AnxA1 Ab treatments. Paracellular transmigration was not significantly different between the treatments. Error bars indicate SEM.

DISCUSSION

Although calpain-truncated annexin A1 had been confirmed as the soluble neutrophil protein responsible for inducing endothelial ERK activation, the down-stream effects of ERK activation and how this mediated neutrophil extravasation was unclear. The experiments performed in this chapter showed that ERK-activation induced by truncated annexin A1 was being mediated through the FPR family and that blocking neutrophil-derived annexin A1 with annexin A1 Abs could reduce neutrophil adhesion and transmigration by impairing endothelial transmigratory cup formation.

Before it was established that annexin A1 was the protein responsible for endothelial ERK activation, the possibility that stimulated neutrophil conditioned medium was augmenting transmigration through increasing endothelial monolayer permeability was initially investigated. A number of studies have suggested that leukocyte transendothelial migration is facilitated by an increase in endothelial permeability chiefly through the "stiffening" and contraction of endothelial cells *via* myosin light chain phosphorylation to introduce paracellular openings in the monolayer [5, 148, 263, 265], although others have suggested there is no effect on permeability [85, 149]. To investigate this concept, the paracellular flux of FITC-conjugated dextran through HUVEC monolayers was monitored following treatment with stimulated neutrophil conditioned medium (Figure 5.1). FITC-dextran flux was unaltered in the conditioned medium treatment, indicating that ERK activation was not involved in increasing endothelial monolayer permeability. Neutrophil transendothelial migration can result in increased permeability of the vasculature to small particles and solutes [266], however the unrestricted leakage of large proteins during transmigration is highly unlikely, with the exception of disease states [267, 268], as the primary function of the endothelium is to act as a selective barrier to protect the blood stream and maintain fluid homeostasis [269]. Thus a non-specific increase in permeability during transmigration would be unlikely as it would contradict the very purpose of the endothelium and the tightly controlled nature of leukocyte extravasation would be pointless.

Although it was known that endothelial ERK-activation promoted neutrophil transmigration, how the downstream signalling events affected the endothelium were unknown. The identification of the endothelial receptor for annexin A1 was sought as it could potentially offer clues into the

function of annexin A1-induced ERK activation. As it had been previously shown that full length annexin A1 and Ac 2-26 are ligands for FPRL-1 and FPR [223, 252], the possibility that truncated annexin A1 was activating ERK through the FPR family was investigated. Furthermore, despite truncated annexin A1 being routinely detected in exudates from a number of inflammatory conditions [198, 242, 270, 271], the binding of this form of annexin A1 to the FPRs has not been investigated. This is presumably due to the assumption it was cleaved owing to elevated protease levels in inflamed tissues or was generated purely as a mechanism to deactivate the anti-inflammatory parental protein and was not thought to be a molecule possessing biological activity. To this end, a nonselective FPR antagonist, Boc2, was used to determine if it could block the activity of truncated annexin A1 on endothelial cells. Boc2 could effectively inhibit ERK-activation induced by neutrophil conditioned medium but not the PMA positive control, indicating that truncated annexin A1, like full length annexin A1 and Ac2-26, could bind to the FPRs to induce ERK activation (Figure 5.2). A study by Hayhoe and colleagues has shown that FPR or FPRL-1 transfected HEK293 cells treated with either recombinant annexin A1 or Ac2-26 resulted in ERK activation, which was postulated to inhibit neutrophil adhesion [252]. However previous results from our laboratory demonstrated that ERK inhibitors had no effect on neutrophil adhesion to TNF-α endothelial monolayers [99]. In further opposition to the above report, results from this study indicated that full length annexin A1 could not activate ERK in HUVEC, although the source of this difference may lie in the cell lines used and the degree of FPR and FPRL-1 overexpression achieved in the HEK293 cells. As Ac2-26 was shown to signal through both FPR and FPRL-1, but full length annexin A1 could only induce ERK activation through binding to FPRL-1 [252], it is likely that truncated annexin A1 mediates its pro-inflammatory effect through FPRL-1, particularly as the N-terminus is absent. However to be completely confident of this, the effect of truncated annexin A1 would need to be determined in a FPRL-1-expressing cell line devoid of other members of the FPR family. In the likelihood that truncated annexin A1 is signalling through FPRL-1, this leads to an interesting scenario where the N- and C-terminus of annexin A1 are competing for the same endothelial receptor to give opposing functions in the context of inflammation, a concept introduced in chapter 4. FPRL-1 is a promiscuous receptor that has been shown to bind a range of structurally unrelated pro-inflammatory and anti-inflammatory agonists. Many of these agonists are of host-origin such as prion protein fragment PrP106-126 [272], LL-37 [273], lipid metabolite lipoxin A4 (LXA4), serum amyloid A (SAA) [274], 42 amino acid amyloid β ($A\beta_{42}$) [275] and annexin A1 [252]. PrP106-126, LL-37, SAA and $A\beta_{42}$ all induce a pro-inflammatory, chemotactic response in

human leukocytes and are present during the inflammatory response in their relevant pathologic states. While LXA4 has been shown to bind and activate FPRL-1, like full length annexin A1, it has anti-inflammatory effects and inhibits neutrophil accumulation by interfering with neutrophil chemotaxis [276], adhesion and transmigration through endothelial and epithelial monolayers respectively [277, 278]. How FPRL-1 differentially responds to these pro- and anti-inflammatory agonists is unclear, though it may be through the inhibition or activation of different signalling cascades. Indeed it has been shown that while SAA can bind FPRL-1 to induce neutrophil secretion of the proinflammatory cytokine IL-8, LXA4 can partially inhibit this secretion by interfering with the activation of signalling pathways stimulated by SAA, including ERK [279]. It is therefore conceivable that as cleaved annexin A1 is the predominant form released from stimulated neutrophils, its interaction with endothelial FPRL-1 to activate ERK and promote neutrophil extravasation could be inhibited by full length annexin A1 released by non-specific mechanisms. This inhibition of neutrophil transmigration would serve as a safety mechanism, as the unprocessed parental protein is not present under normal conditions and may be an indicator of neutrophil hyperactivity, excessive release of intracellular contents or necrosis, as observed in several inflammatory disease states [18, 280-282].

Given the compelling suite of studies detailing the inhibitory properties of full-length annexin A1 and peptide derivatives on neutrophil extravasation (reviewed in [283]), the effect of Ac2-26 and truncated annexin A1 on neutrophil transmigration was investigated *in vitro*. These experiments were performed based on the hypothesis that the addition of exogenous pro-inflammatory annexin A1 would further increase transmigration already driven by a chemoattractant gradient. The net result was partly in line with published data, in that that the addition of the truncated and full length annexin A1 mixture decreased neutrophil transmigration (Figure 5.5). Ac2-26 however displayed no significant inhibition (Figure 5.3), despite it previously being shown to mimic the anti-inflammatory effects of full length protein [211, 215]. With the benefit of hindsight, these experiments were fraught with problems and flawed in design for the hypothesis that was being investigated, particularly after the finding that truncated annexin A1 was signalling through the FPR family to activate endothelial ERK (Figure 5.2). When these experiments were performed, those in Figure 5.2 had not been carried out and there was the preconception at the time that truncated annexin A1 was not signalling through FPRL-1 due to evidence that the anti-inflammatory effects of annexin A1 are mediated through this receptor [284]. The primary factor impacting on the results from these experiments was that annexin A1 is a chemoattractant for neutrophils [189] that binds to FPRL-1 and that FPRL-1 is expressed by

both endothelial cells and neutrophils. Therefore the addition of annexin A1 with neutrophils into the apical chamber of the Transwell would have dual opposing effects; activation of endothelial ERK (pro-inflammatory) and desensitisation of neutrophil FPRL-1 (anti-inflammatory). The final extent of neutrophil transmigration would balance on the degree of activation of endothelial cells and neutrophils by annexin A1 and fMLP respectively, against the desensitisation of neutrophils with annexin A1, which would inhibit chemotaxis into the basolateral chamber. The effect of neutrophil desensitisation by annexin A1 could not be avoided by adding the protein to the basolateral chamber of the Transwell due to the aforementioned chemotactic property of annexin A1 for neutrophils, which would have presumably elevated transmigration regardless of whether endothelial ERK was activated. It was also very likely that the presence of full length annexin A1 was contributing to the inhibition of transmigration observed. Despite the full length form not preventing endothelial ERK activation by truncated annexin A1, the inhibitory effects on the neutrophil were not taken into account at the time and would have almost certainly had an impact on the results. The addition of fMLP to activate neutrophils was also needed so that the necessary adhesion molecules required for transmigration were upregulated, which is also why the addition of annexin A1 alone without chemoattractant was not attempted. It was also interesting that Ac2-26 had no effect on neutrophil transmigration in this system. Much higher amounts of Ac2-26 are generally required to generate the anti-inflammatory effects reminiscent of full length annexin A1 (see [211] and [283] for examples), therefore the concentrations used in these experiments may not have been sufficient to overcome the proinflammatory effects of fMLP and truncated annexin A1.

Several studies detailing the anti-inflammatory effects of Ac2-26 and full length annexin A1 have shown this occurs by interfering with the interaction of neutrophils with the endothelium [223, 228, 252], indicating that annexin A1 signalling through FPRL-1 could be affecting endothelial adhesion molecule function. As it has been shown that ERK activation by neutrophil conditioned medium does not require neutrophil adhesion to HUVEC [99], adhesion molecules primarily involved in the actual transmigration process were investigated. Endothelial ICAM-1 and VCAM-1 have been shown to form structures termed “transmigratory cups”, which project from the endothelium to capture and envelop transmigrating leukocytes [81, 84]. To this end, the effect of the annexin A1 Ab on transmigratory cup formation was investigated. An assay was developed based on that described in Carman *et al*, where the transmigratory cup structure was originally identified [81], which was modified to analyse cup formation around transmigrating neutrophils by monitoring ICAM-1. The key differences in the previously described assay and the conditions

developed in this study were; TNF-α concentration and simulation time (Carman: 100ng/ml for 12h, Williams: 5ng/ml for 4h), neutrophil stimulation (Carman: no stimulation, Williams: 100ng/ml fMLP) and neutrophil incubation time with HUVEC (Carman: 10-60 min, Williams: 2 min). Extended TNF-α stimulation has been shown to induce changes in the morphology of endothelial cells *in vitro* [71], which may have consequences unrelated to the neutrophil extravasation process, therefore TNF-α treatment was limited to a period where ICAM-1 and VCAM-1 were visibly but not maximally upregulated (4h). Neutrophils were also subjected to a short stimulation period with fMLP to ensure that the process of surface adhesion molecule activation and upregulation was initiated, which could potentially be required for the transmigration process. Lastly, it was found that only a 2 min incubation period was required to visualise the formation of endothelial ICAM-1 projections around transmigrating neutrophils. Neutrophils adherent to the apical surface of the monolayer were virtually non-existent following longer incubation periods and a significant proportion were observed under the HUVEC monolayers (data not shown due to difficulty in capturing images). Carman *et al* and other laboratories have described a longer crawling period before transendothelial migration takes place *in vitro* (4 mins, [81]), however by using light microscopy, only a short crawling period of 15-30 seconds was observed using this assay before many neutrophils vanished from sight under the HUVEC monolayer. Possible reasons for the shorter time period required for transmigration to take place may be due to the fact that the neutrophils were pre-stimulated and that the HUVEC were not washed prior to the addition of the neutrophils. The washing step included by Carman *et al* would remove both TNF-α together with IL-8 secreted from the endothelium as a result of the TNF-α stimulation, which would ordinarily stimulate neutrophils. Consequently, the unstimulated neutrophils added to their assay would only become activated by adhesion molecules upregulated on the endothelium induced by TNF-α stimulation. Naturally this process would take longer compared to a system which has all the components required for transmigration already present. It was also noted that holes were formed in the HUVEC monolayers and steps were taken to ensure that these holes were not present before or formed during the neutrophil incubation step. Despite these efforts, holes present in the monolayer would not promote neutrophil transmigration, as transmigration will generally not occur across sub-confluent or unhealthy monolayer. This especially applied in this assay, as fMLP was located on the apical side of the monolayer and was not forming a chemoattractant gradient across the monolayer as established for neutrophil transmigration assays in Transwells. Furthermore, holes were not observed in the middle of cells where neutrophils were not present, even in longer

incubation points, indicating that transcellular pores closed rapidly behind emigrating neutrophils.

As endothelial ICAM-1 projections around transmigrating neutrophils could be visualised, the effect of the annexin A1 Ab on transmigratory cup formation was evaluated. It was immediately apparent that the Ab was inhibiting neutrophil adhesion to HUVEC, with a 45% reduction in total neutrophils adherent in the Ab treated samples compared to the nil treatment (Figure 5.12). Of those neutrophils that did adhere, approximately 90% displayed discernable ICAM-1 staining around the periphery of the neutrophil, similar to that observed in the Nil and irrelevant Ab treated samples (Figure 5.13), however the intensity of this ICAM-1 staining was significantly reduced compared to the control treatment cells (Figure 5.14). The reduction in the intensity was not due to an effect of the Ab on total ICAM-1 staining, as surface ICAM-1 staining quantified to normalise transmigratory cup intensity between images was unchanged across each treatment (Figure 5.15). This result further confirmed those shown in Figures 4.4 and 4.5, where the annexin A1 Ab inhibited neutrophil transmigration towards a fMLP gradient and across TNF-α activated HUVEC. One study has reported a switch from predominantly paracellular migration to transcellular migration following extended (24h) TNF-α activation of HUVEC [71], therefore the transmigratory mechanism employed by neutrophils displaying ICAM-1 staining was examined. It was found that transcellular migration was the principle method employed by transmigrating neutrophils in this assay, as indicated by the Nil and control Ab treated samples (Figure 5.18). In the presence of the annexin A1 Ab however, the number of neutrophils transmigrating through the transcellular route was significantly reduced, whereas the number using the paracellular route was not significantly affected by the addition of the Ab. (Figure 5.18). The formation of transmigratory cups occurs during both transcellular and paracellular neutrophil transendothelial migration [81], which was also observed in this study (Figure 5.13), so the observation that annexin A1 Ab predominantly affected transcellular migration was very interesting. It is possible that there were additional factors present at the HUVEC cell junctions that were assisting neutrophil adhesion to the monolayer at these areas. The weak ICAM-1 staining in the transmigratory cups of Ab-treated samples clearly indicated that neutrophil adhesion to HUVEC monolayers was impaired by interfering with transmigratory cup formation. It is possible that neutrophils that may have weakly adhered could have been washed off and only those migrating at the cellular junctions remained through binding to junctional proteins. This would therefore give the impression that paracellular migration was the preferred method to traverse the endothelial monolayer in the Ab treated samples. A number of proteins have been implicated in

the paracellular transmigration process, including PECAM-1, CD99, JAMS, ICAM-2 and VE-cadherin (reviewed in [34]), so it is possible that neutrophils were binding homo- or heterophilically to these molecules, which assisted in maintaining adherence at paracellular junctions. A single experiment was carried out to investigate the effect of the annexin A1 Ab on PECAM-1 using this assay, however no difference in PECAM-1 levels or distribution was observed across the treatments (data not shown). Whilst this experiment needs to be repeated to confirm these results, it was not surprising that PECAM-1 was unaffected, as it does not mediate leukocyte transmigration in response to TNF-α and leukocytes stimulated with fMLP do not require PECAM-1 for transmigration [285].

Although it was shown that the annexin A1 Ab reduced the intensity of ICAM-1 staining in transmigratory cups, the effect of the Ab was probably greater than Figure 5.14 indicates. Realistically, the quantification of transmigratory cup intensity in this experiment was biased against the Ab having an effect, as the neutrophils remaining adherent to HUVEC were in the minority and probably displayed the minimum baseline level of ICAM-1 staining required to initiate transmigration. However as approximately half of the neutrophils were washed off in the Ab treated samples due to insufficient tethering to endothelial ICAM-1, therefore the true effect of the Ab in reducing ICAM-1 accumulation around transmigrating neutrophils was not reflected accurately in Figure 5.14. Although the effect of the annexin A1 Ab was convincing in this assay, it would be important to determine how the Ab performs in a flow chamber model of transmigration, as shear stress of blood flow exerted on endothelial cell surface molecules has signalling consequences and therefore adds a further dimension to the process of leukocyte extravasation.

It was also intriguing that transcellular migration was the primary method utilised by emigrating neutrophils in this assay. Previous studies have suggested that paracellular migration is the predominant form of transmigration observed *in vitro*, with only 3-7% of neutrophils transmigrating transcellularly across cytokine-activated HUVECs [69, 71, 81]. In contrast, this study has shown that 68% ± 2.5% and 70% ± 5.9% of neutrophils were transmigrating transcellularly in the Nil and control Ab treatments respectively. These figures are in agreement with the numbers observed *in vivo* by examination of inflamed sections by scanning and transmission electron microscopy [63-67], where there is a greater incidence of leukocyte transcellular migration compared to paracellular transmigration. Despite this, the idea that paracellular migration was the preferred method of extravasation employed by leukocytes

became convention [21], which is surprising given reports from leukocyte transmigration studies conducted approximately 50 years ago detailing the phenomenon of transcellular migration *in vivo* [63]. This may be because transcellular migration could not be visualised, was not observed due to the sub-optimal *in vitro* transmigration conditions, or was simply overlooked because a range of incubation periods were not investigated. However this study and other recent reports describe the occurrence of transcellular migration *in vitro*, accompanied by the formation of endothelial ICAM-1 and VCAM-1 enriched transmigratory cups, which together suggest transcellular migration is an important route through the vasculature for emigrating leukocytes.

SUMMARY

After demonstrating that that calpain-cleaved annexin A1 was the ERK-activating protein in stimulated neutrophil conditioned medium, the annexin A1 endothelial receptor and events downstream of ERK activation were investigated to determine how ERK activation influenced neutrophil transendothelial migration. The possibility that ERK activation was promoting neutrophil transmigration by altering endothelial permeability was eliminated, as conditioned medium failed to alter HUVEC monolayer permeability to FITC-conjugated dextran, an assay that measures the integrity of intercellular junctions. To identify the endothelial receptor involved in ERK-signalling, a nonselective FPR antagonist was utilised as annexin A1 and N-terminal peptide derivatives are ligands for the FPR family and have been shown to exert their anti-inflammatory effects through these receptors. The FPR antagonist inhibited endothelial ERK activation elicited by neutrophil conditioned medium, suggesting that annexin A1 in neutrophil conditioned medium was signalling through endothelial FPR to facilitate neutrophil transmigration. As several studies have shown that annexin A1 promotes neutrophil detachment from the endothelium, endothelial adhesion molecules were investigated as potential downstream targets for ERK activation during neutrophil transmigration. Using an assay to examine ICAM-1 in transmigratory cup formation, it was found that the annexin A1 Ab could inhibit neutrophil adhesion to HUVEC monolayers, which was due to the Ab interfering with ICAM-1 clustering around emigrating neutrophils. Quantification of transmigrating neutrophils highlighted that the majority of neutrophils engaged in a transmigratory cup were emigrating *via* a transcellular pathway, which is in agreement with leukocyte transcellular migration observed by several laboratories *in vivo*, however this figure is higher than transcellular migration reported in a number of *in vitro* studies, where paracellular migration was the primary method detected.

This chapter resolves a number of uncertainties relating to how annexin A1 was signalling to the endothelium to modulate neutrophil extravasation. A novel role for annexin A1-induced ERK-activation in transmigratory cup formation was identified and the importance of this signalling pathway was emphasised through the use of annexin A1 blocking Abs, which significantly impaired the levels of neutrophil adhesion and transmigration.

Chapter 6

DISCUSSION

DISCUSSION

The present study identified that stimulated neutrophils secrete a pro-inflammatory form of annexin A1 that binds to the FPR family on endothelial cells to activate ERK, which in turn permits neutrophil passage through the endothelium. Furthermore, it was shown that the administration of annexin A1-blocking Abs impaired neutrophil adhesion and emigration by interfering with the formation of endothelial transmigratory cup structures. These experiments highlighted that annexin A1 is an important mediator in the transendothelial migration of neutrophils.

Leukocyte emigration from blood vessels was originally described in the nineteenth century and is a characteristic feature of inflammation. Theories developed by early pioneers in the field still hold true over 100 years later; that signals emanating from sites of infection attract immune cells [286] and that alterations in the vessel walls are responsible for immune cell emigration [287]. It is now undisputed that both leukocytes and the endothelium play an important, proactive role in the process of leukocyte diapedesis and the classic 3-step model of extravasation involving leukocyte rolling, adhesion and finally transmigration is widely accepted. Additional sub-steps have been added in recent times, including intraluminal crawling of neutrophils and monocytes on the endothelial surface, which occurs briefly before the act of transendothelial migration in a Mac-1 and ICAM-1-dependent manner to increase the efficiency of the process [46, 51]. It has also been recently shown that extravasation can occur *via* two distinct pathways; paracellular migration between the tightly apposed cells of the endothelial sheet and/or transcellular migration whereby leukocytes pass through individual endothelial cells away from the cell borders [81]. Despite the apparent disruptive nature of entire immune cells passing through or between endothelial cells, the migrating leukocyte does so with little disturbance to the integrity of the endothelium through an exquisitely regulated process involving many surface adhesion molecules on both cell types and the activation of corresponding signalling pathways.

