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Intracellular and Cell-surface Neutrophil Proteinase Levels and Distribution: changes following extravasation and microbial infection

Thesis submitted to Cardiff University for the degree of Doctor of Philosophy

By

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Summary of thesis

Neutrophils are efficient phagocytic cells that form the body's first line defence against entry of foreign infectious microorganisms, but also contribute to tissue damage and non-infectious, chronic inflammation. Neutrophils contain a variety of separate classes of proteinase containing granules: primary (azurophilic) granules [containing serine proteinases elastase (NE), proteinase 3 (Pr3), and cathepsin G (Cat G)], secondary granules [containing metalloproteinase 8 (MMP-8)] and tertiary granules [containing metalloproteinase (MMP-9)]. These proteases are important molecules in immune and inflammatory processes. Sustained inflammation is associated with accumulation of these proteinases and is assumed to contribute to normal parenchymal damage and pathology. Regulation of proteolysis induced by these proteases is crucial to avoid self-induced damage.

In the first part of my PhD thesis, I sought to examine the surface expression of Pr3 and CD177 (a surface receptor of Pr3) on neutrophils, in presence of physiological inhibitors of proteases (alpha-1-antitrypsin; AAT). I have demonstrated that membrane-bound Pr3 (mPr3) is still detectable on the surface of neutrophils in the presence of purified inhibitors and autologous serum as a source of physiological inhibitors. The interaction between CD177 and Pr3 was also examined by expressing CD177 cDNA on the surface of non-neutrophil cells (CHO cells), as was the ability of purified AAT and serum to interfere with these interactions. AAT was able to remove Pr3 from the surface of CD177-CHO cells, and similar results were observed for AAT removal of Pr3 binding to CD177-expressing neutrophils.

In the second part of this thesis, I examined the change in neutrophil proteinases (Pr3, MMP-8 and -9) expression following neutrophil transmigration. *In vitro* transmigrated neutrophils showed no significant change in mPr3 expression compared to un-migrated neutrophils and both CD177-positive and CD177-negative subsets were able to migrate across HUVEC cells. In addition, intracellular Pr3 and MMP-8 also showed no change after *in vitro* transmigration. For comparison I also examined the CD177 and proteinase expression in salivary neutrophils (*in vivo* low inflammation transmigration) relative to matched volunteer blood neutrophils. In contrast to the *in vitro* data, I found only CD177-positive (with Pr3 bound to the surface) neutrophils present in the saliva of healthy individuals. I also found that levels of MMP-8 and MMP-9 were completely depleted in salivary neutrophils, relative to the matched levels in blood neutrophils from the same donor.

In the third part of this thesis I used confocal microscopy to examine the intracellular distribution of neutrophil proteinases within different granule subsets. It was found that selected neutrophil proteinases co-localised with two different granule markers; showing their location in either azurophilic granules (CD63) or secondary granules (CD66b). However, the relationship with Pr3 revealed some discrepancies compared to previous reports. Some neutrophil proteins were co-localized both before and after neutrophil stimulation; whereas others were co-localisation before but not after stimulation. This suggested that degranulation of subsets of granules had occurred.

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This work is dedicated to all my family specially my husband, parents and children Omer, Ali and Mohamed

Contents

List of abbreviations

AAT	Alpha1-Antitrypsin
Ab	Antibody
ACT	Alpha-1-Antichymotrypsin
ANCA	Anti-neutrophil cytoplasmic antibodies
BBB	Blood Brain Barrier
BM	Basement Membrane
BPI	Bactericidal Permeability-Increasing protein
BSA	Bovine Serum Albumin
BSS	Balanced Salt Solution
Cat G	Cathepsin G
CD	Cluster of differentiation
CF	Cystic fibrosis
CHO	Chinese Hamster Ovary
CR	Complement surface receptors
Cyto B	Cytochalasin B
DMEM	Many modifications of Eagle's Medium
DNA	Deoxyribonucleic acid
EC	Endothelial cells
ECM	Extracellular matrix
EBM	Endothelial basement membrane
EDTA	Ethylene diamine tetraacetic acid

ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ESAM	Endothelial specific adhesion molecule
ESL-1	E-selectin ligand-1
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-Phenylalanine
G-CSF	Granulocyte colony-stimulating factor
GlyCAM-1	Glycosylated cell adhesion molecule-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HBP	Heparin-binding protein
HBSS	Hank's Balanced Salt Solution
HRPO	Horseradish peroxidise
ICA	Intensity Correlation Analysis
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1β
JACoP	Just Another Co-localisation Plugin
JAMs	Junctional adhesion molecules
LB	Luria Broth
LERs	Low expression regions
LFA-1	Lymphocyte Function-Associated antigen -1