A common phrase encountered in publications on leukocyte extravasation from the 1990s to the early 2000s generally amounts to: “The rolling and adhesion steps of leukocyte transmigration are well characterised, however little is known about the transmigration process…” [21, 129, 202]. Naturally this was the impetus behind the study conducted by Stein *et al* in 2003 which screened a range of pharmacological inhibitors to identify signalling pathways important in

neutrophil transendothelial migration, which ultimately led to the formation of the present study. The transmigration process is still the least characterised step in leukocyte emigration, although a number of recent papers published since the commencement of this study have been pivotal in demystifying the mechanisms of transendothelial migration. One such paper from Timothy Springer's laboratory identified the arrangement of endothelial transmigratory cups structures, enriched in ICAM-1 and VCAM-1, around transmigrating leukocytes to facilitate both paracellular and transcellular transmigration [81]. Until this study, *in vitro* evidence for transcellular migration was scarce, with only a small number of groups reporting on this alternate process. Consequently, the idea that leukocyte transendothelial migration occurred by paracellular mechanisms became dogma, despite a number of compelling studies describing the occurrence of transmigration *via* the transcellular route *in vivo* using EM techniques. It was fortunate that the phenomenon of transmigratory cup formation and transcellular transendothelial migration was published at this time as both features were particularly relevant to this study,

6.1. Involvement of annexin A1 in transmigratory cup formation during neutrophil transendothelial migration

Results from this study have implicated annexin A1 in the functioning of ICAM-1 and VCAM-1 enriched transmigratory cup structures that are formed during the process of neutrophil extravasation. In the static assay used to visualise this process, ICAM-1 and VCAM-1 were upregulated prior to the addition of neutrophils by pre-stimulation with TNF-α. As the annexin A1 Ab inhibited neutrophil adhesion and transmigratory cup formation, this implied that annexin A1 activity was required for the tethering of neutrophils to the surface of the endothelium and/or the clustering of surface ICAM-1 around the adherent neutrophils, rather than affecting adhesion molecule upregulation on the endothelium. ICAM-1 is an essential player in leukocyte transmigration, where it is required for both the tethering and arrest of leukocytes to the endothelium [71, 84] and in the aforementioned role of transmigratory cup formation to promote leukocyte emigration [81]. Endothelial filamin B, an actin cross-linking molecule, has been shown to be crucial for ICAM-1-mediated transmigration as the removal of this protein results in decreased cell adhesion, impaired ICAM-1 trafficking to transmigratory cups and a consequent decrease in total cell emigration [288]. These effects were consistent with those observed regarding neutrophil behaviour and transmigration following treatment with the annexin A1 Ab in this study. Actin remodelling is an important feature in leukocyte transendothelial migration,

as actin polymerisation has been shown to be important in transmigratory cup assembly [81] and transcellular migration occurs through actin-rich regions of endothelial cells [72]. These studies combined with observations that ICAM-1 can signal to components of the cytoskeleton [289, 290] suggest that cytoskeletal remodelling events could potentially be down stream of ERK activation to allow the lateral movement of ICAM-1/VCAM-1 on the endothelial surface and therefore promote neutrophil transmigration [288].

As the assay used was static and lacking the physiological consequences of shear-stress induced by blood flow over endothelial cells [24], the involvement of annexin A1 in this process is purely speculative at this stage. It is crucial this experiment is performed in a flow chamber assay under more relevant conditions before any solid conclusions can be made on the involvement of annexin A1 induced ERK-activation in endothelial transmigratory cup formation.

Despite the obvious short-comings of the assay in terms of physiological relevance, a number of factors were consistent with observations made *in vivo*. For instance, the high incidence of transcellular migration detected was intriguing as these levels were comparable to analyses of leukocyte emigration occurring *in vivo*, but greater than the degree of transcellular transmigration observed in *in vitro* studies. Paracellular migration requires the disruption of adherens junctions necessary for the adhesion and mechanical strength of monolayers of cells [61]. It is therefore conceivable that the 2 min incubation period of neutrophils with endothelial monolayers used in the assay was not sufficient to allow all possible paracellular migration events to occur, as longer time periods are required to destabilise junctional protein interactions [61]. If this is true, the rapid rate at which transcellular migration occurs lends itself to the idea that this transmigration pathway is indeed valid and the preferred method of neutrophil transendothelial migration. Alternatively, and probably very likely, there may also be discrepancies in what various laboratories determine as paracellular migration. In this study, paracellular migration was labelled as such if the borders of neighbouring endothelial cells were both obviously disrupted by the neutrophil migrating between them. However if a neutrophil appeared to be making slight contact with only one cell boundary without disturbing that of adjacent endothelial cells, combined with the observation that the entire neutrophil was moving through the endothelial cell body, this was determined as transcellular migration. This is a grey area and obviously very subjective, and since paracellular and transcellular migration are not mutually exclusive and both processes require transmigratory cup formation, categorising each based solely on surface adhesion molecule expression is difficult.

Endothelial “dome” structures have also been reported that were found to project and envelop transmigrating neutrophils [85]. These are reminiscent of *in vivo* EM images captured by Hoshi and colleagues, where the membranes of endothelial cells were seen to rise and surround neutrophils that were emigrating through transcellular channels in the inflamed mouse lip [291]. Endothelial ICAM-1 projections were also detected closing over neutrophils that had partially migrated through the monolayer using the assay described in this study (see Figures 5.8 and 5.9A for examples).

Although not investigated alongside ICAM-1 in this study, it is likely that endothelial VCAM-1 would behave in the same manner as ICAM-1 following treatment with the annexin A1 Ab, as it is the other predominant component of transmigratory cups [81]. Furthermore, it has been shown that VCAM-1 can partially compensate for ICAM-1 during leukocyte transmigration in an ICAM-1-deficient mouse model [292].

6.2. Annexin A1: pro- or anti-inflammatory?

As stressed throughout this thesis, the observation that annexin A1 was behaving in a pro-inflammatory manner was a perplexing issue, as the vast majority of annexin A1 studies emphasise a potent anti-inflammatory function for this protein. The addition of exogenous annexin A1 and N-terminal peptides negatively modulates neutrophil extravasation in response to inflammatory stimuli, whilst immunisation with annexin A1 neutralising antibodies promotes neutrophil trafficking in models of acute and chronic inflammation [211, 216, 230, 231]. There is no question that full length annexin A1 and Ac2-26 can effectively inhibit neutrophil accumulation at sites of inflammation, however these effects may not directly translate to the normal neutrophil response under early inflammatory events. Upon neutrophil stimulation and adherence to the endothelium, annexin A1 is externalised to the plasma membrane, the majority of which is the intact form [202]. It appears that proteolytic cleavage of annexin A1 is required for secretion from immune cells, as the majority of annexin A1 released from neutrophils is N-terminally cleaved [199, 243]. Given that only N-terminally truncated annexin A1 is secreted from activated neutrophils, studies where exogenous annexin A1 is administered in models of inflammation should be regarded with caution as the neutrophils and endothelial cells within these systems are being exposed to full length protein that would otherwise not be present in the blood stream. This would most likely lead to FPR/FPRL-1 desensitisation and consequently an overall reduction in the degree of neutrophil emigration to sites of inflammation. Although

annexin A1 does not possess classical signal sequences to target the protein for export, both full length and truncated forms are often observed in extracellular fluids and inflammatory exudates from inflamed tissues [198, 242, 270, 271]. As truncated annexin A1 is the predominant form released following neutrophil stimulation, the mechanisms by which full length annexin A1 is generated in these tissues is unknown. As proposed previously, it may be a genuine host response where annexin A1 transcription and expression is upregulated in an effort to control the inflammatory event and minimise tissue damage at the site of inflammation and could be produced by alveolar type II cells or macrophages [293-295]. However an alternative explanation is that the presence of full length annexin A1 may also be the result of secondary necrosis of apoptotic neutrophils at these sites. Apoptotic neutrophils are generally cleared during the resolution of an inflammatory event following phagocytosis by macrophages, which results in the suppression of pro-inflammatory cytokines and the release of additional anti-inflammatory mediators such as TGF-β and IL-10 from surrounding immune cells [296]. When the number of apoptotic neutrophils exceeds the phagocyte-mediated clearance capacity of the organism, the apoptotic bodies disintegrate and secondary necrosis occurs [297]. It is therefore conceivable that full length annexin A1 could be released from neutrophils in inflamed tissues following secondary necrosis, rather than the host organism actively increasing the production of annexin A1 at these sites, which may assist in the resolution of the inflammatory events. This may also provide a reason why neutrophils continue annexin A1 transcription following extravasation [200], as it would be released with the mixture of noxious, pro-inflammatory molecules from neutrophils during secondary necrosis and assist in preventing the ensuing immune response.

6.3. Mechanisms and effects of annexin A1 truncation

The secretion of truncated annexin A1 from stimulated neutrophils has been observed by a number of groups and this study has additionally provided a functional role for this cleavage product in neutrophil extravasation. Aside from the activation of ERK signalling pathway in the vasculature to promote neutrophil transmigration, there is still an unknown purpose for truncated annexin A1 after this process. Prior to extravasation, approximately 50% of annexin A1 mobilised to the surface of neutrophils is intact, however this figure drops to approximately 6% following transendothelial migration, where the majority of the protein is secreted and the remaining cell-associated annexin A1 is the truncated form and is located inside large vacuoles within neutrophils [200]. Since it is believed that annexin A1 is liberated from neutrophils

following proteolytic cleavage at the plasma membrane, it is unclear how or why a portion of the cleaved protein is then internalised following transendothelial migration. It is possible that not all proteolytically cleaved annexin A1 is externalised. A further interesting observation was that a pool of truncated, but not full length, annexin A1 was observed within endothelial cells in the area surrounding the transmigration site [200]. After agonist binding to G-protein coupled receptors, it is often necessary for the receptor/agonist complex to be internalised to activate signalling pathways, such as the MAPK pathway [298, 299]. It is therefore reasonable to propose that receptor internalisation could also be occurring after truncated annexin A1 binds to endothelial FPRL-1 to induce ERK-activation, hence the increased annexin A1 content observed within endothelial cells. An alternative explanation may be that residual annexin A1 is non-specifically deposited during the process of transcellular migration. Endothelial annexin A1 content was not investigated in this study, as the annexin A1 Abs used in the laboratory detected both intact and truncated forms of annexin A1. However this would be an interesting aspect to address, especially considering the high incidence of transcellular migration observed using the assay developed to monitor transmigratory cup formation around emigrating neutrophils.

Whilst there are genuine disorders where uncontrolled protease activity results in tissue damage and disease, the presence of proteolytic cleavage products is frequently dismissed as the result of degradation rather than a specific processing event to yield products with functional activity. Such is the case with annexin A1, where the truncated form observed in inflammatory exudates is often attributed to non-specific cleavage by elevated concentrations of anti-microbial proteases at sites of inflammation. This is in part due to the fact annexin A1 does not a possess a recognisable signal peptide, which are sequences present within some proteins that target the molecule to a specific location and are later removed by proteolysis to generate a mature, active form of the protein. A common theory to explain the presence of proteolytically cleaved annexin A1 in inflamed sites is that proteolytic cleavage of full length annexin A1 ablates the anti-inflammatory property of the protein [215] and therefore contributes to the elevated influx of neutrophils observed in affected tissues in a number of inflammatory diseases. This is despite a number of reports highlighting a functional effect following annexin A1 proteolysis such as reduced Ca^{2+} requirement for membrane aggregation, where cleavage of annexin A1 after residue 26 (calpain I cleavage site) resulted in a 4-fold increase in Ca^{2+} sensitivity for membrane binding in a chromaffin granule aggregation assay, but cleavage after residues 12 (cathepsin D cleavage site) and 29 (plasmin cleavage site) resulted in a 3-fold decrease and no change in Ca^{2+} sensitivity respectively [222]. Another study showed that all three truncated forms had a uniform reduction

in Ca^{2+} required for phospholipid binding, indicating that membrane aggregation and phospholipid binding were mediated by separate regions of annexin A1 [194]. A further report showed that the intracellular distribution of annexin A1 changed following truncation of the first 26 amino acids from the N-terminus, which caused a shift in its association from early to late endosomes and multivesicular endosomes in baby hamster kidney cells [300]. These studies highlighted that small changes to the N-terminus of annexin A1 can significantly alter the behaviour of the remaining portion of the protein. Therefore whilst proteolytic cleavage of annexin A1 may result in the attenuation of its anti-inflammatory activity as suggested, it does not appear that this encompasses the entire story of its involvement in the neutrophil-mediated inflammatory response, particularly given the ERK-activating capacity of the calpain cleavage product observed in this study.

Several proteases have been shown to recognise and cleave at sites within the N-terminal domain of annexin A1 including cathepsin D [222], calpain I [222], plasmin [222], neutrophil elastase [242, 243], proteinase 3 [244] and a matrix metalloprotease [245]. Although many of the reported annexin A1 cleavage products do not have an obvious functional role in inflammation, the sole purpose of some enzymes involved in its proteolysis may simply be to inactivate anti-inflammatory annexin A1, whilst other proteases may generate a cleavage product with biological activity. It appears that the latter is the case for calpain I in neutrophils, where it was found to be involved in the secretion of truncated annexin A1 with the capacity to activate endothelial ERK from stimulated neutrophils. The calpains are a family of ubiquitous, calcium-dependent serine proteases with a number of functions including cell migration, proliferation and apoptosis [301, 302]. Neutrophils express two calpain isoforms in particular; calpain I and calpain II, also designated as μ-calpain and *m*-calpain for their micromolar and millimolar Ca^{2+} requirements for activation *in vitro*, respectively [303]. Calpain I is constitutively active in human neutrophils and is expressed at a much higher level than calpain II [304]. Calpain I inhibition has been shown to markedly attenuate neutrophil activity and consequently inflammation and disease progression in a number of experimentally induced disorders including myocardial, intestinal and renal ischaemia reperfusion injury, ulcerative colitis, multiple organ failure and acute pancreatitis [305-310], however the calpain inhibitors administered in these models were most likely acting on a range of different cell types to achieve this effect. Anderson *et al* have also shown that a calpain inhibitor, calpeptin, could promote the detachment of rolling fMLP-stimulated neutrophils from monolayers of activated platelets [311]. Following stimulation with fMLP or PMA of neutrophils *in vitro*, neutrophil calpain has been observed to translocate to the plasma membrane and become

"activated" in a sense, as it displayed a reduced Ca^{2+} requirement for proteolytic activity [248, 312]. A further study from the same laboratory showed that neutrophil internalisation of a calpain monoclonal antibody resulted in decreased calpain activity and a concomitant reduction in granule exocytosis following fMLP and PMA stimulation, thus suggesting a role for this protease in the secretion of neutrophil granule contents [313]. Calpain I has also been implicated in neutrophil chemotaxis, where the inhibition of basal calpain I activity induced neutrophil polarisation, spreading and random migration [304]. However this inhibition also impaired neutrophil chemotaxis towards both fMLP and IL-8 chemoattractant gradients, highlighting that calpain I activity is required for a specific, directional response to chemotactic stimuli. Following neutrophil stimulation and adhesion to endothelial monolayers, annexin A1 is likewise mobilised from neutrophil granules and translocates to the plasma membrane [201], a process which has been shown to be dependent on phosphorylation in the N-terminal region in other cell lines [209]. Both phosphorylation and membrane association have previously been shown to predispose annexin A1 to proteolytic processing [207, 314]. Therefore as calpain I and annexin A1 both translocate to the plasma membrane following neutrophil stimulation, it is possible that calpain-mediated annexin A1 proteolysis may occur at this site and result in the secretion of pro-inflammatory truncated annexin A1.

A truncated form of annexin A1 has also been detected on the membranes of specific granules and secretory vesicles in neutrophils, but not azurophil granules, where the binding is calcium-dependent [249, 315]. The binding characteristics of truncated annexin A1 differed to the intact protein, which bound to all three neutrophil organelles regardless of the presence of calcium [249, 315]. This indicated that either the proteolytic activity resided within the membranes of specific granules and secretory vesicles, or that the phospholipid bound form of annexin A1 was more susceptible to proteolytic cleavage. Annexin A1 has also been found localised to gelatinase granules and is mobilised to the neutrophil plasma membrane following adhesion to the endothelium, however the ratio of intact to cleaved protein in these granules was not evaluated [201]. This raises the possibility that the different forms of annexin A1 may be subject to differential exocytosis from the neutrophil, which may be important in regulating the apparent opposing roles exerted by the different forms of annexin A1, especially given the hierarchy of neutrophil granule exocytosis following stimulation. Neutrophils regulate the secretion of the various granules and secretory vesicles in a specific manner, which ensures a targeted inflammatory response and avoids the problem of extensive tissue damage by the release of destructive granule contents. Indeed secretory vesicles and specific granules are more readily

mobilised following neutrophil stimulation than azurophil granules, which only undergo limited exocytosis following stimulation [316], even with potent agonists such as PMA [317]. The primary role for azurophil granules is the elimination of engulfed microorganisms in the phagolysosome, which is facilitated by the high content of cytotoxic enzymes such as myeloperoxidase and neutrophil elastase contained within these organelles [316]. Azurophil granule exocytosis generally only occurs when the neutrophil has reached the site of infection and is completely activated following extravasation. Alternatively, the majority of secretory vesicles can be rapidly discharged from neutrophils following treatment with nanomolar concentrations of fMLP, without the release of the remaining neutrophil granules [318]. The presence of truncated annexin A1 within these granules is consistent with observations made in this study, where low doses of fMLP resulted in the rapid secretion of truncated annexin A1 from neutrophils. If truncated annexin A1 is secreted from intravascular neutrophils to promote transendothelial migration *in vivo,* efficient mobilisation would seemingly be an important feature to facilitate neutrophil emigration in the immediate vicinity where the inflammatory cue was received. It is also significant that annexin A1 was localised to these organelles as the contents are less noxious when compared to that of azurophil granules, which would damage the vasculature if exocytosed at this early stage. The finding that IL-8 could induce the secretion of truncated annexin A1 further lends credence to this hypothesis, as IL-8 also stimulates secretory vesicle and specific granule exocytosis [319] and it shows that truncated annexin A1 release can be induced by two very different forms of proinflammatory stimuli relevant in the neutrophil-mediated inflammatory response. It would be interesting to investigate additional stimuli, such as LPS to determine if these also have a similar effect. It still needs to be determined when and where annexin A1 is cleaved in the neutrophil. Annexin A1 proteolysis could occur in neutrophil granules and then be released upon stimulation with other granule contents, or the full length protein may possibly be cleaved at the plasma membrane following co-translocation of annexin A1 and calpain I to facilitate the secretion of truncated annexin A1.

Truncated forms of annexin A1 have been particularly noted in bronchoalveolar lavage fluid (BALF) from patients with lung diseases such as cystic fibrosis, idiopathic pulmonary fibrosis and interstitial lung disease, among others [242, 293, 320, 321]. A number of forms of annexin A1 have been detected in BALFs [293, 320], however 33 kDa annexin A1 receives the most attention in particular, as unpublished observations suggest that this product loses the capacity to suppress inflammation [320]. The presence of truncated annexin A1 is also closely correlated with the severity of lung disease and neutrophil infiltration, with no full length protein observable

in some patients. The addition of BALF from cystic fibrosis patients to purified annexin A1 resulted in the cleavage to a 33kDa form, suggesting a protease within the BALF was responsible for generating the cleavage product. N-terminal sequencing of the truncated annexin A1 species identified Ser-37 as the N-terminus, which was attributed to neutrophil elastase activity, a component of azurophil granules, as the addition of exogenous elastase generated a product of similar molecular weight [242]. As much of the lung damage that occurs in cystic fibrosis is the result of massive neutrophil infiltration, it is not surprising that neutrophil elastase activity was observed in BALF from these patients and was responsible for annexin A1 cleavage [322]. These experiments do not preclude the possibility that other forms of annexin A1 were present before full neutrophil degranulation and elastase cleavage, although the results do suggest that the lack of anti-inflammatory, full length annexin A1 in inflammatory disorders is due to increased protease content and may exacerbate the inflammatory response. Alternatively, elastase-cleaved annexin A1 may also possess the ability to activate endothelial ERK and should have been investigated alongside calpain I, cathepsin D and plasmin, as a truncated form of annexin A1 with Ser-37 was also found from mass spectrometry analysis of fMLP-stimulated neutrophil conditioned medium.

6.4. Regulation of annexin A1 by glucocorticoids

Many of the studies focussing on the anti-inflammatory and antimigratory effects of annexin A1 have been formulated based on the involvement of annexin A1 in glucocorticoid-mediated anti-inflammation. Annexin A1 is abundantly expressed in the anterior pituitary gland and localises specifically to folliculostellate cells [323], but it is also found in other areas of the brain in lower amounts, such as the hypothalamus [324]. Annexin A1 synthesis is induced in a number of cell types following treatment with glucocorticoids [325-327] and has itself been shown to mimic the anti-inflammatory effects of glucocorticoids [328]. These findings were further supported by studies utilising the annexin A1 knockout mouse, where cycloxygenase-2 levels were elevated and glucocorticoids lost their efficacy in inhibiting inflammation in several models of acute inflammation [219, 220]. In light of these findings, combined with the observation that leukocytes from adrenalectomised rats displayed reduced levels of annexin A1 protein [329], it was postulated that annexin A1 was potentially involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Cumulatively, these studies suggested that annexin A1 was a key molecule involved in mediating the anti-inflammatory effects of glucocorticoids. However in an

interesting study performed by Solito *et al*, it was shown that phosphorylation on residue 27 was required for the lipidation and translocation of annexin A1 to the plasma membrane in a murine pituitary folliculostellate cell line [209]. Whilst the phosphorylation event was the primary finding of the study, it was intriguing that the process was induced by LPS. In a previous study by the same group, annexin A1 was similarly translocated to the plasma membrane following treatment of the same pituitary folliculostellate cell line with glucocorticoids [330]. This effectively suggested that the pro-inflammatory molecule, LPS, was mimicking the actions of glucocorticoids by promoting the release of anti-inflammatory annexin A1 from this cell line. This of course is entirely paradoxical, as recognised by the authors and it was reasoned that the temporal responses of annexin A1 activation by LPS and glucocorticoids are different and therefore may provide an explanation for this inconsistency. LPS rapidly induced maximal annexin A1 translocation 5 min post-treatment [209], but the response induced by glucocorticoids was slow-onset and sustained for several hours. Although not addressed by the authors, it would be interesting to investigate whether LPS-stimulated annexin A1 was processed differently at the plasma membrane compared to annexin A1 induced by glucocorticoids, as these cell types are known to secrete proteins to facilitate paracrine/juxtacrine signalling in the HPA axis. An early study investigating the anti-inflammatory effects of annexin A1 showed that dexamethasone treatment of lung adenocarcinoma cells caused the translocation of full length and a truncated form to the plasma membrane, however it was not determined which forms if any were secreted [331]. This was yet another study that highlighted the ambiguous nature of annexin A1 mobilisation in inflammation, however the suggestion that this could be the result of temporal regulation by opposing stimuli may provide an explanation for inconsistencies in the literature, particularly in relation to FPRL-1 signalling, as addressed in section 6.5.

Not all regulation of annexin A1 expression and secretion is mediated by glucocorticoids however. One group in particular has shown that increased levels of annexin A1 present in colon samples correlated with the degree of inflammation induced by trinitrobenzenesulfonic acid (TNBS) in a rat experimental model of colitis [198]. This occurred regardless of glucocorticoid treatment or whether the animals had been adrenalectomised or hypophysectomised [197]. This indicated that glucocorticoids and other pituitary factors were not involved in the regulation of annexin A1 in this setting. It was observed that along with the ~9-fold increase in annexin A1 expression in inflamed colon specimens, a soluble, truncated form of annexin A1 was also detected. This truncated form was only found in the inflamed colon and was not present in non-inflamed control samples. The presence of the truncated form was also unaffected by

glucocorticoid treatment [197], indicating that another process independent of the HPA axis was involved in the generation of this product. A further study carried out by the same group found that annexin A1 levels were similar in resident colonic cells from control and inflamed colon samples, therefore the increased annexin A1 expression was not due to these structural cell types [332]. However it was found that neutrophils, along with a small portion of macrophages, were the major type of infiltrating leukocytes in TNBS-induced colitis in rats and were responsible for the increased expression and secretion of annexin A1 in these areas [332]. The lack of effect of glucocorticoids on the generation of truncated annexin A1 expression was interesting, as another study showed that in adrenalectomised rats the annexin A1 content was reduced in all subsets of leukocytes with the exception of neutrophils, suggesting that neutrophil annexin A1 may be regulated by factors distinct from the HPA axis [329]. Therefore the truncated annexin A1 observed in the inflamed colon of TNBS-treated rats may have been the result of neutrophil activity and provides a mechanism for the heightened degree of inflammation and lack of effect of glucocorticoids on annexin A1 levels in this model.

6.5. Pleiotropic signalling through the FPRs

All FPRL-1 agonists identified to date have been shown to elicit a Ca^{2+} flux upon binding to the receptor, however the ultimate effect of this signalling is as varied due to the range of molecules to which it can bind. FPRL-1 was originally described as a low-affinity receptor for the formylated fMLP peptide, requiring micromolar amounts as opposed to nanomolar amounts needed for FPR activation. Since its identification, many agonists and antagonists have been shown transduce both pro- and anti-inflammatory effects through this receptor [190]. LL-37 and SAA are both pro-inflammatory FPRL-1 agonists that induce a chemotactic response in neutrophils and serum levels of both proteins have been shown to dramatically increase during inflammatory events. SAA has the capacity to induce NFκB activity, which results in the activation of the ERK and p38 MAPK pathways and ultimately transcription of the pro-inflammatory cytokine IL-8 [279]. However LXA4, an anti-inflammatory protein that is also a FPRL-1 agonist, had the ability to inhibit ERK 1/2 and p38 phosphorylation induced by SAA, suggesting that inflammation could be modulated by competitive binding of pro- and anti-inflammatory molecules at this receptor. Another study has shown that LXA4 could inhibit TNF-α induced pro-inflammatory effects despite TNF-α not being a FPRL-1 agonist [333], indicating that LXA4 might also use a receptor distinct from the FPRs. SAA has also been shown to

stimulate the generation of TNF-α and IL-10 through FPRL-1, which are pro- and anti-inflammatory cytokines respectively [334]. It was found however that the upregulation of TNF-α was the result of activation of the ERK signalling pathway, whereas IL-10 generation was dependent on the activation of MAPK p38 pathway. The generation of these two cytokines appeared to be regulated temporally however, and suggests that different agonists to FPRL-1 can induce the activation of various signalling cascades, highlighting that this receptor is pivotal in mediating different signals in the inflammatory response.

A similar contradictory theme is also observed with annexin A1, where the anti-inflammatory effects of annexin A1 and N-terminal peptides are mediated through FPRL-1 signalling events [252], however some observations have also been made where annexin A1 induces pro-inflammatory responses in cells. Ac2-26 was shown to activate monocytes and induce a chemotactic response through the FPRs [189], which ultimately desensitised the cells to fMLP-stimulated chemotaxis and resulted in decreased leukocyte accumulation. Following TNF-α stimulation, annexin A1 has also been shown to induce matrix metalloprotease secretion in rheumatoid arthritis synovial fibroblasts following FPRL-1 binding and induction of the ERK signalling pathway, among others [335]. Adding a further layer of complexity to the annexin A1 story, an N-terminal peptide Ac9-25 was found to activate neutrophil NADPH oxidase *and* inhibit this signal at the same time, however the latter was postulated to be mediated by a receptor to the distinct from the FPRs [336]. Annexin A1 has also been shown to activate FPR in human lung cells, which resulted in the production of the acute phase protein, fibrinogen, in a similar manner to IL-6 [337].

The wide range of effects observed following FPRL-1 activation may also be augmented due to the cell types expressing the receptor. In addition to the phagocytic leukocytes, FPRL-1 expression has been found in microvascular endothelial cells, epithelial cells, lymphocytes, folliculostellate cells and astrocytes [275]. Therefore, the assortment of agonists shown to transduce pro- and anti-inflammatory signals through FPRL-1 in a temporal manner, combined with the diverse tissue distribution of this receptor, highlights why such diverse signals can be achieved through FPRL-1. The exact mechanisms by which FPRL-1 differentially responds to its various agonists still remains to be elucidated.

6.6 Annexin A1-deficient mice

Despite our findings, there is an abundance of literature detailing increased inflammatory activity occurring in the annexin A1 KO mouse [220] and other annexin A1 knock-out studies where neutrophils isolated from the annexin A1 KO mouse demonstrated spontaneous migratory behaviour and an exaggerated response to inflammatory stimuli *in vitro* [338]. One would assume that if annexin A1 was a vital protein involved in facilitating neutrophil extravasation, its removal would result in the opposite to that published and a degree of neutropenia would be observed. However, an upregulation of other annexins, namely annexins 2, 4, 5 and 6, were detected in the annexin A1 KO mouse [220], hence it is possible that one of these annexins or other annexin A1-independent mechanisms of neutrophil transendothelial migration are utilised when annexin A1 is unavailable. If this is the case, the degree of transendothelial migration would also be increased by the lack of anti-inflammatory annexin A1 regulated by glucocorticoids and the HPA axis to assist in resolving the inflammatory event. Given the high level of annexin A3 expression in neutrophils (approximately 1% total cytosolic protein [169]) and that annexin A3 was detected in our screen of stimulated neutrophil conditioned medium together with annexin A1, it would be interesting to determine if this member of the annexin family can compensate for annexin A1 loss and whether it can be processed and released in a similar manner from neutrophils. Indeed it has been shown that annexin A3 expression was increased in the inflamed colon of rats treated with TNBS, in a similar manner as annexin A1 [198]. To attempt to address this possibility, HEK293 cells stably overexpressing StrepII-tagged annexin A3 were generated, however the purification of recombinant annexin A3 proved problematic. The fact that annexin A3 is present in stimulated neutrophil conditioned medium indicates that it is secreted, which is consistent with the findings from studies showing calcium-dependent annexin A3 association with neutrophil granules and translocation to the plasma membrane upon cell activation [171, 339]. Therefore annexin A3 would be an interesting candidate to investigate for a potential role in leukocyte transendothelial migration, however it may require processing reminiscent of annexin A1 to be active.

Another issue to take into consideration is the differences between the mouse and human immune systems. Whilst the murine immune system mirrors the human system remarkably well, there have been a number of reports that describe subtle immunological variations between the two [340-342], which have been extensively reviewed by Mestas and Hughes [343]. One study identified that mice treated with interferon-γ antibodies displayed an exacerbation of experimental allergic encephalomyelitis and equally, systemic treatment with interferon-γ

delayed disease onset [344]. This suggested a protective anti-inflammatory role for the cytokine, however it was found that the administration of interferon-γ to human subjects presenting with multiple sclerosis led to a worsening of the disease state [345]. This highlights that while mouse models are important for the preclinical modelling of human disease, direct extrapolation to human conditions should be done so with caution. This is of particular relevance to the area of annexin A1 modulation of immune function, as there are significant differences in the FPR family of receptors in humans and mice and variations in the neutrophil-mediated inflammatory response. The FPR family in humans currently consists of FPR, FPRL-1 and FPRL-2, however there are seven structurally divergent members in mice which display different binding affinities for ligand [190, 346]. A difference in blood leukocyte composition between humans and mice has also been found, with neutrophils comprising 50-70% of total leukocytes in humans but only 10-25% in mice [347]. It is unclear if this deviation has any functional consequences, however given the importance of neutrophils in the early stages of the human inflammatory response, this would be an interesting aspect to investigate. This may indicate that murine models of inflammation have an increased reliance on lymphocyte-mediated inflammation, therefore neutrophil-mediated inflammation in mouse models would not be entirely comparable to that in humans. Consequently the data from annexin A1 deficient mice, the effects on neutrophils and relevance to the human immune system are probably not as straightforward as has previously been suggested, although it does highlight that annexin A1-targeted therapies for the treatment of aberrant inflammation warrant further investigation.

6.7. Proposed model for annexin A1-mediated neutrophil transendothelial migration

The results generated in this study have expanded upon the current knowledge of annexin A1 proteolysis and secretion from neutrophils and the effect of this cleavage product on endothelial cells to modulate neutrophil extravasation.

6.7.1. Secretion of annexin A1 from neutrophils

Secretion of truncated annexin A1 can be induced by either fMLP or IL-8, indicating that signalling through the FPRs and IL-8 receptor can transduce annexin A1 mobilisation in activated neutrophils (Figure 6.1). As shown by previous studies, FPR and IL-8 receptor ligation results in an intracellular Ca^{2+} flux, releasing truncated annexin A1 as a consequence, however it

is still unclear what the precise sequence of events are that lie between the Ca^{2+} signalling and annexin A1 secretion. In view of the current literature, it appears that annexin A1 is phosphorylated following cell stimulation, which then causes its translocation to the plasma membrane. Phospholipid-bound annexin A1 has an increased susceptibility to proteolysis, which is most likely the key event that liberates the truncated protein from the neutrophil plasma membrane. Annexin A1 proteolysis may also occur in the membranes of certain neutrophil granules, resulting in localised production of truncated annexin A1 within the granules. Truncated annexin A1 could then be secreted in a regulated manner following neutrophil activation and granule exocytosis. Results from this study indicate that calpain I is responsible for annexin A1 cleavage, as its inhibition prevents the release of active annexin A1 from neutrophils.

6.7.2. Annexin A1 induced endothelial ERK-activation

Truncated annexin A1 secreted from activated neutrophils binds to FPR/s present on the endothelium, most likely FPRL-1. This results in the activation of the ERK 1/2 signalling pathway, which is essential for neutrophil transendothelial migration. ERK 1/2 activation possibly signals to components of the cytoskeleton, which then impacts on the formation and lateral movement of ICAM-enriched projections on the endothelial surface to capture neutrophils and guide their transmigration through the vasculature (Figure 6.2).

This and other studies discussed in chapter 6 highlight contradictory roles of annexin A1 and its endothelial receptor in the context of inflammation. Annexin A1 appears to display opposing functions depending on the cell type, factor that induced its activation and the period of time following the activation event. It is therefore proposed that annexin A1 is functioning in two distinct capacities;

1) *Pro-inflammatory annexin A1*. This form of annexin A1 is rapidly mobilised, is generated in response to pro-inflammatory mediators and promotes leukocyte trafficking to areas of inflammation through effects on vasculature and chemotactic activation of additional leukocytes expressing FPRs. Annexin A1 displaying pro-inflammatory properties is modified by proteolysis and is most likely limited to immune cells containing such proteases and are capable of rapid exocytosis of granular or vesicular contents, such as granulocytes.

2) *Anti-inflammatory annexin A1*. This species of annexin A1 is inducible by glucocorticoids and is involved in mediating the anti-inflammatory effects of these molecules. Annexin A1 can also

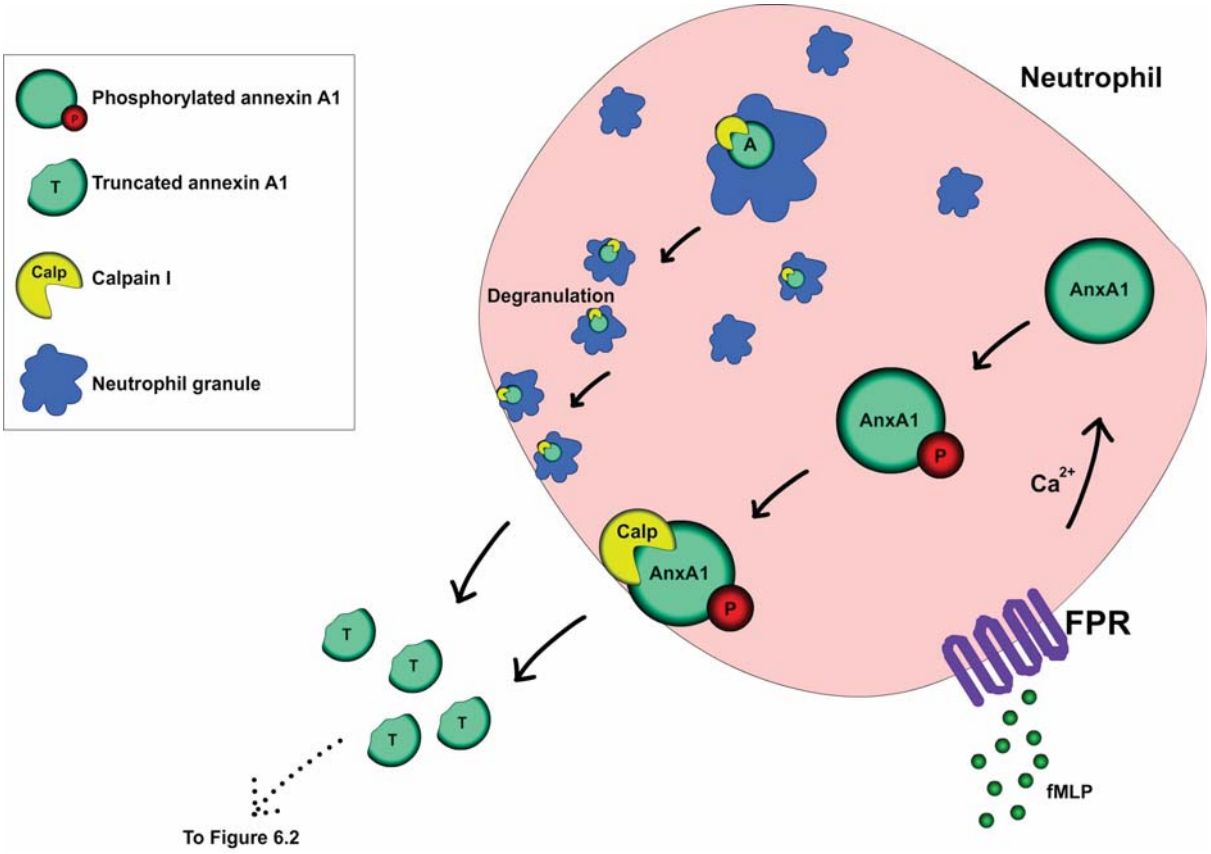


Figure 6.1: Proposed mechanism of truncated annexin A1 secretion from stimulated neutrophils.

Secretion of truncated annexin A1 can be induced by either fMLP or IL-8 (not shown), where receptor ligation results in an intracellular Ca^{2+} flux. Cytosolic annexin A1 is then phosphorylated and translocated to the plasma membrane. Phospholipid-bound annexin A1 has an increased susceptibility to proteolysis and is cleaved at the plasma membrane by calpain I, resulting in its secretion. Annexin A1 proteolysis may also occur in the membranes of neutrophil granules, resulting in localised production of truncated annexin A1 within the granules. Truncated annexin A1 could then be secreted following neutrophil activation and granule exocytosis.

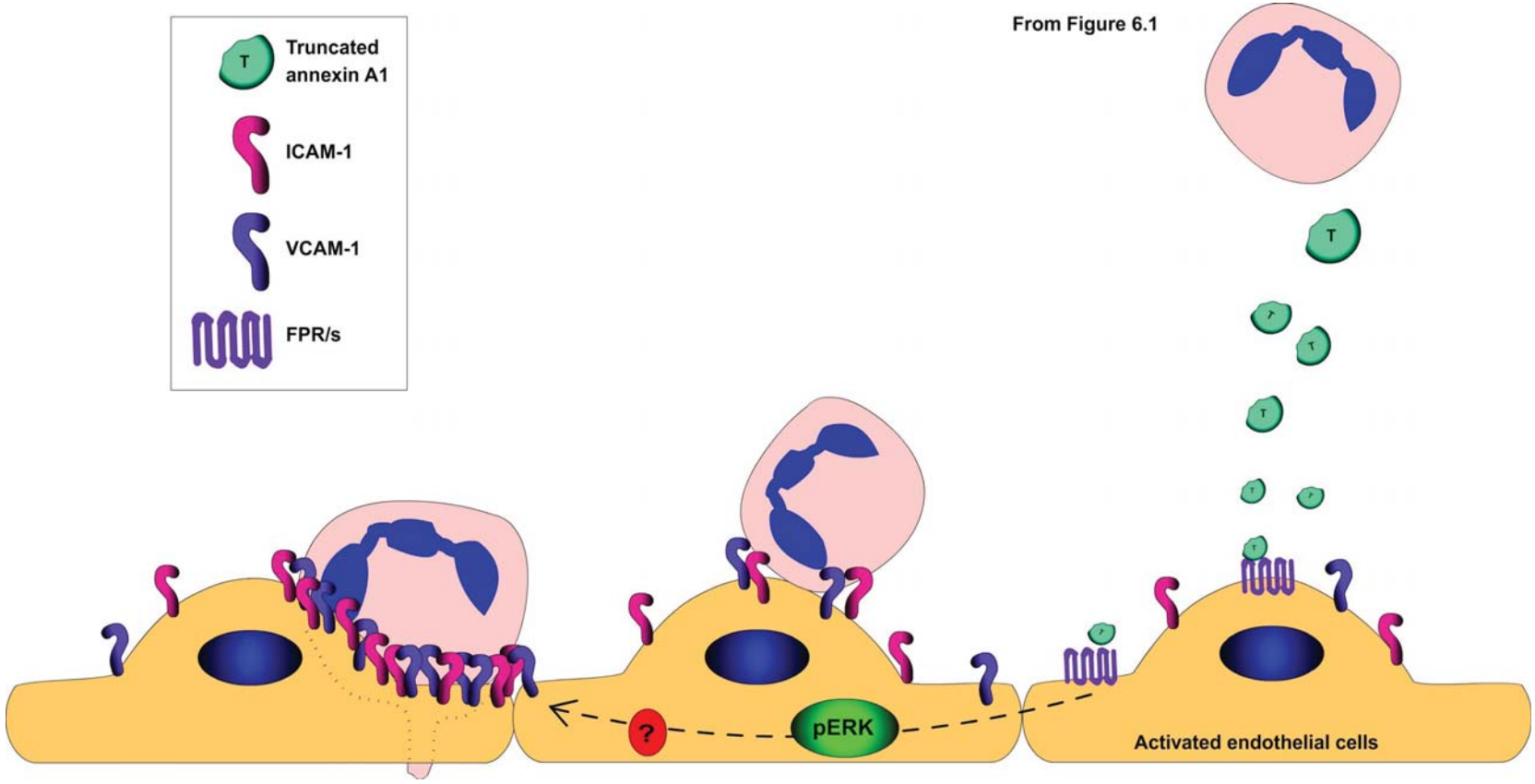


Figure 6.2: Proposed mechanism of truncated annexin A1 effects on the endothelium.
Truncated annexin A1 secreted from stimulated neutrophils binds to FPR/s present on activated endothelial cells, most likely FPRL-1. This results in the activation of the ERK1/2 signalling pathway, which possibly signals to components of the cytoskeleton, indicated by (?). This then modulates the formation and lateral movement of ICAM-1 and VCAM-1-enriched transmigratory cups on the endothelial surface to capture neutrophils and facilitate transmigration through the vasculature

behave in an anti-inflammatory capacity in areas where the inflammatory response is hyperactive and functions to desensitise the FPRs and inhibit the trafficking of cells expressing this receptor family to the area. Anti-inflammatory annexin A1 is full length and is primarily secreted by cells involved in hormone regulation and signalling within the HPA axis.