LPS	Lipopolysaccharide
LTB4	Leukotriene B4
Mac-1	Macrophage-1 antigen
MCF	Mean cellular fluorescence
MMP	Matrix metalloprotease
MMP-8	Matrix metalloprotease-8
MMP-9	Matrix metalloprotease-9
MPO	Myeloperoxidase
mPr3	Membrane-bound proteinase 3
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NSPs	Neutrophil serine proteases
PAF	Platelet activating factor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate Buffered Saline Tween 20
PC	Pericyte
PCR	Polymerase Chain Reaction
PECAM-1	Platelet endothelial cell adhesion molecule
PGRN	Progranulin
PMA	Phorbol Myristate Acetate

PMNs	Polymorphonuclear
PNH	Paroxysmal nocturnal hemoglobinuria
PPP	Platelet Poor Plasma
Pr3	Proteinase 3
PRRs	Pattern recognition receptors
PRV-1	Polycytemia rubra vera-1
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
REs	Restriction Enzymes
ROS	Reactive oxygen species
RPE-GAM	R. Phycoerythin-conjugated goat anti-mouse antibody
RT	Room temperature
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the mean
SLPI	Secretory Leukocyte Protease Inhibitor
SPR	Surface plasmon resonance
TBE	Tris/Borate/EDTA
TEM	Transendothelial migration
TGF-β	Transforming growth factor β
TIMPs	Tissue inhibitor of metalloproteinase
TLRs	Toll-like receptors
TNF-α	Tumor Necrosis Factor- alpha
UV	Ultraviolet

VCAM-1	Vascular cell adhesion molecule-1
Vit B12-bp	Vitamin B12 binding proteins
VVO	Vesiculo-vacuolar organelles
WG	Wegener’s granulomatosis

CHAPTER 1

GENERAL INTRODUCTION

1.1 General characteristics of the immune system

The immune system is a very precisely organised system that has the capacity to identify foreign substances (e.g. pathogens), and to act against them. The immune system contains complex and vital networks of molecules, cells, tissues, and organs which work in concert to protect the body against invasive agents and cancerous growth. It also plays a crucial role in tissue repair after infection or trauma. Initially, the immune system is able to distinguish the body's own components from foreign molecules. In addition, the immune system can detect minute differences that distinguish one pathogen from another. It is divided into two major branches: the innate immune system (broadly the "non-specific" immune system) and the adaptive immune system (the specific immune system). The process of detecting and removing non-self involves both innate and adaptive immune systems.

The innate immune system provides the first line of defence against microbial pathogens, while the adaptive immune system serves to provide a more specific response, which is improved with increasing exposure to pathogens. Both elements of the immune system have cellular and humoral components through which they achieve their protective functions. Moreover there is an extensive association between these two branches. Cells and the components of the innate immune system instruct the adaptive immune system and the adaptive immune system can improve the potency of aspects of the innate immune system. The main components of the innate immune system are the external physical epithelial barriers of the body, phagocytic cells (neutrophils, macrophages and dendritic cells that engulf and destroy pathogenic microorganisms), a special type of lymphocyte called a natural killer (NK) cell, and circulating plasma proteins. The adaptive immune system, on the other hand, is mediated by lymphocytes (B cells and T cells). All cellular components of both branches have their origin in the bone marrow (fig1.1).