Post-translational modifications of annexin A1, such as proteolysis or lipidation, are important features that provide an additional degree of control. Such post-translational modifications are most likely the defining process that determines the pro-inflammatory or anti-inflammatory effects of annexin A1, a concept particularly emphasised by the results generated in this study.

6.8. Annexin A1-related therapies to treat inflammatory disorders

The findings from this study and others have revealed a number of aspects related to annexin A1 signalling that could be exploited in the development of therapies to treat inflammatory disorders. In line with the concept that has been extensively covered in the literature, full length annexin A1 could be administered at sites of inflammation to exert a localised anti-inflammatory effect, or it could be introduced systemically to desensitise the FPRs and consequently reduce the level of neutrophil extravasation overall. Similarly, FPR receptor antagonists could also be employed to interfere with FPR-ligand binding, which would ultimately achieve an anti-inflammatory effect by reducing neutrophil activation and prevent truncated annexin A1 from activating endothelial ERK. An alternative option may be to develop mAbs or other compounds targeted specifically to truncated annexin A1. This could potentially be a more effective approach, as the pro-inflammatory form of annexin A1 would be rendered inactive whilst the full length anti-inflammatory protein will remain and be able to assist in the resolution of the inflammatory event.

CONCLUSION

The experiments conducted in this study have identified a novel role for annexin A1 in the transendothelial migration of neutrophils. Although previously thought to be an inactive degradation product observed in a number of disease states, truncated annexin A1 secreted from stimulated neutrophils was found to possess pro-inflammatory activity and could activate endothelial ERK to promote neutrophil transmigration. Full length annexin A1, which has been previously shown to possess potent anti-inflammatory activity, could not activate endothelial ERK in the same manner as the truncated form of the protein. Calpain I was also shown to have an important function in this process, as the proteolysis of recombinant annexin A1 gave rise to an unidentified cleavage product with the capacity to activate endothelial ERK. Furthermore, the specific inhibition of neutrophil calpain I during neutrophil stimulation prevented the release of active annexin A1, thus supporting a role for calpain I in generating annexin A1 with pro-inflammatory activity. It was also shown that pro-inflammatory annexin A1 activated endothelial ERK through the FPR family, which was an interesting finding given that previous studies have demonstrated that full length annexin A1 exerts its anti-inflammatory effects through this receptor family. The inhibition of annexin A1 activity with blocking Abs was also found to decrease the number of transmigratory cups and the intensity of ICAM-1 staining within them, consequently reducing the level of neutrophil adhesion and emigration across endothelial monolayers *in vitro*. This implicated annexin A1 in the formation of endothelial transmigratory cups and highlighted that it was required for efficient neutrophil transendothelial migration.

FUTURE DIRECTIONS

- Before investigating additional endothelial effects induced by annexin A1, the experiments outlined in this thesis should be repeated using a flow chamber model to mimic the effects of blood flow and shear stress encountered *in vivo*. It has already been shown that endothelial ERK activation is required for neutrophil transmigration under shear; however it would be interesting to determine how fMLP-stimulated neutrophils would perform in this assay and the effects on the ratio of paracellular to transcellular transmigration. The inclusion of annexin A1 Ab in the flow chamber model would confirm whether annexin A1 is inhibiting neutrophil transmigration or promoting neutrophil extravasation, as widely reported in the literature. The rolling velocity and amount of adherent neutrophils should also be determined, as the annexin A1 Ab could potentially interfere with one of these steps in the leukocyte recruitment cascade. Monitoring the real-time movement of ICAM-1 and VCAM-1 in the presence of the annexin A1 Ab would also determine if it is indeed affecting the ability of these proteins to cluster around adherent neutrophils. This could be achieved by using fluorophore-conjugated antibodies or by expressing ICAM-1/VCAM-1 tagged with fluorescent proteins in an endothelial cell line that is more amenable to transfection than HUVEC.
- If it is found that the annexin A1 Ab inhibits ICAM-1/VCAM-1 clustering around adherent neutrophils, potential cytoskeletal remodelling events induced by ERK activation should be investigated, along with signalling molecules such as Rac1 and Rho.
- Many studies describing anti-inflammatory effects of annexin A1 have used full length protein and N-terminal peptides. Administering calpain-cleaved annexin A1 or annexin A1 devoid of the first 26 amino acids to mice in the same manner should be performed to determine if this increases neutrophil accumulation in inflamed tissues.
- As annexin A1-deficient mice display an exaggerated response to pro-inflammatory stimuli and also show an upregulation of other annexins, it would be worthwhile determining if other members of the annexin family of proteins can compensate for annexin A1 loss, initially using experiments described in this study. Annexin A3 should be the initial candidate, as it was detected in the analysis of secreted proteins in fMLP-stimulated neutrophil conditioned medium and appears to be mobilised similarly to annexin A1. Annexin A3 deficient mice,

although not yet described, would also be useful for investigating a potential role for annexin A3 in leukocyte transmigration.

- It would be interesting to compare levels of secreted proteins from fMLP-stimulated neutrophils purified from wild-type and annexin A1-deficient mice to determine if the lack of endogenous annexin A1 alters expression and secretion of other neutrophil proteins. This experiment will also be useful in identifying if neutrophils isolated from mice and humans respond differently to proinflammatory stimuli.
- Since eosinophils have been shown to activate endothelial ERK during transendothelial migration, the possibility that this occurs through annexin A1-mediated events should be more thoroughly investigated using similar experiments to those carried out in this PhD project. Monocytes should also be examined alongside eosinophils.
- The presence of S100A8 and S100A9 proteins have been closely linked to inflammatory disorders. As S100A8, S100A9 and annexin A1 have been shown to bind to an endothelial receptor required for neutrophil transmigration [164], it would be interesting to determine if these proteins form a complex that is relevant in inflammation. The endothelial receptor should at least be given further consideration as it may provide a new therapeutic target to treat inflammatory diseases.

Chapter 7

APPENDIX

Band	Accession number	Name	Description	MW (Da)	pI	Score	Coverage (%)	Matches	Details
1	P30740	ILEU_HUMAN	Monocyte neutrophil elastase inhibitor MNEI, Leukocyte elastase inhibitor	42714.702	6.196	9.3	2.9	5	Appendix 1A, page 178
2	P04083	ANX1_HUMAN	Annexin A1, Annexin I, Lipocortin I, Calpactin I	38.558.938	7	9.3	28.4	7	Appendix 1B, page 179
3	P12429	ANX3_HUMAN	Annexin A3, Annexin III, Lipocortin III	36352.658	5.818	9.3	26.3	8	Appendix 1C, page 180
4	O00299	CLI1_HUMAN	Chloride intracellular channel protein 1	26905.748	5.135	9.3	24.5	5	Appendix 1D, page 181
5	115444	S109_HUMAN	S100 calcium binding protein A9, Calgranulin B	13233.508	6.042	9.3	51.8	5	Appendix 1E, page 182
6	115442	S108_HUMAN	S100 calcium binding protein A8, Calgranulin A	10827.649	6.987	9.3	32.3	3	Appendix 1F, page 183

Appendix 1: Mass spectrometry analysis of candidate proteins present in fMLP stimulated neutrophil conditioned medium.

Candidate bands marked in Figure 3.6 were excised, digested with trypsin and analysed by mass spectrometry. Detailed peptide analysis can be found in the indicated appendices.

Peptides	Submitted mass	Submitted charge	Experimental mass	Retention time	MW	Delta (Da)	Delta (PPM)	Start (residue)	End (residue)	Score	Ladder
TFHFNTVEEVHSR	534.902	3.0	1601.683	49.5	1601.759	0.076	47.737	57	69	14.0	41.8
IPELLASGMVDNMTK	817.894	2.0	1633.772	48.5	1633.806	0.034	20.59	144	158	7.7	34.2
IEEQLTLEK	551.787	2.0	1101.558	40.9	1101.592	0.034	30.539	246	254	90.4	79.1
LEESYTLNSDLAR	755.845	2.0	1509.674	45.8	1509.731	0.057	37.518	278	290	122.4	86.6
LGVQDLFNSSK	604.3	2.0	1206.584	48.5	1206.625	0.041	33.682	291	301	103.8	87.3

Appendix 1A: Mass spectrometry analysis of band 1– MNEI.

Details of monocyte neutrophil elastase inhibitor peptides generated during mass spectrometry analysis of Band 1 in Figure 3.6.

Peptides	Submitted mass	Submitted charge	Experimental mass	Retention time	MW	Delta (Da)	Delta (PPM)	Start (residue)	End (residue)	Score	Ladder
GGPGSAVSPYPTFNPSSDVAALHK	786.028	3.0	2355.061	44.2	2355.149	0.088	37.562	29	52	185.9	74.4
GVDEATIIDILTK	694.377	2.0	1386.738	45.9	1386.761	0.023	16.326	58	70	-1.2	38.8
GLGTDEDTLIEILASR	851.919	2.0	1701.822	48.4	1701.879	0.057	33.282	128	143	96.2	68.2
TPAQFDADELR	631.787	2.0	1261.558	25.3	1261.594	0.036	28.251	113	123	222.9	85.5
DITSDTSGDFR	607.255	2.0	1212.494	21.8	1212.526	0.032	26.095	166	176	151.8	60
SEDFGVNEDLADSDAR	870.352	2.0	1738.688	26.1	1738.728	0.04	22.799	188	203	171.7	81.2
GTDVNVFNTILTTR	775.889	2.0	1549.762	44.7	1549.81	0.048	30.74	214	227	86.1	83.6
SEIDMNDIK	532.74	2.0	1063.464	23.8	1063.486	0.022	20.349	303	311	78.4	79.1

Appendix 1B: Mass spectrometry analysis of band 2- Annexin A1.

Details of annexin A1 peptides generated during mass spectrometry analysis of Band 2 in Figure 3.6.

Peptides	Submitted mass	Submitted charge	Experimental mass	Retention time	MW	Delta (Da)	Delta (PPM)	Start (residue)	End (residue)	Score	Ladder
DYPDFSPSVDAEAIQK	891.389	2.0	1780.762	40.4	1780.816	0.054	30.122	14	29	275.5	74.1
MLISILTER	538.293	2.0	1074.57	49.6	1074.611	0.041	37.82	40	48	59.0	62.8
DISQAYYTVYK	675.81	2.0	1349.604	41.8	1349.65	0.046	33.817	127	137	138.1	78.2
SLGDDISSETSGDFR	793.328	2.0	1584.64	40.4	1584.69	0.05	31.326	139	153	128.8	68.4
SLGDDISSETSGDFRK	857.377	2.0	1712.738	49	1712.785	0.047	27.231	139	154	49.6	55.3
NTPAFLAER	509.751	2.0	1017.486	45	1017.524	0.038	36.993	249	257	132.0	76.7
GIGTDEFTLNR	611.785	2.0	1221.554	45.7	1221.599	0.045	36.544	264	274	164.9	74.5
SDTSGDYEITLLK	721.334	2.0	1440.652	47.1	1440.698	0.046	31.68	305	317	177.9	83.6

Appendix 1C: Mass spectrometry analysis of band 3- Annexin A3.

Details of annexin A3 peptides generated during mass spectrometry analysis of Band 3 in Figure 3.6.

Peptides	Submitted mass	Submitted charge	Experimental mass	Retention time	MW	Delta (Da)	Delta (PPM)	Start (residue)	End (residue)	Score	Ladder
IGNCPFSQR	511.232	2.0	1020.488	34.2	1020.481	0.033	31.986	21	29	44.7	62.8
GVTFNVTTVDTK	641.316	2.0	1280.616	37.2	1280.661	0.045	34.858	38	49	130.7	75.4
LAALNPESNTAGLDIFAK	922.965	2.0	1843.914	42.8	1843.968	0.054	29.09	96	113	222.6	72.2
NSNPALNDNLEK	664.804	2.0	1327.592	30.4	1327.637	0.045	33.625	120	131	184.0	75.4
YLSNAYAR	479.229	2.0	956.442	29.9	956.472	0.03	30.99	209	216	57.5	59.5

Appendix 1D: Mass spectrometry analysis of band 4- Chloride intracellular channel protein.

Details of chloride intracellular channel protein peptides generated during mass spectrometry analysis of Band 4 in Figure 3.6.

Peptides	Submitted mass	Submitted charge	Experimental mass	Retention time	MW	Delta (Da)	Delta (PPM)	Start (residue)	End (residue)	Score	Ladder
NIETIINTFHQYSVK	903.95	2.0	1805.884	30.9	1805.931	0.047	25.827	11	25	319.4	78.5
LGHPDTLNQGEFK	728.351	2.0	1454.686	17.8	1454.715	0.029	19.688	26	38	277.8	97
KDLQNFLK	503.28	2.0	1004.544	20.5	1004.565	0.021	20.547	43	50	137.9	105.4
VIEHIMEDLDTNADK	871.891	2.0	1741.766	23.2	1741.819	0.053	30.222	58	72	309.1	100
LTWASHEK	486.241	2.0	970.466	16.1	970.487	0.021	21.268	86	93	53.4	83.8

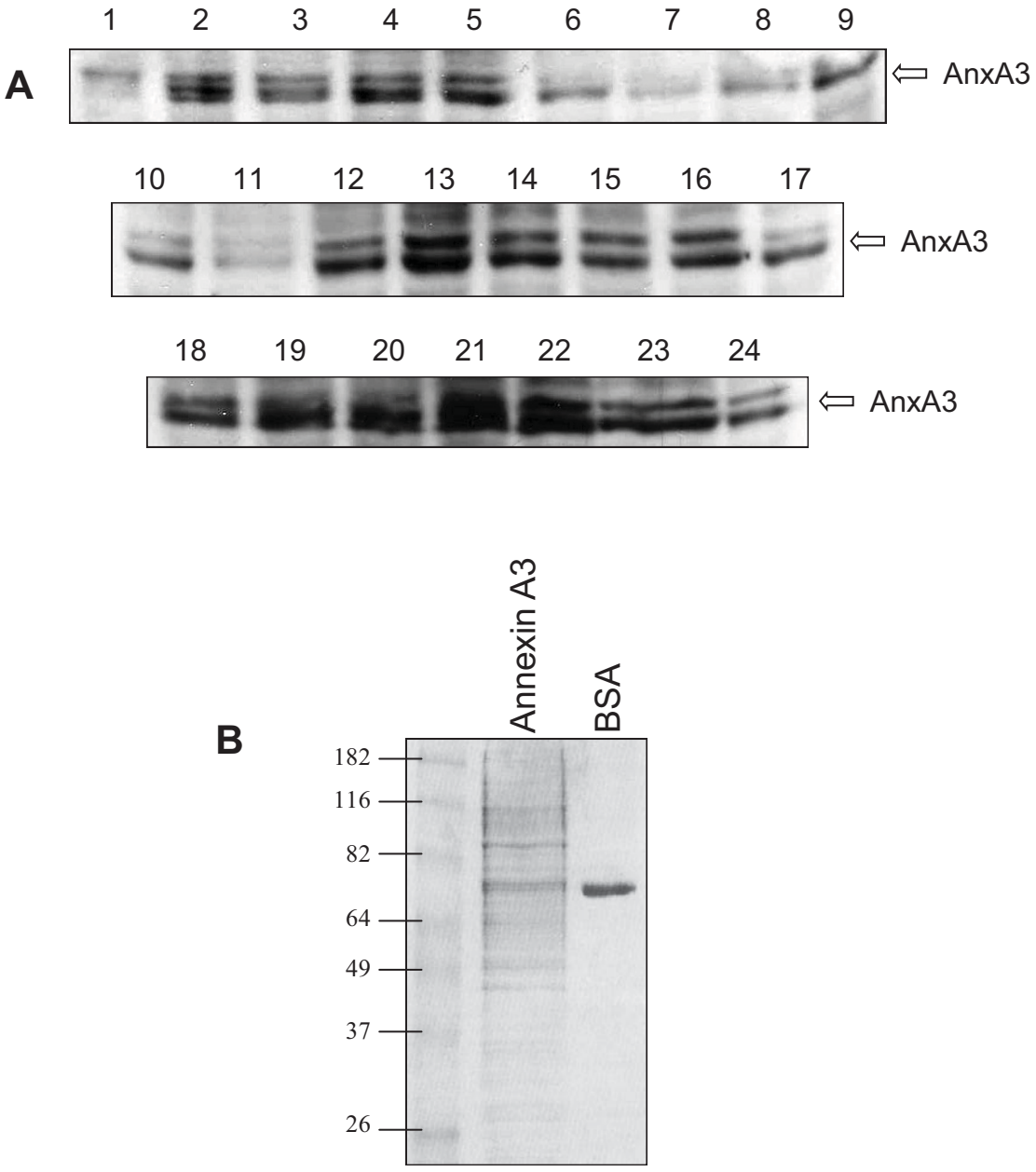
Appendix 1E: Mass spectrometry analysis of band 5- S100A9.

Details of S100A9 peptides generated during mass spectrometry analysis of Band 5 in Figure 3.6.

Peptides	Submitted mass	Submitted charge	Experimental mass	Retention time	MW	Delta (Da)	Delta (PPM)	Start (residue)	End (residue)	Score	Ladder
ALNSIIDVYHK	636.838	2.0	1271.66	23	1271.687	0.027	20.949	8	18	231.3	85.5
GNFHAVYR	482.229	2.0	962.442	15.9	962.472	0.03	30.797	24	31	103.8	73
LLETECPQYIR	682.829	2.0	1363.642	23.6	1363.681	0.039	28.336	37	47	139.6	80

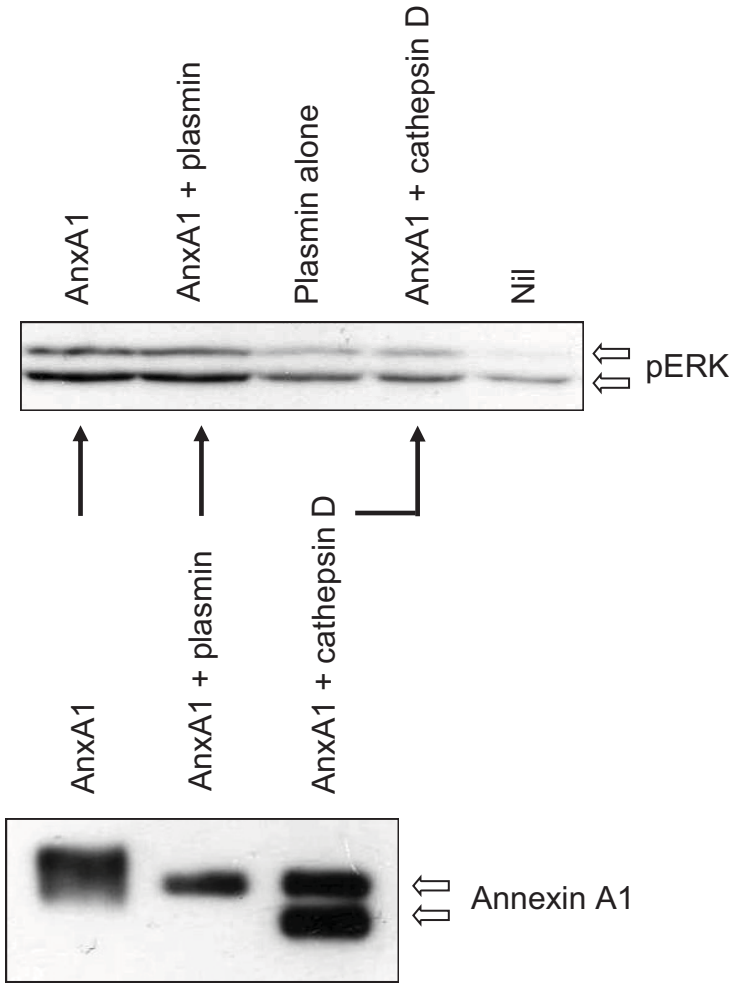
Appendix 1F: Mass spectrometry analysis of band 6- S100A8.

Details of S100A8 peptides generated during mass spectrometry analysis of Band 6 in Figure 3.6.



Appendix 2: Overexpression and purification of recombinant StrepII-tagged annexin A3.

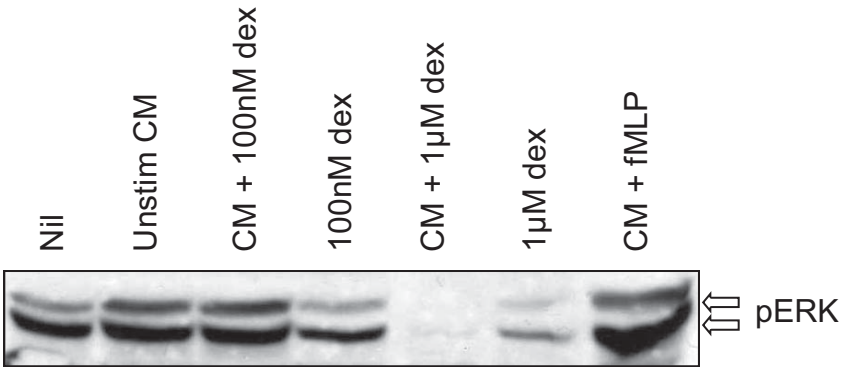
A) Strep-II-tagged annexin A3 was cloned into pcDNA3 and transfected into HEK293 cells, then grown in the presence of G418 to select for stable transformants. Isolated colonies were propagated individually and equal amounts of total protein from each clone were analysed for annexin A3 expression by western blotting using an anti-StrepII-HRP conjugated antibody.
B) Protein purified using StrepII purification spin columns (Merck, Germany), compared to 1µg BSA protein standard.



Appendix 3: Cathepsin D and plasmin-cleaved annexin A1 do not activate endothelial ERK.

Upper panel- Resting HUVEC monolayers were treated with 20µg partially purified annexin A1 (AnxA1), 20µg plasmin-cleaved purified annexin A1 (AnxA1 + plasmin), plasmin alone, 20µg cathepsin D-cleaved purified annexin A1 (AnxA1 + cathepsin D) and PBS (Nil). HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.

Lower panel– Samples tested on HUVEC were analysed for annexin A1 content by western blotting with an Ab to annexin A1.



Appendix 4: Dexamethasone inhibits basal phospho-ERK levels in HUVEC.

Resting HUVEC monolayers were treated with PBS (Nil), unstimulated-neutrophil conditioned medium (Unstim CM), fMLP-stimulated neutrophil conditioned medium (CM+fMLP), or dexamethasone at the indicated concentrations and combination with fMLP-stimulated neutrophil conditioned medium. Unstim CM and Nil highlight high basal phospho-ERK levels. HUVEC ERK activation was determined by western blotting whole cell lysates with an Ab to phosphorylated ERK.

REFERENCES

1. Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A. (1999) Phylogenetic perspectives in innate immunity. *Science*. **284**(5418): p. 1313-8.
2. Fox, A. and Harrison, L.C. (2000) Innate immunity and graft rejection. *Immunol Rev*. **173**: p. 141-7.
3. Faurschou, M. and Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*. **5**(14): p. 1317-27.
4. Albelda, S.M., Smith, C.W., and Ward, P.A. (1994) Adhesion molecules and inflammatory injury. *Faseb J*. **8**(8): p. 504-12.
5. Edens, H.A. and Parkos, C.A. (2003) Neutrophil transendothelial migration and alteration in vascular permeability: focus on neutrophil-derived azurocidin. *Curr Opin Hematol*. **10**(1): p. 25-30.
6. Roos, D., van Bruggen, R., and Meischl, C. (2003) Oxidative killing of microbes by neutrophils. *Microbes Infect*. **5**(14): p. 1307-15.
7. Bicknell, S., van Eeden, S., Hayashi, S., Hards, J., English, D., and Hogg, J.C. (1994) A non-radioisotopic method for tracing neutrophils in vivo using 5'-bromo-2'-deoxyuridine. *Am J Respir Cell Mol Biol*. **10**(1): p. 16-23.
8. Coxon, A., Tang, T., and Mayadas, T.N. (1999) Cytokine-activated endothelial cells delay neutrophil apoptosis in vitro and in vivo. A role for granulocyte/macrophage colony-stimulating factor. *J Exp Med*. **190**(7): p. 923-34.
9. Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M., and Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest*. **83**(3): p. 865-75.
10. Bazzoni, F., Cassatella, M.A., Rossi, F., Ceska, M., Dewald, B., and Baggiolini, M. (1991) Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J Exp Med*. **173**(3): p. 771-4.
11. Djeu, J.Y., Serbousek, D., and Blanchard, D.K. (1990) Release of tumor necrosis factor by human polymorphonuclear leukocytes. *Blood*. **76**(7): p. 1405-9.
12. Heyworth, P.G., Cross, A.R., and Curnutte, J.T. (2003) Chronic granulomatous disease. *Curr Opin Immunol*. **15**(5): p. 578-84.
13. Zigmond, S.H. (1977) Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J Cell Biol*. **75**(2 Pt 1): p. 606-16.
14. Lee, J., Ishihara, A., and Jacobson, K. (1993) How do cells move along surfaces? *Trends Cell Biol*. **3**(11): p. 366-70.
15. Maxfield, F.R. (1993) Regulation of leukocyte locomotion by Ca2+. *Trends Cell Biol*. **3**(11): p. 386-91.
16. Devreotes, P.N. and Zigmond, S.H. (1988) Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. *Annu Rev Cell Biol*. **4**: p. 649-86.
17. Sengelov, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C., and Borregaard, N. (1995) Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J Immunol*. **154**(8): p. 4157-65.
18. Cowburn, A.S., Condliffe, A.M., Farahi, N., Summers, C., and Chilvers, E.R. (2008) Advances in neutrophil biology: clinical implications. *Chest*. **134**(3): p. 606-12.

19. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science*. **303**(5663): p. 1532-5.
20. Liu, Y., Shaw, S.K., Ma, S., Yang, L., Luscinskas, F.W., and Parkos, C.A. (2004) Regulation of leukocyte transmigration: cell surface interactions and signaling events. *J Immunol*. **172**(1): p. 7-13.
21. Muller, W.A. (2003) Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol*. **24**(6): p. 327-34.
22. Sica, A., Matsushima, K., Van Damme, J., Wang, J.M., Polentarutti, N., Dejana, E., Colotta, F., and Mantovani, A. (1990) IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. *Immunology*. **69**(4): p. 548-53.
23. Woolf, E., Grigorova, I., Sagiv, A., Grabovsky, V., Feigelson, S.W., Shulman, Z., Hartmann, T., Sixt, M., Cyster, J.G., and Alon, R. (2007) Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat Immunol*. **8**(10): p. 1076-85.
24. Hu, X., Zhang, Y., Cheng, D., Ding, Y., Yang, D., Jiang, F., Zhou, C., Ying, B., and Wen, F. (2008) Mechanical stress upregulates intercellular adhesion molecule-1 in pulmonary epithelial cells. *Respiration*. **76**(3): p. 344-50.
25. Simon, S.I. and Goldsmith, H.L. (2002) Leukocyte adhesion dynamics in shear flow. *Ann Biomed Eng*. **30**(3): p. 315-32.
26. Kansas, G.S. (1996) Selectins and their ligands: current concepts and controversies. *Blood*. **88**(9): p. 3259-87.
27. Bevilacqua, M.P., Stengelin, S., Gimbrone, M.A., Jr., and Seed, B. (1989) Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science*. **243**(4895): p. 1160-5.
28. Patel, K.D., Nollert, M.U., and McEver, R.P. (1995) P-selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. *J Cell Biol*. **131**(6 Pt 2): p. 1893-902.
29. Bullard, D.C., Kunkel, E.J., Kubo, H., Hicks, M.J., Lorenzo, I., Doyle, N.A., Doerschuk, C.M., Ley, K., and Beaudet, A.L. (1996) Infectious susceptibility and severe deficiency of leukocyte rolling and recruitment in E-selectin and P-selectin double mutant mice. *J Exp Med*. **183**(5): p. 2329-36.
30. Pan, J., Xia, L., and McEver, R.P. (1998) Comparison of promoters for the murine and human P-selectin genes suggests species-specific and conserved mechanisms for transcriptional regulation in endothelial cells. *J Biol Chem*. **273**(16): p. 10058-67.
31. Kunkel, E.J. and Ley, K. (1996) Distinct phenotype of E-selectin-deficient mice. E-selectin is required for slow leukocyte rolling in vivo. *Circ Res*. **79**(6): p. 1196-204.
32. Stein, J.V., Cheng, G., Stockton, B.M., Fors, B.P., Butcher, E.C., and von Andrian, U.H. (1999) L-selectin-mediated leukocyte adhesion in vivo: microvillous distribution determines tethering efficiency, but not rolling velocity. *J Exp Med*. **189**(1): p. 37-50.
33. Walcheck, B., Moore, K.L., McEver, R.P., and Kishimoto, T.K. (1996) Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1. A mechanism that amplifies initial leukocyte accumulation of P-selectin in vitro. *J Clin Invest*. **98**(5): p. 1081-7.
34. Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. **7**(9): p. 678-89.
35. Thomas, W. (2008) Catch bonds in adhesion. *Annu Rev Biomed Eng*. **10**: p. 39-57.
36. Springer, T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. **76**(2): p. 301-14.

37. Tonnesen, M.G. (1989) Neutrophil-endothelial cell interactions: mechanisms of neutrophil adherence to vascular endothelium. *J Invest Dermatol*. **93**(2 Suppl): p. 53S-58S.
38. Rainger, G.E., Fisher, A.C., and Nash, G.B. (1997) Endothelial-borne platelet-activating factor and interleukin-8 rapidly immobilize rolling neutrophils. *Am J Physiol*. **272**(1 Pt 2): p. H114-22.
39. Lorenzon, P., Vecile, E., Nardon, E., Ferrero, E., Harlan, J.M., Tedesco, F., and Dobrina, A. (1998) Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. *J Cell Biol*. **142**(5): p. 1381-91.
40. Simon, S.I., Burns, A.R., Taylor, A.D., Gopalan, P.K., Lynam, E.B., Sklar, L.A., and Smith, C.W. (1995) L-selectin (CD62L) cross-linking signals neutrophil adhesive functions via the Mac-1 (CD11b/CD18) beta 2-integrin. *J Immunol*. **155**(3): p. 1502-14.
41. Gopalan, P.K., Smith, C.W., Lu, H., Berg, E.L., McIntire, L.V., and Simon, S.I. (1997) Neutrophil CD18-dependent arrest on intercellular adhesion molecule 1 (ICAM-1) in shear flow can be activated through L-selectin. *J Immunol*. **158**(1): p. 367-75.
42. Zollner, O., Lenter, M.C., Blanks, J.E., Borges, E., Steegmaier, M., Zerwes, H.G., and Vestweber, D. (1997) L-selectin from human, but not from mouse neutrophils binds directly to E-selectin. *J Cell Biol*. **136**(3): p. 707-16.
43. Green, C.E., Pearson, D.N., Camphausen, R.T., Staunton, D.E., and Simon, S.I. (2004) Shear-dependent capping of L-selectin and P-selectin glycoprotein ligand 1 by E-selectin signals activation of high-avidity beta2-integrin on neutrophils. *J Immunol*. **172**(12): p. 7780-90.
44. Shimaoka, M., Takagi, J., and Springer, T.A. (2002) Conformational regulation of integrin structure and function. *Annu Rev Biophys Biomol Struct*. **31**: p. 485-516.
45. Henderson, R.B., Lim, L.H., Tessier, P.A., Gavins, F.N., Mathies, M., Perretti, M., and Hogg, N. (2001) The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. *J Exp Med*. **194**(2): p. 219-26.
46. Schenkel, A.R., Mamdouh, Z., and Muller, W.A. (2004) Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat Immunol*. **5**(4): p. 393-400.
47. Staunton, D.E., Dustin, M.L., Erickson, H.P., and Springer, T.A. (1990) The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell*. **61**(2): p. 243-54.
48. Diamond, M.S., Staunton, D.E., Marlin, S.D., and Springer, T.A. (1991) Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell*. **65**(6): p. 961-71.
49. Altieri, D.C., Bader, R., Mannucci, P.M., and Edgington, T.S. (1988) Oligospecificity of the cellular adhesion receptor Mac-1 encompasses an inducible recognition specificity for fibrinogen. *J Cell Biol*. **107**(5): p. 1893-900.
50. Zen, K., Babbin, B.A., Liu, Y., Whelan, J.B., Nusrat, A., and Parkos, C.A. (2004) JAM-C is a component of desmosomes and a ligand for CD11b/CD18-mediated neutrophil transepithelial migration. *Mol Biol Cell*. **15**(8): p. 3926-37.
51. Phillipson, M., Heit, B., Colarusso, P., Liu, L., Ballantyne, C.M., and Kubes, P. (2006) Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med*. **203**(12): p. 2569-75.
52. Anderson, D.C. and Springer, T.A. (1987) Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu Rev Med*. **38**: p. 175-94.
53. Hibbs, M.L., Wardlaw, A.J., Stacker, S.A., Anderson, D.C., Lee, A., Roberts, T.M., and Springer, T.A. (1990) Transfection of cells from patients with leukocyte adhesion

deficiency with an integrin beta subunit (CD18) restores lymphocyte function-associated antigen-1 expression and function. *J Clin Invest*. **85**(3): p. 674-81.
54. Furie, M.B., Tancinco, M.C., and Smith, C.W. (1991) Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration in vitro. *Blood*. **78**(8): p. 2089-97.
55. Morland, C.M., Morland, B.J., Darbyshire, P.J., and Stockley, R.A. (2000) Migration of CD18-deficient neutrophils in vitro: evidence for a CD18-independent pathway induced by IL-8. *Biochim Biophys Acta*. **1500**(1): p. 70-6.
56. Mackarel, A.J., Plant, B.J., FitzGerald, M.X., O'Connor, C.M., Martin, L., Elborn, J.S., and Gallagher, C.G. (2005) Cystic fibrosis sputum stimulates CD18-independent neutrophil migration across endothelial cells. *Exp Lung Res*. **31**(4): p. 377-90.
57. Berlin, C., Bargatze, R.F., Campbell, J.J., von Andrian, U.H., Szabo, M.C., Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L., and Butcher, E.C. (1995) alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell*. **80**(3): p. 413-22.
58. Alon, R., Kassner, P.D., Carr, M.W., Finger, E.B., Hemler, M.E., and Springer, T.A. (1995) The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J Cell Biol*. **128**(6): p. 1243-53.
59. Johnston, B., Issekutz, T.B., and Kubes, P. (1996) The alpha 4-integrin supports leukocyte rolling and adhesion in chronically inflamed postcapillary venules in vivo. *J Exp Med*. **183**(5): p. 1995-2006.
60. Allport, J.R., Muller, W.A., and Luscinskas, F.W. (2000) Monocytes induce reversible focal changes in vascular endothelial cadherin complex during transendothelial migration under flow. *J Cell Biol*. **148**(1): p. 203-16.
61. Shaw, S.K., Bamba, P.S., Perkins, B.N., and Luscinskas, F.W. (2001) Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. *J Immunol*. **167**(4): p. 2323-30.
62. Su, W.H., Chen, H.I., and Jen, C.J. (2002) Differential movements of VE-cadherin and PECAM-1 during transmigration of polymorphonuclear leukocytes through human umbilical vein endothelium. *Blood*. **100**(10): p. 3597-603.
63. Marchesi, V.T. and Gowans, J.L. (1964) The Migration of Lymphocytes through the Endothelium of Venules in Lymph Nodes: an Electron Microscope Study. *Proc R Soc Lond B Biol Sci*. **159**: p. 283-90.
64. Feng, D., Nagy, J.A., Pyne, K., Dvorak, H.F., and Dvorak, A.M. (1998) Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med*. **187**(6): p. 903-15.
65. Feng, D., Nagy, J.A., Dvorak, H.F., and Dvorak, A.M. (2002) Ultrastructural studies define soluble macromolecular, particulate, and cellular transendothelial cell pathways in venules, lymphatic vessels, and tumor-associated microvessels in man and animals. *Microsc Res Tech*. **57**(5): p. 289-326.
66. Cho, Y. and De Bruyn, P.P. (1986) Internal structure of the postcapillary high-endothelial venules of rodent lymph nodes and Peyer's patches and the transendothelial lymphocyte passage. *Am J Anat*. **177**(4): p. 481-90.
67. Greenwood, J., Howes, R., and Lightman, S. (1994) The blood-retinal barrier in experimental autoimmune uveoretinitis. Leukocyte interactions and functional damage. *Lab Invest*. **70**(1): p. 39-52.

68. Migliorisi, G., Folkes, E., Pawlowski, N., and Cramer, E.B. (1987) In vitro studies of human monocyte migration across endothelium in response to leukotriene B4 and f-Met-Leu-Phe. *Am J Pathol*. **127**(1): p. 157-67.
69. Cinamon, G., Shinder, V., Shamri, R., and Alon, R. (2004) Chemoattractant signals and beta 2 integrin occupancy at apical endothelial contacts combine with shear stress signals to promote transendothelial neutrophil migration. *J Immunol*. **173**(12): p. 7282-91.
70. Nieminen, M., Henttinen, T., Merinen, M., Marttila-Ichihara, F., Eriksson, J.E., and Jalkanen, S. (2006) Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol*. **8**(2): p. 156-62.
71. Yang, L., Froio, R.M., Sciuto, T.E., Dvorak, A.M., Alon, R., and Luscinskas, F.W. (2005) ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood*. **106**(2): p. 584-92.
72. Millan, J., Hewlett, L., Glyn, M., Toomre, D., Clark, P., and Ridley, A.J. (2006) Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains. *Nat Cell Biol*. **8**(2): p. 113-23.
73. Pober, J.S., Gimbrone, M.A., Jr., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R., and Springer, T.A. (1986) Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J Immunol*. **137**(6): p. 1893-6.
74. Mulligan, M.S., Vaporciyan, A.A., Miyasaka, M., Tamatani, T., and Ward, P.A. (1993) Tumor necrosis factor alpha regulates in vivo intrapulmonary expression of ICAM-1. *Am J Pathol*. **142**(6): p. 1739-49.
75. Nakashima, Y., Raines, E.W., Plump, A.S., Breslow, J.L., and Ross, R. (1998) Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol*. **18**(5): p. 842-51.
76. Sans, E., Delachanal, E., and Duperray, A. (2001) Analysis of the roles of ICAM-1 in neutrophil transmigration using a reconstituted mammalian cell expression model: implication of ICAM-1 cytoplasmic domain and Rho-dependent signaling pathway. *J Immunol*. **166**(1): p. 544-51.
77. Imhof, B.A. and Aurrand-Lions, M. (2004) Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol*. **4**(6): p. 432-44.
78. Sligh, J.E., Jr., Ballantyne, C.M., Rich, S.S., Hawkins, H.K., Smith, C.W., Bradley, A., and Beaudet, A.L. (1993) Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A*. **90**(18): p. 8529-33.
79. Jun, C.D., Shimaoka, M., Carman, C.V., Takagi, J., and Springer, T.A. (2001) Dimerization and the effectiveness of ICAM-1 in mediating LFA-1-dependent adhesion. *Proc Natl Acad Sci U S A*. **98**(12): p. 6830-5.
80. Hibbs, M.L., Xu, H., Stacker, S.A., and Springer, T.A. (1991) Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. *Science*. **251**(5001): p. 1611-3.
81. Carman, C.V. and Springer, T.A. (2004) A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol*. **167**(2): p. 377-88.
82. Shaw, S.K., Ma, S., Kim, M.B., Rao, R.M., Hartman, C.U., Froio, R.M., Yang, L., Jones, T., Liu, Y., Nusrat, A., Parkos, C.A., and Luscinskas, F.W. (2004) Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration. *J Exp Med*. **200**(12): p. 1571-80.