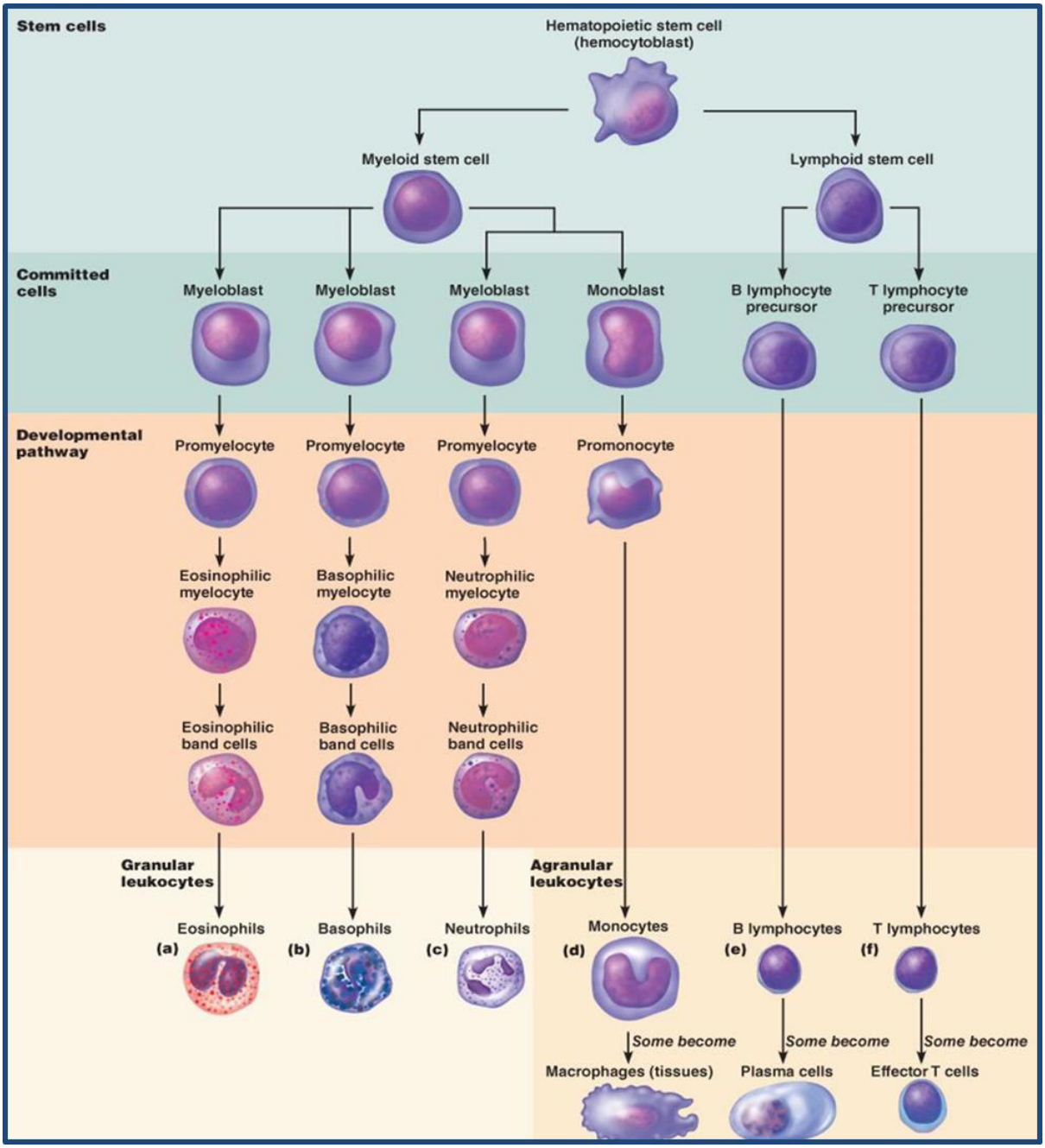


Figure 1.1 Production and development of blood cells from hematopoietic stem cells. Available from: www. classes.midlandstech.com

After initial activation of innate immunity, cells of the adaptive immune system are recruited to further tailor and augment the power of innate immunity mechanisms. Communication between the different types of immune cells critically depends on the production of soluble mediators termed cytokines and chemokines, of which a large number have been identified, providing an additional layer of complexity to the system.

1.2 Inflammation

Inflammation is the first response of the immune system to tissue damage or infection. Inflammatory processes involve innate and adaptive immune responses. The processes lead to elimination of the causative agent and to disposal of damaged and dead tissue, followed by repair and regeneration of new tissue. The classic signs of inflammation are redness, heat, swelling, pain and loss of function in the affected area. These signs are caused by augmented blood flow to the inflamed tissues which cause heat and redness, whereas changes in vascular permeability are associated with swelling leading to oedema and pain. The inflammatory response causes secretion of a number of mediators, such as cytokines and acute phase proteins. These substances work as signals between the immune system cells. They bind to specific receptors on target cells and alter cell behaviour, typically activation, division, apoptosis and movement. Cytokines can be pro-inflammatory molecules that up-regulate the immune response, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Kuby, 1997) or they can be anti-inflammatory that down-regulate the immune response such as interleukin 4 (IL-4) and interleukin 10 (IL-10) (Smith & Miles, 2000). They are synthesised and secreted from a variety of immune cells (e.g. monocytes) and non-immune cells (e.g. endothelial cells), after stimulation by microbes or other cytokines. In addition to the production of these mediators, inflammation results in the activation and recruitment of inflammatory cells (leukocytes) from the blood to the inflammatory focus (extravasation) (Medzhitov, 2008). The accumulation and activation of leukocytes (initially neutrophils and

later monocytes) are essential events in the pathogenesis of most forms of inflammatory disease.