83. Barreiro, O., Yanez-Mo, M., Serrador, J.M., Montoya, M.C., Vicente-Manzanares, M., Tejedor, R., Furthmayr, H., and Sanchez-Madrid, F. (2002) Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *J Cell Biol*. **157**(7): p. 1233-45.
84. Carman, C.V., Jun, C.D., Salas, A., and Springer, T.A. (2003) Endothelial cells proactively form microvilli-like membrane projections upon intercellular adhesion molecule 1 engagement of leukocyte LFA-1. *J Immunol*. **171**(11): p. 6135-44.
85. Phillipson, M., Kaur, J., Colarusso, P., Ballantyne, C.M., and Kubes, P. (2008) Endothelial domes encapsulate adherent neutrophils and minimize increases in vascular permeability in paracellular and transcellular emigration. *PLoS ONE*. **3**(2): p. e1649.
86. Barreiro, O., Vicente-Manzanares, M., Urzainqui, A., Yanez-Mo, M., and Sanchez-Madrid, F. (2004) Interactive protrusive structures during leukocyte adhesion and transendothelial migration. *Front Biosci*. **9**: p. 1849-63.
87. Greenwood, J., Amos, C.L., Walters, C.E., Couraud, P.O., Lyck, R., Engelhardt, B., and Adamson, P. (2003) Intracellular domain of brain endothelial intercellular adhesion molecule-1 is essential for T lymphocyte-mediated signaling and migration. *J Immunol*. **171**(4): p. 2099-108.
88. McLaughlin, F., Hayes, B.P., Horgan, C.M., Beesley, J.E., Campbell, C.J., and Randi, A.M. (1998) Tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta down-regulate intercellular adhesion molecule (ICAM)-2 expression on the endothelium. *Cell Adhes Commun*. **6**(5): p. 381-400.
89. Lyck, R., Reiss, Y., Gerwin, N., Greenwood, J., Adamson, P., and Engelhardt, B. (2003) T-cell interaction with ICAM-1/ICAM-2 double-deficient brain endothelium in vitro: the cytoplasmic tail of endothelial ICAM-1 is necessary for transendothelial migration of T cells. *Blood*. **102**(10): p. 3675-83.
90. Elices, M.J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M.E., and Lobb, R.R. (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell*. **60**(4): p. 577-84.
91. van Buul, J.D., Kanters, E., and Hordijk, P.L. (2007) Endothelial signaling by Ig-like cell adhesion molecules. *Arterioscler Thromb Vasc Biol*. **27**(9): p. 1870-6.
92. Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., Tsukita, S., and Tsukita, S. (1998) Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. *J Cell Biol*. **140**(4): p. 885-95.
93. Cook-Mills, J.M. (2002) VCAM-1 signals during lymphocyte migration: role of reactive oxygen species. *Mol Immunol*. **39**(9): p. 499-508.
94. van Wetering, S., van den Berk, N., van Buul, J.D., Mul, F.P., Lommerse, I., Mous, R., ten Klooster, J.P., Zwaginga, J.J., and Hordijk, P.L. (2003) VCAM-1-mediated Rac signaling controls endothelial cell-cell contacts and leukocyte transmigration. *Am J Physiol Cell Physiol*. **285**(2): p. C343-52.
95. Abdala-Valencia, H. and Cook-Mills, J.M. (2006) VCAM-1 signals activate endothelial cell protein kinase Calpha via oxidation. *J Immunol*. **177**(9): p. 6379-87.
96. Deem, T.L. and Cook-Mills, J.M. (2004) Vascular cell adhesion molecule 1 (VCAM-1) activation of endothelial cell matrix metalloproteinases: role of reactive oxygen species. *Blood*. **104**(8): p. 2385-93.
97. Cuvelier, S.L., Paul, S., Shariat, N., Colarusso, P., and Patel, K.D. (2005) Eosinophil adhesion under flow conditions activates mechanosensitive signaling pathways in human endothelial cells. *J Exp Med*. **202**(6): p. 865-76.

98. Cuvelier, S.L. and Patel, K.D. (2001) Shear-dependent eosinophil transmigration on interleukin 4-stimulated endothelial cells: a role for endothelium-associated eotaxin-3. *J Exp Med*. **194**(12): p. 1699-709.
99. Stein, B.N., Gamble, J.R., Pitson, S.M., Vadas, M.A., and Khew-Goodall, Y. (2003) Activation of endothelial extracellular signal-regulated kinase is essential for neutrophil transmigration: potential involvement of a soluble neutrophil factor in endothelial activation. *J Immunol*. **171**(11): p. 6097-104.
100. Muller, W.A., Weigl, S.A., Deng, X., and Phillips, D.M. (1993) PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med*. **178**(2): p. 449-60.
101. DeLisser, H.M., Newman, P.J., and Albelda, S.M. (1994) Molecular and functional aspects of PECAM-1/CD31. *Immunol Today*. **15**(10): p. 490-5.
102. Watt, S.M., Gschmeissner, S.E., and Bates, P.A. (1995) PECAM-1: its expression and function as a cell adhesion molecule on hemopoietic and endothelial cells. *Leuk Lymphoma*. **17**(3-4): p. 229-44.
103. Newman, P.J., Berndt, M.C., Gorski, J., White, G.C., 2nd, Lyman, S., Paddock, C., and Muller, W.A. (1990) PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science*. **247**(4947): p. 1219-22.
104. Liao, F., Ali, J., Greene, T., and Muller, W.A. (1997) Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. *J Exp Med*. **185**(7): p. 1349-57.
105. Vaporciyan, A.A., DeLisser, H.M., Yan, H.C., Mendiguren, II, Thom, S.R., Jones, M.L., Ward, P.A., and Albelda, S.M. (1993) Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. *Science*. **262**(5139): p. 1580-2.
106. Bogen, S., Pak, J., Garifallou, M., Deng, X., and Muller, W.A. (1994) Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation in vivo. *J Exp Med*. **179**(3): p. 1059-64.
107. Christofidou-Solomidou, M., Nakada, M.T., Williams, J., Muller, W.A., and DeLisser, H.M. (1997) Neutrophil platelet endothelial cell adhesion molecule-1 participates in neutrophil recruitment at inflammatory sites and is down-regulated after leukocyte extravasation. *J Immunol*. **158**(10): p. 4872-8.
108. Elias, C.G., 3rd, Spellberg, J.P., Karan-Tamir, B., Lin, C.H., Wang, Y.J., McKenna, P.J., Muller, W.A., Zukowski, M.M., and Andrew, D.P. (1998) Ligation of CD31/PECAM-1 modulates the function of lymphocytes, monocytes and neutrophils. *Eur J Immunol*. **28**(6): p. 1948-58.
109. Wakelin, M.W., Sanz, M.J., Dewar, A., Albelda, S.M., Larkin, S.W., Boughton-Smith, N., Williams, T.J., and Nourshargh, S. (1996) An anti-platelet-endothelial cell adhesion molecule-1 antibody inhibits leukocyte extravasation from mesenteric microvessels in vivo by blocking the passage through the basement membrane. *J Exp Med*. **184**(1): p. 229-39.
110. Duncan, G.S., Andrew, D.P., Takimoto, H., Kaufman, S.A., Yoshida, H., Spellberg, J., Luis de la Pompa, J., Elia, A., Wakeham, A., Karan-Tamir, B., Muller, W.A., Senaldi, G., Zukowski, M.M., and Mak, T.W. (1999) Genetic evidence for functional redundancy of Platelet/Endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. *J Immunol*. **162**(5): p. 3022-30.
111. Dangerfield, J., Larbi, K.Y., Huang, M.T., Dewar, A., and Nourshargh, S. (2002) PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate

leukocyte migration through the perivascular basement membrane. *J Exp Med*. **196**(9): p. 1201-11.
112. Watanabe, H., Hattori, S., Katsuda, S., Nakanishi, I., and Nagai, Y. (1990) Human neutrophil elastase: degradation of basement membrane components and immunolocalization in the tissue. *J Biochem (Tokyo)*. **108**(5): p. 753-9.
113. Heck, L.W., Blackburn, W.D., Irwin, M.H., and Abrahamson, D.R. (1990) Degradation of basement membrane laminin by human neutrophil elastase and cathepsin G. *Am J Pathol*. **136**(6): p. 1267-74.
114. Bonnefoy, A. and Legrand, C. (2000) Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase. *Thromb Res*. **98**(4): p. 323-32.
115. O'Brien, C.D., Lim, P., Sun, J., and Albelda, S.M. (2003) PECAM-1-dependent neutrophil transmigration is independent of monolayer PECAM-1 signaling or localization. *Blood*. **101**(7): p. 2816-25.
116. Mamdouh, Z., Chen, X., Pierini, L.M., Maxfield, F.R., and Muller, W.A. (2003) Targeted recycling of PECAM from endothelial surface-connected compartments during diapedesis. *Nature*. **421**(6924): p. 748-53.
117. Mamdouh, Z., Kreitzer, G.E., and Muller, W.A. (2008) Leukocyte transmigration requires kinesin-mediated microtubule-dependent membrane trafficking from the lateral border recycling compartment. *J Exp Med*. **205**(4): p. 951-66.
118. Huang, M.T., Larbi, K.Y., Scheiermann, C., Woodfin, A., Gerwin, N., Haskard, D.O., and Nourshargh, S. (2006) ICAM-2 mediates neutrophil transmigration in vivo: evidence for stimulus specificity and a role in PECAM-1-independent transmigration. *Blood*. **107**(12): p. 4721-7.
119. Schenkel, A.R., Chew, T.W., Chlipala, E., Harbord, M.W., and Muller, W.A. (2006) Different susceptibilities of PECAM-deficient mouse strains to spontaneous idiopathic pneumonitis. *Exp Mol Pathol*. **81**(1): p. 23-30.
120. Schenkel, A.R., Mamdouh, Z., Chen, X., Liebman, R.M., and Muller, W.A. (2002) CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat Immunol*. **3**(2): p. 143-50.
121. Bixel, M.G., Petri, B., Khandoga, A.G., Khandoga, A., Wolburg-Buchholz, K., Wolburg, H., Marz, S., Krombach, F., and Vestweber, D. (2007) A CD99-related antigen on endothelial cells mediates neutrophil, but not lymphocyte extravasation in vivo. *Blood*.
122. Lou, O., Alcaide, P., Luscinskas, F.W., and Muller, W.A. (2007) CD99 is a key mediator of the transendothelial migration of neutrophils. *J Immunol*. **178**(2): p. 1136-43.
123. Bazzoni, G. (2003) The JAM family of junctional adhesion molecules. *Curr Opin Cell Biol*. **15**(5): p. 525-30.
124. Bradfield, P.F., Nourshargh, S., Aurrand-Lions, M., and Imhof, B.A. (2007) JAM family and related proteins in leukocyte migration (Vestweber series). *Arterioscler Thromb Vasc Biol*. **27**(10): p. 2104-12.
125. Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., and Dejana, E. (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol*. **142**(1): p. 117-27.
126. Del Maschio, A., De Luigi, A., Martin-Padura, I., Brockhaus, M., Bartfai, T., Fruscella, P., Adorini, L., Martino, G., Furlan, R., De Simoni, M.G., and Dejana, E. (1999) Leukocyte recruitment in the cerebrospinal fluid of mice with experimental meningitis is

inhibited by an antibody to junctional adhesion molecule (JAM). *J Exp Med*. **190**(9): p. 1351-6.
127. Ozaki, H., Ishii, K., Horiuchi, H., Arai, H., Kawamoto, T., Okawa, K., Iwamatsu, A., and Kita, T. (1999) Cutting edge: combined treatment of TNF-alpha and IFN-gamma causes redistribution of junctional adhesion molecule in human endothelial cells. *J Immunol*. **163**(2): p. 553-7.
128. Ostermann, G., Weber, K.S., Zernecke, A., Schroder, A., and Weber, C. (2002) JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol*. **3**(2): p. 151-8.
129. Johnson-Leger, C.A., Aurrand-Lions, M., Beltraminelli, N., Fasel, N., and Imhof, B.A. (2002) Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. *Blood*. **100**(7): p. 2479-86.
130. Santoso, S., Sachs, U.J., Kroll, H., Linder, M., Ruf, A., Preissner, K.T., and Chavakis, T. (2002) The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. *J Exp Med*. **196**(5): p. 679-91.
131. Aurrand-Lions, M., Lamagna, C., Dangerfield, J.P., Wang, S., Herrera, P., Nourshargh, S., and Imhof, B.A. (2005) Junctional adhesion molecule-C regulates the early influx of leukocytes into tissues during inflammation. *J Immunol*. **174**(10): p. 6406-15.
132. Arrate, M.P., Rodriguez, J.M., Tran, T.M., Brock, T.A., and Cunningham, S.A. (2001) Cloning of human junctional adhesion molecule 3 (JAM3) and its identification as the JAM2 counter-receptor. *J Biol Chem*. **276**(49): p. 45826-32.
133. Bradfield, P.F., Scheiermann, C., Nourshargh, S., Ody, C., Luscinskas, F.W., Rainger, G.E., Nash, G.B., Miljkovic-Licina, M., Aurrand-Lions, M., and Imhof, B.A. (2007) JAM-C regulates unidirectional monocyte transendothelial migration in inflammation. *Blood*. **110**(7): p. 2545-55.
134. Buckley, C.D., Ross, E.A., McGettrick, H.M., Osborne, C.E., Haworth, O., Schmutz, C., Stone, P.C., Salmon, M., Matharu, N.M., Vohra, R.K., Nash, G.B., and Rainger, G.E. (2006) Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukoc Biol*. **79**(2): p. 303-11.
135. Wegmann, F., Petri, B., Khandoga, A.G., Moser, C., Khandoga, A., Volkery, S., Li, H., Nasdala, I., Brandau, O., Fassler, R., Butz, S., Krombach, F., and Vestweber, D. (2006) ESAM supports neutrophil extravasation, activation of Rho, and VEGF-induced vascular permeability. *J Exp Med*. **203**(7): p. 1671-7.
136. Ebnet, K., Suzuki, A., Ohno, S., and Vestweber, D. (2004) Junctional adhesion molecules (JAMs): more molecules with dual functions? *J Cell Sci*. **117**(Pt 1): p. 19-29.
137. Gotsch, U., Borges, E., Bosse, R., Boggemeyer, E., Simon, M., Mossmann, H., and Vestweber, D. (1997) VE-cadherin antibody accelerates neutrophil recruitment in vivo. *J Cell Sci*. **110 (Pt 5)**: p. 583-8.
138. Del Maschio, A., Zanetti, A., Corada, M., Rival, Y., Ruco, L., Lampugnani, M.G., and Dejana, E. (1996) Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *J Cell Biol*. **135**(2): p. 497-510.
139. Allport, J.R., Ding, H., Collins, T., Gerritsen, M.E., and Luscinskas, F.W. (1997) Endothelial-dependent mechanisms regulate leukocyte transmigration: a process involving the proteasome and disruption of the vascular endothelial-cadherin complex at endothelial cell-to-cell junctions. *J Exp Med*. **186**(4): p. 517-27.
140. Moll, T., Dejana, E., and Vestweber, D. (1998) In vitro degradation of endothelial catenins by a neutrophil protease. *J Cell Biol*. **140**(2): p. 403-7.

141. Burns, A.R., Bowden, R.A., MacDonell, S.D., Walker, D.C., Odebunmi, T.O., Donnachie, E.M., Simon, S.I., Entman, M.L., and Smith, C.W. (2000) Analysis of tight junctions during neutrophil transendothelial migration. *J Cell Sci*. **113 (Pt 1)**: p. 45-57.
142. Burns, A.R., Walker, D.C., Brown, E.S., Thurmon, L.T., Bowden, R.A., Keese, C.R., Simon, S.I., Entman, M.L., and Smith, C.W. (1997) Neutrophil transendothelial migration is independent of tight junctions and occurs preferentially at tricellular corners. *J Immunol*. **159**(6): p. 2893-903.
143. Huang, A.J., Manning, J.E., Bandak, T.M., Ratau, M.C., Hanser, K.R., and Silverstein, S.C. (1993) Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *J Cell Biol*. **120**(6): p. 1371-80.
144. Su, W.H., Chen, H.I., Huang, J.P., and Jen, C.J. (2000) Endothelial [Ca(2+)](i) signaling during transmigration of polymorphonuclear leukocytes. *Blood*. **96**(12): p. 3816-22.
145. Majno, G. and Palade, G.E. (1961) Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. *J Biophys Biochem Cytol*. **11**: p. 571-605.
146. Wysolmerski, R.B. and Lagunoff, D. (1990) Involvement of myosin light-chain kinase in endothelial cell retraction. *Proc Natl Acad Sci U S A*. **87**(1): p. 16-20.
147. Saito, H., Minamiya, Y., Kitamura, M., Saito, S., Enomoto, K., Terada, K., and Ogawa, J. (1998) Endothelial myosin light chain kinase regulates neutrophil migration across human umbilical vein endothelial cell monolayer. *J Immunol*. **161**(3): p. 1533-40.
148. Hixenbaugh, E.A., Goeckeler, Z.M., Papaiya, N.N., Wysolmerski, R.B., Silverstein, S.C., and Huang, A.J. (1997) Stimulated neutrophils induce myosin light chain phosphorylation and isometric tension in endothelial cells. *Am J Physiol*. **273**(2 Pt 2): p. H981-8.
149. Haselton, F.R., Woodall, J.H., and Alexander, J.S. (1996) Neutrophil-endothelial interactions in a cell-column model of the microvasculature: effects of fMLP. *Microcirculation*. **3**(3): p. 329-42.
150. Hu, Y., Kiely, J.M., Szente, B.E., Rosenzweig, A., and Gimbrone, M.A., Jr. (2000) E-selectin-dependent signaling via the mitogen-activated protein kinase pathway in vascular endothelial cells. *J Immunol*. **165**(4): p. 2142-8.
151. Hu, Y., Szente, B., Kiely, J.M., and Gimbrone, M.A., Jr. (2001) Molecular events in transmembrane signaling via E-selectin. SHP2 association, adaptor protein complex formation and ERK1/2 activation. *J Biol Chem*. **276**(51): p. 48549-53.
152. Hordijk, P.L. (2006) Endothelial signalling events during leukocyte transmigration. *Febs J*. **273**(19): p. 4408-15.
153. Sano, H., Nakagawa, N., Chiba, R., Kurasawa, K., Saito, Y., and Iwamoto, I. (1998) Cross-linking of intercellular adhesion molecule-1 induces interleukin-8 and RANTES production through the activation of MAP kinases in human vascular endothelial cells. *Biochem Biophys Res Commun*. **250**(3): p. 694-8.
154. Gamble, J.R., Khew-Goodall, Y., and Vadas, M.A. (1993) Transforming growth factor-beta inhibits E-selectin expression on human endothelial cells. *J Immunol*. **150**(10): p. 4494-503.
155. Wall, R.T., Harker, L.A., Quadracci, L.J., and Striker, G.E. (1978) Factors influencing endothelial cell proliferation in vitro. *J Cell Physiol*. **96**(2): p. 203-13.
156. Sambrook, J., Russell, D. *Molecular cloning: a laboratory manual*. 2001: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
157. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**(5259): p. 680-5.

158. van den Bos, C., Rammes, A., Vogl, T., Boynton, R., Zaia, J., Sorg, C., and Roth, J. (1998) Copurification of P6, MRP8, and MRP14 from human granulocytes and separation of individual proteins. *Protein Expr Purif*. **13**(3): p. 313-8.
159. Rothhut, B., Comera, C., Prieur, B., Errasfa, M., Minassian, G., and Russo-Marie, F. (1987) Purification and characterization of a 32-kDa phospholipase A2 inhibitory protein (lipocortin) from human peripheral blood mononuclear cells. *FEBS Lett*. **219**(1): p. 169-75.
160. Smith, W.B., Gamble, J.R., Clark-Lewis, I., and Vadas, M.A. (1991) Interleukin-8 induces neutrophil transendothelial migration. *Immunology*. **72**(1): p. 65-72.
161. Lefer, D.J. (2002) Do neutrophils contribute to myocardial reperfusion injury? *Basic Res Cardiol*. **97**(4): p. 263-7.
162. Vuorte, J., Jansson, S.E., and Repo, H. (2001) Evaluation of red blood cell lysing solutions in the study of neutrophil oxidative burst by the DCFH assay. *Cytometry*. **43**(4): p. 290-6.
163. Roth, J., Sunderkotter, C., Goebeler, M., Gutwald, J., and Sorg, C. (1992) Expression of the calcium-binding proteins MRP8 and MRP14 by early infiltrating cells in experimental contact dermatitis. *Int Arch Allergy Immunol*. **98**(2): p. 140-5.
164. Srikrishna, G., Panneerselvam, K., Westphal, V., Abraham, V., Varki, A., and Freeze, H.H. (2001) Two proteins modulating transendothelial migration of leukocytes recognize novel carboxylated glycans on endothelial cells. *J Immunol*. **166**(7): p. 4678-88.
165. Korndorfer, I.P., Brueckner, F., and Skerra, A. (2007) The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. *J Mol Biol*. **370**(5): p. 887-98.
166. Busetto, S., Trevisan, E., Decleva, E., Dri, P., and Menegazzi, R. (2007) Chloride movements in human neutrophils during phagocytosis: characterization and relationship to granule release. *J Immunol*. **179**(6): p. 4110-24.
167. Horwitz, M., Benson, K.F., Person, R.E., Aprikyan, A.G., and Dale, D.C. (1999) Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. *Nat Genet*. **23**(4): p. 433-6.
168. Young, R.E., Voisin, M.B., Wang, S., Dangerfield, J., and Nourshargh, S. (2007) Role of neutrophil elastase in LTB4-induced neutrophil transmigration in vivo assessed with a specific inhibitor and neutrophil elastase deficient mice. *Br J Pharmacol*. **151**(5): p. 628-37.
169. Ernst, J.D., Hoye, E., Blackwood, R.A., and Jaye, D. (1990) Purification and characterization of an abundant cytosolic protein from human neutrophils that promotes Ca2(+)-dependent aggregation of isolated specific granules. *J Clin Invest*. **85**(4): p. 1065-71.
170. Ernst, J.D. (1991) Annexin III translocates to the periphagosomal region when neutrophils ingest opsonized yeast. *J Immunol*. **146**(9): p. 3110-4.
171. Rosales, J.L. and Ernst, J.D. (1997) Calcium-dependent neutrophil secretion: characterization and regulation by annexins. *J Immunol*. **159**(12): p. 6195-202.
172. Kerkhoff, C., Klempt, M., and Sorg, C. (1998) Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). *Biochim Biophys Acta*. **1448**(2): p. 200-11.
173. Brun, J.G., Jonsson, R., and Haga, H.J. (1994) Measurement of plasma calprotectin as an indicator of arthritis and disease activity in patients with inflammatory rheumatic diseases. *J Rheumatol*. **21**(4): p. 733-8.