1.3 Overview of the neutrophil

Neutrophils (also known as neutrophilic granulocytes or polymorphonuclear leukocytes; PMNs) are a key component of the innate immune system and may represent an essential link between the innate and adaptive immune system (Silva MT, 2010). PMNs play a principal role in establishing an inflammatory response. They were first discovered by a Russian zoologist Elie Metchnikoff who recognized the important function of neutrophils in inflammation and transmigration. These cells were named neutrophilic granulocytes by Paul Ehrlich based on their staining by neutral dyes. In 1908, the Nobel Prize in Medicine was awarded jointly to Metchnikoff and Ehrlich for their work. PMNs are the major type of circulating leukocyte in human peripheral blood and they represent 40-80 percent of total leukocytes under normal conditions. They are short-lived, with a circulating half-life of 6–8 h and 1-4 days in tissue. However, a study by Pillay *et al.,* (2010) suggested that the average circulatory lifespan of neutrophils is 5.4 days and this is reduced when activated, as the cells will die soon after completing phagocytosis. However, this has been questioned as an artefact of the labelling method used (Li KW *et al.,* 2011). In the circulation of healthy adults, neutrophils exist in a resting state, which ensures that their toxic intracellular contents are not accidentally released to damage host tissue. Their principle functions are to ingest and kill micro-organisms through a series of antimicrobial strategies (phagocytosis, degranulation and the formation of neutrophil extracellular traps; fig1.2). They also produce inflammatory mediators that allow the resolution of infection and the establishment of long-lasting, adaptive immunity via other cells of the immune system. Under physiological conditions, neutrophils can be found in the bone marrow, spleen, liver and lung with the lung being enriched with mature, terminally differentiated neutrophils (Summers *et al.,* 2010).