174. Roth, J., Teigelkamp, S., Wilke, M., Grun, L., Tummler, B., and Sorg, C. (1992) Complex pattern of the myelo-monocytic differentiation antigens MRP8 and MRP14 during chronic airway inflammation. *Immunobiology*. **186**(3-4): p. 304-14.
175. Goebeler, M., Roth, J., Burwinkel, F., Vollmer, E., Bocker, W., and Sorg, C. (1994) Expression and complex formation of S100-like proteins MRP8 and MRP14 by macrophages during renal allograft rejection. *Transplantation*. **58**(3): p. 355-61.
176. Rugtveit, J., Brandtzaeg, P., Halstensen, T.S., Fausa, O., and Scott, H. (1994) Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. *Gut*. **35**(5): p. 669-74.
177. Edgeworth, J., Gorman, M., Bennett, R., Freemont, P., and Hogg, N. (1991) Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem*. **266**(12): p. 7706-13.
178. Odink, K., Cerletti, N., Bruggen, J., Clerc, R.G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., and Sorg, C. (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. *Nature*. **330**(6143): p. 80-2.
179. Zwadlo, G., Schlegel, R., and Sorg, C. (1986) A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues. *J Immunol*. **137**(2): p. 512-8.
180. Foell, D., Frosch, M., Sorg, C., and Roth, J. (2004) Phagocyte-specific calcium-binding S100 proteins as clinical laboratory markers of inflammation. *Clin Chim Acta*. **344**(1-2): p. 37-51.
181. Frosch, M., Strey, A., Vogl, T., Wulffraat, N.M., Kuis, W., Sunderkotter, C., Harms, E., Sorg, C., and Roth, J. (2000) Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum*. **43**(3): p. 628-37.
182. Boussac, M. and Garin, J. (2000) Calcium-dependent secretion in human neutrophils: a proteomic approach. *Electrophoresis*. **21**(3): p. 665-72.
183. Rammes, A., Roth, J., Goebeler, M., Klempt, M., Hartmann, M., and Sorg, C. (1997) Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem*. **272**(14): p. 9496-502.
184. Hogg, N., Allen, C., and Edgeworth, J. (1989) Monoclonal antibody 5.5 reacts with p8,14, a myeloid molecule associated with some vascular endothelium. *Eur J Immunol*. **19**(6): p. 1053-61.
185. Robinson, M.J., Tessier, P., Poulsom, R., and Hogg, N. (2002) The S100 family heterodimer, MRP-8/14, binds with high affinity to heparin and heparan sulfate glycosaminoglycans on endothelial cells. *J Biol Chem*. **277**(5): p. 3658-65.
186. Rintala-Dempsey, A.C., Rezvanpour, A., and Shaw, G.S. (2008) S100-annexin complexes--structural insights. *Febs J*. **275**(20): p. 4956-66.
187. Mailliard, W.S., Haigler, H.T., and Schlaepfer, D.D. (1996) Calcium-dependent binding of S100C to the N-terminal domain of annexin I. *J Biol Chem*. **271**(2): p. 719-25.
188. Uebi, T., Miwa, N., and Kawamura, S. (2007) Comprehensive interaction of dicalcin with annexins in frog olfactory and respiratory cilia. *Febs J*. **274**(18): p. 4863-76.
189. Ernst, S., Lange, C., Wilbers, A., Goebeler, V., Gerke, V., and Rescher, U. (2004) An annexin 1 N-terminal peptide activates leukocytes by triggering different members of the formyl peptide receptor family. *J Immunol*. **172**(12): p. 7669-76.

190. Migeotte, I., Communi, D., and Parmentier, M. (2006) Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine Growth Factor Rev*. **17**(6): p. 501-19.
191. Ogawa, H., Kunkel, S.L., Fantone, J.C., and Ward, P.A. (1981) Comparative study of eosinophil and neutrophil chemotaxis and enzyme release. *Am J Pathol*. **105**(2): p. 149-55.
192. Perretti, M. and Flower, R.J. (2004) Annexin 1 and the biology of the neutrophil. *J Leukoc Biol*. **76**(1): p. 25-9.
193. Gerke, V. and Moss, S.E. (2002) Annexins: from structure to function. *Physiol Rev*. **82**(2): p. 331-71.
194. Ando, Y., Imamura, S., Hong, Y.M., Owada, M.K., Kakunaga, T., and Kannagi, R. (1989) Enhancement of calcium sensitivity of lipocortin I in phospholipid binding induced by limited proteolysis and phosphorylation at the amino terminus as analyzed by phospholipid affinity column chromatography. *J Biol Chem*. **264**(12): p. 6948-55.
195. Barnes, J.A. and Gomes, A.V. (2002) Proteolytic signals in the primary structure of annexins. *Mol Cell Biochem*. **231**(1-2): p. 1-7.
196. Parente, L. and Solito, E. (2004) Annexin 1: more than an anti-phospholipase protein. *Inflamm Res*. **53**(4): p. 125-32.
197. Vergnolle, N., Comera, C., More, J., Alvinerie, M., and Bueno, L. (1997) Expression and secretion of lipocortin 1 in gut inflammation are not regulated by pituitary-adrenal axis. *Am J Physiol*. **273**(2 Pt 2): p. R623-9.
198. Vergnolle, N., Comera, C., and Bueno, L. (1995) Annexin 1 is overexpressed and specifically secreted during experimentally induced colitis in rats. *Eur J Biochem*. **232**(2): p. 603-10.
199. Perretti, M. (1997) Endogenous mediators that inhibit the leukocyte-endothelium interaction. *Trends Pharmacol Sci*. **18**(11): p. 418-25.
200. Oliani, S.M., Paul-Clark, M.J., Christian, H.C., Flower, R.J., and Perretti, M. (2001) Neutrophil interaction with inflamed postcapillary venule endothelium alters annexin 1 expression. *Am J Pathol*. **158**(2): p. 603-15.
201. Perretti, M., Christian, H., Wheller, S.K., Aiello, I., Mugridge, K.G., Morris, J.F., Flower, R.J., and Goulding, N.J. (2000) Annexin I is stored within gelatinase granules of human neutrophil and mobilized on the cell surface upon adhesion but not phagocytosis. *Cell Biol Int*. **24**(3): p. 163-74.
202. Perretti, M., Croxtall, J.D., Wheller, S.K., Goulding, N.J., Hannon, R., and Flower, R.J. (1996) Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nat Med*. **2**(11): p. 1259-62.
203. Ando, Y., Imamura, S., Owada, M.K., and Kannagi, R. (1991) Calcium-induced intracellular cross-linking of lipocortin I by tissue transglutaminase in A431 cells. Augmentation by membrane phospholipids. *J Biol Chem*. **266**(2): p. 1101-8.
204. Varticovski, L., Chahwala, S.B., Whitman, M., Cantley, L., Schindler, D., Chow, E.P., Sinclair, L.K., and Pepinsky, R.B. (1988) Location of sites in human lipocortin I that are phosphorylated by protein tyrosine kinases and protein kinases A and C. *Biochemistry*. **27**(10): p. 3682-90.
205. Dorovkov, M.V. and Ryazanov, A.G. (2004) Phosphorylation of annexin I by TRPM7 channel-kinase. *J Biol Chem*. **279**(49): p. 50643-6.
206. Skouteris, G.G. and Schroder, C.H. (1996) The hepatocyte growth factor receptor kinase-mediated phosphorylation of lipocortin-1 transduces the proliferating signal of the hepatocyte growth factor. *J Biol Chem*. **271**(44): p. 27266-73.

207. Haigler, H.T., Schlaepfer, D.D., and Burgess, W.H. (1987) Characterization of lipocortin I and an immunologically unrelated 33-kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A2 inhibitors. *J Biol Chem*. **262**(14): p. 6921-30.
208. Solito, E., de Coupade, C., Parente, L., Flower, R.J., and Russo-Marie, F. (1998) IL-6 stimulates annexin 1 expression and translocation and suggests a new biological role as class II acute phase protein. *Cytokine*. **10**(7): p. 514-21.
209. Solito, E., Christian, H.C., Festa, M., Mulla, A., Tierney, T., Flower, R.J., and Buckingham, J.C. (2006) Post-translational modification plays an essential role in the translocation of annexin A1 from the cytoplasm to the cell surface. *Faseb J*. **20**(9): p. 1498-500.
210. Schlaepfer, D.D. and Haigler, H.T. (1987) Characterization of Ca2+-dependent phospholipid binding and phosphorylation of lipocortin I. *J Biol Chem*. **262**(14): p. 6931-7.
211. Perretti, M., Ahluwalia, A., Harris, J.G., Goulding, N.J., and Flower, R.J. (1993) Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse. A qualitative comparison with an anti-CD11b monoclonal antibody. *J Immunol*. **151**(8): p. 4306-14.
212. Perretti, M., Ahluwalia, A., Harris, J.G., Harris, H.J., Wheller, S.K., and Flower, R.J. (1996) Acute inflammatory response in the mouse: exacerbation by immunoneutralization of lipocortin 1. *Br J Pharmacol*. **117**(6): p. 1145-54.
213. Flower, R.J. (1988) Eleventh Gaddum memorial lecture. Lipocortin and the mechanism of action of the glucocorticoids. *Br J Pharmacol*. **94**(4): p. 987-1015.
214. Cirino, G., Peers, S.H., Flower, R.J., Browning, J.L., and Pepinsky, R.B. (1989) Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. *Proc Natl Acad Sci U S A*. **86**(9): p. 3428-32.
215. Cirino, G., Cicala, C., Sorrentino, L., Ciliberto, G., Arpaia, G., Perretti, M., and Flower, R.J. (1993) Anti-inflammatory actions of an N-terminal peptide from human lipocortin 1. *Br J Pharmacol*. **108**(3): p. 573-4.
216. Perretti, M., Wheller, S.K., Choudhury, Q., Croxtall, J.D., and Flower, R.J. (1995) Selective inhibition of neutrophil function by a peptide derived from lipocortin 1 N-terminus. *Biochem Pharmacol*. **50**(7): p. 1037-42.
217. Roviezzo, F., Getting, S.J., Paul-Clark, M.J., Yona, S., Gavins, F.N., Perretti, M., Hannon, R., Croxtall, J.D., Buckingham, J.C., and Flower, R.J. (2002) The annexin-1 knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol*. **53**(4 Pt 1): p. 541-53.
218. Damazo, A.S., Yona, S., D'Acquisto, F., Flower, R.J., Oliani, S.M., and Perretti, M. (2005) Critical protective role for annexin 1 gene expression in the endotoxemic murine microcirculation. *Am J Pathol*. **166**(6): p. 1607-17.
219. Yang, Y.H., Morand, E.F., Getting, S.J., Paul-Clark, M., Liu, D.L., Yona, S., Hannon, R., Buckingham, J.C., Perretti, M., and Flower, R.J. (2004) Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis Rheum*. **50**(3): p. 976-84.
220. Hannon, R., Croxtall, J.D., Getting, S.J., Roviezzo, F., Yona, S., Paul-Clark, M.J., Gavins, F.N., Perretti, M., Morris, J.F., Buckingham, J.C., and Flower, R.J. (2003) Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *Faseb J*. **17**(2): p. 253-5.
221. Lim, L.H. and Pervaiz, S. (2007) Annexin 1: the new face of an old molecule. *Faseb J*. **21**(4): p. 968-75.

222. Wang, W. and Creutz, C.E. (1994) Role of the amino-terminal domain in regulating interactions of annexin I with membranes: effects of amino-terminal truncation and mutagenesis of the phosphorylation sites. *Biochemistry*. **33**(1): p. 275-82.
223. Gavins, F.N., Yona, S., Kamal, A.M., Flower, R.J., and Perretti, M. (2003) Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. *Blood*. **101**(10): p. 4140-7.
224. Mancuso, F., Flower, R.J., and Perretti, M. (1995) Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule. Involvement of endogenous lipocortin 1. *J Immunol*. **155**(1): p. 377-86.
225. Ritchie, R.H., Gordon, J.M., Woodman, O.L., Cao, A.H., and Dusting, G.J. (2005) Annexin-1 peptide Anx-1(2-26) protects adult rat cardiac myocytes from cellular injury induced by simulated ischaemia. *Br J Pharmacol*. **145**(4): p. 495-502.
226. Wu, C.C., Croxtall, J.D., Perretti, M., Bryant, C.E., Thiemermann, C., Flower, R.J., and Vane, J.R. (1995) Lipocortin 1 mediates the inhibition by dexamethasone of the induction by endotoxin of nitric oxide synthase in the rat. *Proc Natl Acad Sci U S A*. **92**(8): p. 3473-7.
227. Getting, S.J., Flower, R.J., and Perretti, M. (1997) Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1. *Br J Pharmacol*. **120**(6): p. 1075-82.
228. Lim, L.H., Solito, E., Russo-Marie, F., Flower, R.J., and Perretti, M. (1998) Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc Natl Acad Sci U S A*. **95**(24): p. 14535-9.
229. Ferreira, S.H., Cunha, F.Q., Lorenzetti, B.B., Michelin, M.A., Perretti, M., Flower, R.J., and Poole, S. (1997) Role of lipocortin-1 in the anti-hyperalgesic actions of dexamethasone. *Br J Pharmacol*. **121**(5): p. 883-8.
230. Cuzzocrea, S., Tailor, A., Zingarelli, B., Salzman, A.L., Flower, R.J., Szabo, C., and Perretti, M. (1997) Lipocortin 1 protects against splanchnic artery occlusion and reperfusion injury by affecting neutrophil migration. *J Immunol*. **159**(10): p. 5089-97.
231. Harris, J.G., Flower, R.J., and Perretti, M. (1995) Alteration of neutrophil trafficking by a lipocortin 1 N-terminus peptide. *Eur J Pharmacol*. **279**(2-3): p. 149-57.
232. Goulet, F., Moore, K.G., and Sartorelli, A.C. (1992) Glycosylation of annexin I and annexin II. *Biochem Biophys Res Commun*. **188**(2): p. 554-8.
233. Taylor, K.E., Giddings, J.C., and van den Berg, C.W. (2005) C-reactive protein-induced in vitro endothelial cell activation is an artefact caused by azide and lipopolysaccharide. *Arterioscler Thromb Vasc Biol*. **25**(6): p. 1225-30.
234. Nerurkar, S.S., McDevitt, P.J., Scott, G.F., Johanson, K.O., Willette, R.N., and Yue, T.L. (2005) Lipopolysaccharide (LPS) contamination plays the real role in C-reactive protein-induced IL-6 secretion from human endothelial cells in vitro. *Arterioscler Thromb Vasc Biol*. **25**(9): p. e136.
235. Movitz, C. and Dahlgren, C. (2000) Endogenous cleavage of annexin I generates a truncated protein with a reduced calcium requirement for binding to neutrophil secretory vesicles and plasma membrane. *Biochim Biophys Acta*. **1468**(1-2): p. 231-8.
236. Hollenberg, M.D., Valentine-Braun, K.A., and Northup, J.K. (1988) Protein tyrosine kinase substrates: Rosetta stones or simply structural elements? *Trends Pharmacol Sci*. **9**(2): p. 63-6.
237. John, C., Cover, P., Solito, E., Morris, J., Christian, H., Flower, R., and Buckingham, J. (2002) Annexin 1-dependent actions of glucocorticoids in the anterior pituitary gland: roles of the N-terminal domain and protein kinase C. *Endocrinology*. **143**(8): p. 3060-70.

238. Stoehr, S.J., Smolen, J.E., and Suchard, S.J. (1990) Lipocortins are major substrates for protein kinase C in extracts of human neutrophils. *J Immunol*. **144**(10): p. 3936-45.
239. Chuah, S.Y. and Pallen, C.J. (1989) Calcium-dependent and phosphorylation-stimulated proteolysis of lipocortin I by an endogenous A431 cell membrane protease. *J Biol Chem*. **264**(35): p. 21160-6.
240. Glenney, J. (1986) Phospholipid-dependent Ca2+ binding by the 36-kDa tyrosine kinase substrate (calpactin) and its 33-kDa core. *J Biol Chem*. **261**(16): p. 7247-52.
241. Hall, S.C., Smith, D.M., Masiarz, F.R., Soo, V.W., Tran, H.M., Epstein, L.B., and Burlingame, A.L. (1993) Mass spectrometric and Edman sequencing of lipocortin I isolated by two-dimensional SDS/PAGE of human melanoma lysates. *Proc Natl Acad Sci U S A*. **90**(5): p. 1927-31.
242. Tsao, F.H., Meyer, K.C., Chen, X., Rosenthal, N.S., and Hu, J. (1998) Degradation of annexin I in bronchoalveolar lavage fluid from patients with cystic fibrosis. *Am J Respir Cell Mol Biol*. **18**(1): p. 120-8.
243. Rescher, U., Goebeler, V., Wilbers, A., and Gerke, V. (2006) Proteolytic cleavage of annexin 1 by human leukocyte elastase. *Biochim Biophys Acta*. **1763**(11): p. 1320-4.
244. Vong, L., D'Acquisto, F., Pederzoli-Ribeil, M., Lavagno, L., Flower, R.J., Witko-Sarsat, V., and Perretti, M. (2007) Annexin 1 cleavage in activated neutrophils: a pivotal role for proteinase 3. *J Biol Chem*. **282**(41): p. 29998-30004.
245. Movitz, C., Sjolin, C., and Dahlgren, C. (1999) Cleavage of annexin I in human neutrophils is mediated by a membrane-localized metalloprotease. *Biochim Biophys Acta*. **1416**(1-2): p. 101-8.
246. Kelher, M.R., Ambruso, D.R., Elzi, D.J., Anderson, S.M., Paterson, A.J., Thurman, G.W., and Silliman, C.C. (2003) Formyl-Met-Leu-Phe induces calcium-dependent tyrosine phosphorylation of Rel-1 in neutrophils. *Cell Calcium*. **34**(6): p. 445-55.
247. Schaff, U.Y., Yamayoshi, I., Tse, T., Griffin, D., Kibathi, L., and Simon, S.I. (2008) Calcium flux in neutrophils synchronizes beta2 integrin adhesive and signaling events that guide inflammatory recruitment. *Ann Biomed Eng*. **36**(4): p. 632-46.
248. Pontremoli, S., Melloni, E., Salamino, F., Patrone, M., Michetti, M., and Horecker, B.L. (1989) Activation of neutrophil calpain following its translocation to the plasma membrane induced by phorbol ester or fMet-Leu-Phe. *Biochem Biophys Res Commun*. **160**(2): p. 737-43.
249. Sjolin, C. and Dahlgren, C. (1996) Diverse effects of different neutrophil organelles on truncation and membrane-binding characteristics of annexin I. *Biochim Biophys Acta*. **1281**(2): p. 227-34.
250. Tompa, P., Buzder-Lantos, P., Tantos, A., Farkas, A., Szilagyi, A., Banoczi, Z., Hudecz, F., and Friedrich, P. (2004) On the sequential determinants of calpain cleavage. *J Biol Chem*. **279**(20): p. 20775-85.
251. Rosengarth, A. and Luecke, H. (2003) A calcium-driven conformational switch of the N-terminal and core domains of annexin A1. *J Mol Biol*. **326**(5): p. 1317-25.
252. Hayhoe, R.P., Kamal, A.M., Solito, E., Flower, R.J., Cooper, D., and Perretti, M. (2006) Annexin 1 and its bioactive peptide inhibit neutrophil-endothelium interactions under flow: indication of distinct receptor involvement. *Blood*. **107**(5): p. 2123-30.
253. Bandeira-Melo, C., Bonavita, A.G., Diaz, B.L., PM, E.S., Carvalho, V.F., Jose, P.J., Flower, R.J., Perretti, M., and Martins, M.A. (2005) A novel effect for annexin 1-derived peptide ac2-26: reduction of allergic inflammation in the rat. *J Pharmacol Exp Ther*. **313**(3): p. 1416-22.

254. Walther, A., Riehemann, K., and Gerke, V. (2000) A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Mol Cell*. **5**(5): p. 831-40.
255. Furst, R., Zahler, S., and Vollmar, A.M. (2008) Dexamethasone-induced expression of endothelial mitogen-activated protein kinase phosphatase-1 involves activation of the transcription factors activator protein-1 and 3',5'-cyclic adenosine 5'-monophosphate response element-binding protein and the generation of reactive oxygen species. *Endocrinology*. **149**(7): p. 3635-42.
256. Schiffmann, E., Corcoran, B.A., and Wahl, S.M. (1975) N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci U S A*. **72**(3): p. 1059-62.
257. Murphy, P.M., Ozcelik, T., Kenney, R.T., Tiffany, H.L., McDermott, D., and Francke, U. (1992) A structural homologue of the N-formyl peptide receptor. Characterization and chromosome mapping of a peptide chemoattractant receptor family. *J Biol Chem*. **267**(11): p. 7637-43.
258. Bao, L., Gerard, N.P., Eddy, R.L., Jr., Shows, T.B., and Gerard, C. (1992) Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. *Genomics*. **13**(2): p. 437-40.
259. Ye, R.D., Cavanagh, S.L., Quehenberger, O., Prossnitz, E.R., and Cochrane, C.G. (1992) Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. *Biochem Biophys Res Commun*. **184**(2): p. 582-9.
260. Prossnitz, E.R. and Ye, R.D. (1997) The N-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function. *Pharmacol Ther*. **74**(1): p. 73-102.
261. Christophe, T., Karlsson, A., Dugave, C., Rabiet, M.J., Boulay, F., and Dahlgren, C. (2001) The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J Biol Chem*. **276**(24): p. 21585-93.
262. Becker, E.L., Forouhar, F.A., Grunnet, M.L., Boulay, F., Tardif, M., Bormann, B.J., Sodja, D., Ye, R.D., Woska, J.R., Jr., and Murphy, P.M. (1998) Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues, and cells. *Cell Tissue Res*. **292**(1): p. 129-35.
263. Garcia, J.G., Verin, A.D., Herenyiova, M., and English, D. (1998) Adherent neutrophils activate endothelial myosin light chain kinase: role in transendothelial migration. *J Appl Physiol*. **84**(5): p. 1817-21.
264. Malik, A.B., Lo, S.K., and Bizios, R. (1986) Thrombin-induced alterations in endothelial permeability. *Ann N Y Acad Sci*. **485**: p. 293-309.
265. Wong, D., Prameya, R., and Dorovini-Zis, K. (2007) Adhesion and migration of polymorphonuclear leukocytes across human brain microvessel endothelial cells are differentially regulated by endothelial cell adhesion molecules and modulate monolayer permeability. *J Neuroimmunol*. **184**(1-2): p. 136-48.
266. Sumagin, R., Lomakina, E., and Sarelius, I.H. (2008) Leukocyte-endothelial cell interactions are linked to vascular permeability via ICAM-1-mediated signaling. *Am J Physiol Heart Circ Physiol*. **295**(3): p. H969-H977.
267. Dejana, E., Orsenigo, F., and Lampugnani, M.G. (2008) The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci*. **121**(Pt 13): p. 2115-22.

268. Hu, G., Vogel, S.M., Schwartz, D.E., Malik, A.B., and Minshall, R.D. (2008) Intercellular adhesion molecule-1-dependent neutrophil adhesion to endothelial cells induces caveolae-mediated pulmonary vascular hyperpermeability. *Circ Res*. **102**(12): p. e120-31.
269. Mehta, D. and Malik, A.B. (2006) Signaling mechanisms regulating endothelial permeability. *Physiol Rev*. **86**(1): p. 279-367.
270. Vishwanatha, J.K., Davis, R.G., Rubinstein, I., and Floreani, A. (1998) Annexin I degradation in bronchoalveolar lavage fluids from healthy smokers: a possible mechanism of inflammation. *Clin Cancer Res*. **4**(10): p. 2559-64.
271. Chung, Y.W., Oh, H.Y., Kim, J.Y., Kim, J.H., and Kim, I.Y. (2004) Allergen-induced proteolytic cleavage of annexin-1 and activation of cytosolic phospholipase A2 in the lungs of a mouse model of asthma. *Proteomics*. **4**(11): p. 3328-34.
272. Le, Y., Yazawa, H., Gong, W., Yu, Z., Ferrans, V.J., Murphy, P.M., and Wang, J.M. (2001) The neurotoxic prion peptide fragment PrP(106-126) is a chemotactic agonist for the G protein-coupled receptor formyl peptide receptor-like 1. *J Immunol*. **166**(3): p. 1448-51.
273. De, Y., Chen, Q., Schmidt, A.P., Anderson, G.M., Wang, J.M., Wooters, J., Oppenheim, J.J., and Chertov, O. (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med*. **192**(7): p. 1069-74.
274. Su, S.B., Gong, W., Gao, J.L., Shen, W., Murphy, P.M., Oppenheim, J.J., and Wang, J.M. (1999) A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J Exp Med*. **189**(2): p. 395-402.
275. Le, Y., Gong, W., Tiffany, H.L., Tumanov, A., Nedospasov, S., Shen, W., Dunlop, N.M., Gao, J.L., Murphy, P.M., Oppenheim, J.J., and Wang, J.M. (2001) Amyloid (beta)42 activates a G-protein-coupled chemoattractant receptor, FPR-like-1. *J Neurosci*. **21**(2): p. RC123.
276. Lee, T.H., Horton, C.E., Kyan-Aung, U., Haskard, D., Crea, A.E., and Spur, B.W. (1989) Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. *Clin Sci (Lond)*. **77**(2): p. 195-203.
277. Colgan, S.P., Serhan, C.N., Parkos, C.A., Delp-Archer, C., and Madara, J.L. (1993) Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. *J Clin Invest*. **92**(1): p. 75-82.
278. Papayianni, A., Serhan, C.N., and Brady, H.R. (1996) Lipoxin A4 and B4 inhibit leukotriene-stimulated interactions of human neutrophils and endothelial cells. *J Immunol*. **156**(6): p. 2264-72.
279. He, R., Sang, H., and Ye, R.D. (2003) Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R. *Blood*. **101**(4): p. 1572-81.
280. Tirouvanziam, R., Gernez, Y., Conrad, C.K., Moss, R.B., Schrijver, I., Dunn, C.E., Davies, Z.A., Herzenberg, L.A., and Herzenberg, L.A. (2008) Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci U S A*. **105**(11): p. 4335-9.
281. Sakane, T., Takeno, M., Suzuki, N., and Inaba, G. (1999) Behcet's disease. *N Engl J Med*. **341**(17): p. 1284-91.
282. Hanai, H., Watanabe, F., Saniabadi, A.R., Matsushitai, I., Takeuchi, K., and Iida, T. (2002) Therapeutic efficacy of granulocyte and monocyte adsorption apheresis in severe active ulcerative colitis. *Dig Dis Sci*. **47**(10): p. 2349-53.

283. D'Acquisto, F., Perretti, M., and Flower, R.J. (2008) Annexin-A1: a pivotal regulator of the innate and adaptive immune systems. *Br J Pharmacol*. **155**(2): p. 152-69.
284. Perretti, M., Chiang, N., La, M., Fierro, I.M., Marullo, S., Getting, S.J., Solito, E., and Serhan, C.N. (2002) Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat Med*. **8**(11): p. 1296-302.
285. Nourshargh, S., Krombach, F., and Dejana, E. (2006) The role of JAM-A and PECAM-1 in modulating leukocyte infiltration in inflamed and ischemic tissues. *J Leukoc Biol*. **80**(4): p. 714-8.
286. Metchnikoff, E. *Lectures on the comparative pathology of inflammation: Delivered at the Pasteur Institute in 1891*. 1968: Reprinted by Dover Press.
287. Cohnheim, J. *Lectures on general pathology.* 1889: The New Sydenham Society, London.
288. Kanters, E., van Rijssel, J., Hensbergen, P.J., Hondius, D., Mul, F.P., Deelder, A.M., Sonnenberg, A., van Buul, J.D., and Hordijk, P.L. (2008) Filamin B Mediates ICAM-1-driven Leukocyte Transendothelial Migration. *J Biol Chem*. **283**(46): p. 31830-9.
289. van Buul, J.D., Allingham, M.J., Samson, T., Meller, J., Boulter, E., Garcia-Mata, R., and Burridge, K. (2007) RhoG regulates endothelial apical cup assembly downstream from ICAM1 engagement and is involved in leukocyte trans-endothelial migration. *J Cell Biol*. **178**(7): p. 1279-93.
290. Etienne-Manneville, S., Manneville, J.B., Adamson, P., Wilbourn, B., Greenwood, J., and Couraud, P.O. (2000) ICAM-1-coupled cytoskeletal rearrangements and transendothelial lymphocyte migration involve intracellular calcium signaling in brain endothelial cell lines. *J Immunol*. **165**(6): p. 3375-83.
291. Hoshi, O. and Ushiki, T. (1999) Scanning electron microscopic studies on the route of neutrophil extravasation in the mouse after exposure to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). *Arch Histol Cytol*. **62**(3): p. 253-60.
292. Sumagin, R. and Sarelius, I.H. (2007) A role for ICAM-1 in maintenance of leukocyte-endothelial cell rolling interactions in inflamed arterioles. *Am J Physiol Heart Circ Physiol*. **293**(5): p. H2786-98.
293. Ambrose, M.P. and Hunninghake, G.W. (1990) Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. *J Appl Physiol*. **68**(4): p. 1668-71.
294. Ambrose, M.P. and Hunninghake, G.W. (1990) Corticosteroids increase lipocortin I in alveolar epithelial cells. *Am J Respir Cell Mol Biol*. **3**(4): p. 349-53.
295. Ambrose, M.P., Bahns, C.L., and Hunninghake, G.W. (1992) Lipocortin I production by human alveolar macrophages. *Am J Respir Cell Mol Biol*. **6**(1): p. 17-21.
296. Byrne, A. and Reen, D.J. (2002) Lipopolysaccharide induces rapid production of IL-10 by monocytes in the presence of apoptotic neutrophils. *J Immunol*. **168**(4): p. 1968-77.
297. Silva, M.T., do Vale, A., and dos Santos, N.M. (2008) Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. *Apoptosis*. **13**(4): p. 463-82.
298. Daaka, Y., Luttrell, L.M., Ahn, S., Della Rocca, G.J., Ferguson, S.S., Caron, M.G., and Lefkowitz, R.J. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem*. **273**(2): p. 685-8.
299. Brandenburg, L.O., Koch, T., Sievers, J., and Lucius, R. (2007) Internalization of PrP106-126 by the formyl-peptide-receptor-like-1 in glial cells. *J Neurochem*. **101**(3): p. 718-28.
300. Seemann, J., Weber, K., Osborn, M., Parton, R.G., and Gerke, V. (1996) The association of annexin I with early endosomes is regulated by Ca2+ and requires an intact N-terminal domain. *Mol Biol Cell*. **7**(9): p. 1359-74.

301. Croall, D.E. and DeMartino, G.N. (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol Rev*. **71**(3): p. 813-47.
302. Franco, S.J. and Huttenlocher, A. (2005) Regulating cell migration: calpains make the cut. *J Cell Sci*. **118**(Pt 17): p. 3829-38.
303. Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y., and Imahori, K. (1981) Limited autolysis of Ca2+-activated neutral protease (CANP) changes its sensitivity to Ca2+ ions. *J Biochem*. **90**(1): p. 275-8.
304. Lokuta, M.A., Nuzzi, P.A., and Huttenlocher, A. (2003) Calpain regulates neutrophil chemotaxis. *Proc Natl Acad Sci U S A*. **100**(7): p. 4006-11.
305. Ikeda, Y., Young, L.H., and Lefer, A.M. (2002) Attenuation of neutrophil-mediated myocardial ischemia-reperfusion injury by a calpain inhibitor. *Am J Physiol Heart Circ Physiol*. **282**(4): p. H1421-6.
306. Cuzzocrea, S., McDonald, M.C., Mazzon, E., Mota-Filipe, H., Centorrino, T., Terranova, M.L., Ciccolo, A., Britti, D., Caputi, A.P., and Thiemermann, C. (2001) Calpain inhibitor I reduces colon injury caused by dinitrobenzene sulphonic acid in the rat. *Gut*. **48**(4): p. 478-88.
307. Chatterjee, P.K., Brown, P.A., Cuzzocrea, S., Zacharowski, K., Stewart, K.N., Mota-Filipe, H., McDonald, M.C., and Thiemermann, C. (2001) Calpain inhibitor-1 reduces renal ischemia/reperfusion injury in the rat. *Kidney Int*. **59**(6): p. 2073-83.
308. Cuzzocrea, S., Chatterjee, P.K., Mazzon, E., Serraino, I., Dugo, L., Centorrino, T., Barbera, A., Ciccolo, A., Fulia, F., McDonald, M.C., Caputi, A.P., and Thiemermann, C. (2002) Effects of calpain inhibitor I on multiple organ failure induced by zymosan in the rat. *Crit Care Med*. **30**(10): p. 2284-94.
309. Marzocco, S., Di Paola, R., Autore, G., Mazzon, E., Pinto, A., Caputi, A.P., Thiemermann, C., and Cuzzocrea, S. (2004) Calpain inhibitor I reduces intestinal ischemia-reperfusion injury in the rat. *Shock*. **21**(1): p. 38-44.
310. Virlos, I., Mazzon, E., Serraino, I., Genovese, T., Di Paola, R., Thiemerman, C., Siriwardena, A., and Cuzzocrea, S. (2004) Calpain I inhibitor ameliorates the indices of disease severity in a murine model of cerulein-induced acute pancreatitis. *Intensive Care Med*. **30**(8): p. 1645-51.
311. Anderson, S.I., Hotchin, N.A., and Nash, G.B. (2000) Role of the cytoskeleton in rapid activation of CD11b/CD18 function and its subsequent downregulation in neutrophils. *J Cell Sci*. **113 (Pt 15)**: p. 2737-45.
312. Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., Sacco, O., and Melloni, E. (1985) Reversible activation of human neutrophil calpain promoted by interaction with plasma membranes. *Biochem Int*. **11**(1): p. 35-44.
313. Pontremoli, S., Melloni, E., Damiani, G., Salamino, F., Sparatore, B., Michetti, M., and Horecker, B.L. (1988) Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. *J Biol Chem*. **263**(4): p. 1915-9.
314. Liu, L., Fisher, A.B., and Zimmerman, U.J. (1995) Regulation of annexin I by proteolysis in rat alveolar epithelial type II cells. *Biochem Mol Biol Int*. **36**(2): p. 373-81.
315. Sjolin, C., Stendahl, O., and Dahlgren, C. (1994) Calcium-induced translocation of annexins to subcellular organelles of human neutrophils. *Biochem J*. **300 (Pt 2)**: p. 325-30.
316. Borregaard, N. and Cowland, J.B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*. **89**(10): p. 3503-21.

317. Parmley, R.T., Rice, W.G., Kinkade, J.M., Jr., Gilbert, C., and Barton, J.C. (1987) Peroxidase-containing microgranules in human neutrophils: physical, morphological, cytochemical, and secretory properties. *Blood*. **70**(5): p. 1630-8.
318. Sengelov, H., Kjeldsen, L., Diamond, M.S., Springer, T.A., and Borregaard, N. (1993) Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. *J Clin Invest*. **92**(3): p. 1467-76.
319. Baggiolini, M. and Clark-Lewis, I. (1992) Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett*. **307**(1): p. 97-101.
320. Smith, S.F., Tetley, T.D., Guz, A., and Flower, R.J. (1990) Detection of lipocortin 1 in human lung lavage fluid: lipocortin degradation as a possible proteolytic mechanism in the control of inflammatory mediators and inflammation. *Environ Health Perspect*. **85**: p. 135-44.
321. Kurosu, K., Takiguchi, Y., Okada, O., Yumoto, N., Sakao, S., Tada, Y., Kasahara, Y., Tanabe, N., Tatsumi, K., Weiden, M., Rom, W.N., and Kuriyama, T. (2008) Identification of annexin 1 as a novel autoantigen in acute exacerbation of idiopathic pulmonary fibrosis. *J Immunol*. **181**(1): p. 756-67.
322. Griese, M., Kappler, M., Gaggar, A., and Hartl, D. (2008) Inhibition of airway proteases in cystic fibrosis lung disease. *Eur Respir J*. **32**(3): p. 783-95.
323. Traverso, V., Christian, H.C., Morris, J.F., and Buckingham, J.C. (1999) Lipocortin 1 (annexin 1): a candidate paracrine agent localized in pituitary folliculo-stellate cells. *Endocrinology*. **140**(9): p. 4311-9.
324. Woods, M.D., Kiss, J.Z., Smith, T., Buckingham, J.C., Flower, R., and Antoni, F.A. (1990) Localization of lipocortin-1 in rat hypothalamus and pituitary gland. *Biochem Soc Trans*. **18**(6): p. 1236-7.
325. McLeod, J.D., Goodall, A., Jelic, P., and Bolton, C. (1995) Changes in the cellular distribution of lipocortin-1 (Annexin-1) in C6 glioma cells after exposure to dexamethasone. *Biochem Pharmacol*. **50**(7): p. 1103-7.
326. Peers, S.H., Smillie, F., Elderfield, A.J., and Flower, R.J. (1993) Glucocorticoid-and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes in vivo. *Br J Pharmacol*. **108**(1): p. 66-72.
327. Solito, E., Nuti, S., and Parente, L. (1994) Dexamethasone-induced translocation of lipocortin (annexin) 1 to the cell membrane of U-937 cells. *Br J Pharmacol*. **112**(2): p. 347-8.
328. Errasfa, M. and Russo-Marie, F. (1989) A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids in vivo in mice. *Br J Pharmacol*. **97**(4): p. 1051-8.
329. Yang, Y.H., Hutchinson, P., Leech, M., and Morand, E.F. (1997) Exacerbation of adjuvant arthritis by adrenalectomy is associated with reduced leukocyte lipocortin 1. *J Rheumatol*. **24**(9): p. 1758-64.
330. Tierney, T., Christian, H.C., Morris, J.F., Solito, E., and Buckingham, J.C. (2003) Evidence from studies on co-cultures of TtT/GF and AtT20 cells that Annexin 1 acts as a paracrine or juxtacrine mediator of the early inhibitory effects of glucocorticoids on ACTH release. *J Neuroendocrinol*. **15**(12): p. 1134-43.
331. Croxtall, J.D. and Flower, R.J. (1992) Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. *Proc Natl Acad Sci U S A*. **89**(8): p. 3571-5.
332. Comera, C., Brousset, P., More, J., Vergnolle, N., and Bueno, L. (1999) Inflammatory neutrophils secrete annexin 1 during experimentally induced colitis in rats. *Dig Dis Sci*. **44**(7): p. 1448-57.

333. Chiang, N., Fierro, I.M., Gronert, K., and Serhan, C.N. (2000) Activation of lipoxin A(4) receptors by aspirin-triggered lipoxins and select peptides evokes ligand-specific responses in inflammation. *J Exp Med*. **191**(7): p. 1197-208.
334. Lee, H.Y., Kim, M.K., Park, K.S., Shin, E.H., Jo, S.H., Kim, S.D., Jo, E.J., Lee, Y.N., Lee, C., Baek, S.H., and Bae, Y.S. (2006) Serum amyloid A induces contrary immune responses via formyl peptide receptor-like 1 in human monocytes. *Mol Pharmacol*. **70**(1): p. 241-8.
335. Tagoe, C.E., Marjanovic, N., Park, J.Y., Chan, E.S., Abeles, A.M., Attur, M., Abramson, S.B., and Pillinger, M.H. (2008) Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts. *J Immunol*. **181**(4): p. 2813-20.
336. Karlsson, J., Fu, H., Boulay, F., Dahlgren, C., Hellstrand, K., and Movitz, C. (2005) Neutrophil NADPH-oxidase activation by an annexin AI peptide is transduced by the formyl peptide receptor (FPR), whereas an inhibitory signal is generated independently of the FPR family receptors. *J Leukoc Biol*. **78**(3): p. 762-71.
337. Rescher, U., Danielczyk, A., Markoff, A., and Gerke, V. (2002) Functional activation of the formyl peptide receptor by a new endogenous ligand in human lung A549 cells. *J Immunol*. **169**(3): p. 1500-4.
338. Chatterjee, B.E., Yona, S., Rosignoli, G., Young, R.E., Nourshargh, S., Flower, R.J., and Perretti, M. (2005) Annexin 1-deficient neutrophils exhibit enhanced transmigration in vivo and increased responsiveness in vitro. *J Leukoc Biol*. **78**(3): p. 639-46.
339. Le Cabec, V. and Maridonneau-Parini, I. (1994) Annexin 3 is associated with cytoplasmic granules in neutrophils and monocytes and translocates to the plasma membrane in activated cells. *Biochem J*. **303 (Pt 2)**: p. 481-7.
340. Copeland, S., Warren, H.S., Lowry, S.F., Calvano, S.E., and Remick, D. (2005) Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol*. **12**(1): p. 60-7.
341. Schneemann, M. and Schoeden, G. (2007) Macrophage biology and immunology: man is not a mouse. *J Leukoc Biol*. **81**(3): p. 579; discussion 580.
342. Matute-Bello, G., Frevert, C.W., and Martin, T.R. (2008) Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. **295**(3): p. L379-99.
343. Mestas, J. and Hughes, C.C. (2004) Of mice and not men: differences between mouse and human immunology. *J Immunol*. **172**(5): p. 2731-8.
344. Lublin, F.D., Knobler, R.L., Kalman, B., Goldhaber, M., Marini, J., Perrault, M., D'Imperio, C., Joseph, J., Alkan, S.S., and Korngold, R. (1993) Monoclonal anti-gamma interferon antibodies enhance experimental allergic encephalomyelitis. *Autoimmunity*. **16**(4): p. 267-74.
345. Panitch, H.S., Hirsch, R.L., Haley, A.S., and Johnson, K.P. (1987) Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet*. **1**(8538): p. 893-5.
346. Gao, J.L. and Murphy, P.M. (1993) Species and subtype variants of the N-formyl peptide chemotactic receptor reveal multiple important functional domains. *J Biol Chem*. **268**(34): p. 25395-401.
347. Doeing, D.C., Borowicz, J.L., and Crockett, E.T. (2003) Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin Pathol*. **3**(1): p. 3.