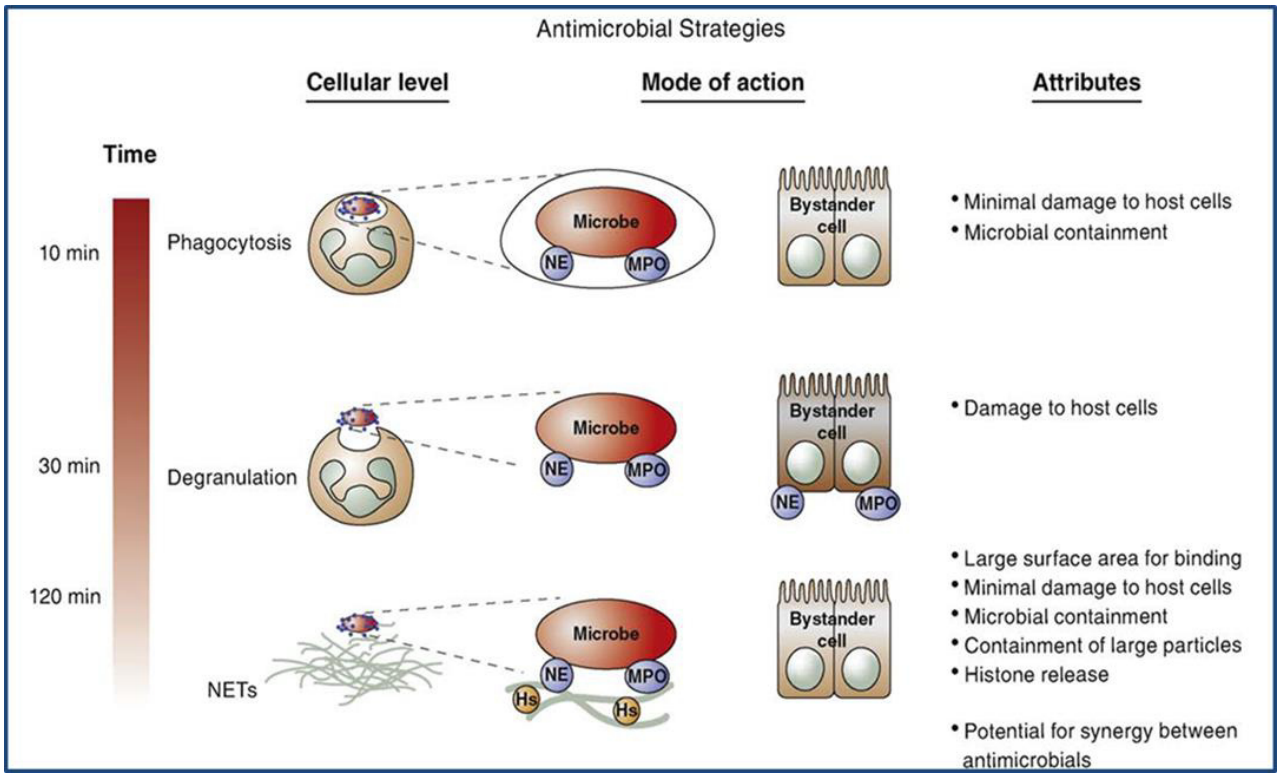


Figure 1.2 Microbial killing by neutrophils antimicrobial strategies. Neutrophil fight and clear microbes via three major strategies: phagocytosis, degranulation, and Neutrophil extracellular traps (NET) formation. These strategies operate over different timescales and cause bystander damage to host cells. Phagocytosis occurs within minutes and causes little damage to host cells whereas, neutrophil degranulation occurs within 30 minutes and causes major damage to the surrounding tissue. Finally, NET formation takes between 2-3 hours to complete. The incorporation of granule proteases (such as neutrophil elastase) into NETs limits their diffusion and hence the potential for host cell damage, increases their effective local concentration, and entraps microbes. Red: Microbe, Blue: granular proteins in the phagolysosome, extracellular space, or tethered within the NET, Gray: decondensed DNA of the NET. Abbreviations: Hs, histones; MPO, myeloperoxidase; NE, neutrophil elastase. Reproduced from: (Papayannopoulos and Zychlinsky 2009), with permission from the publisher Elsevier.

In the bone marrow neutrophils can be subdivided into three pools: the stem cell pool (undifferentiated haematopoietic stem cells), the mitotic pool (cells that are undergoing proliferation and differentiation) and the post-mitotic pool (fully differentiated mature neutrophils) (Summers *et al.,* 2010).

1.3.1 Differentiation of neutrophils

The differentiation of neutrophils occurs in a multi-step process from multi-potential hematopoietic stem cells by a process called granulopoiesis (Anastassova-Kristeva, 2003). In the first developmental stage, the cell divides and differentiates from myeloblasts to promyelocytes. In the next differentiation step, myelocytes start to appear and in the final stages, myelocytes differentiate into metamyelocytes and band cells (as seen in fig 1.1). The differentiation of neutrophils is controlled by numbers of cytokines and growth factors particularly Granulocyte colony-stimulating factor (G-CSF) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lieschke and Burgess 1992). PMN development takes about 2 weeks and their daily production can reach up to 2×10^{11} cells.

This number may increase several fold in the face of systemic infection (Borregaard 2010; Segal and Holland 2000). Neutrophil production is achieved by the control of three central processes: neutrophil production in the bone marrow, neutrophil egress from the bone marrow into the circulation, and neutrophil clearance from the blood by the reticuloendothelial phagocytic system in the spleen, liver and bone marrow. As a result of infection, neutrophils are quickly released from the bone marrow, generating a blood neutrophilia, which is essential to supply enough neutrophils for recruitment locally to sites of infection or inflammation (Sato *et al.,* 1998). Fully differentiated or mature neutrophils are about 10 μm in diameter and characterized by their lobulated nucleus and their granular appearance. Moreover, they contain very few mitochondria, and only small amounts of the

endoplasmic reticulum (ER) and Golgi apparatus. PMNs are no longer capable of growth or division, have little capacity to synthesize mRNA or proteins and are fully equipped with the necessary proteins and peptides, receptors and effectors molecules stored in their granules and vesicles. Any minor proteins synthesized in neutrophils after their exit from the bone marrow are not packed in granules, which are formed during differentiation (Theilgaard-Mönch *et al.,* 2004; Lapinet *et al.,* 2000).

1.3.2 Neutrophil receptors

Neutrophils express a large variety of receptors on their surface and in subcellular organelles (granules and secretory vesicles) which can be mobilized to the cell surface following exposure of the cell to a stimulus. Neutrophils respond to infection and tissue injury through the interaction of these receptors with their targets. Neutrophils recognize pathogen-derived molecules known as pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and flagellin by interacting with pattern recognition receptors (PRRs) expressed on their surface. Neutrophils express a variety of PRRs including, all members of the Toll-like receptor (TLRs) family with the exception of TLR3 (Hayashi *et al.,* 2003), but Bellochio *et al.,* 2004 describe functional response by human blood neutrophils to poly I: C which is a TLR3 ligand. Neutrophils express also Peptidoglycan-recognition protein (PGRP; Liu *et al.,* 2000; Kobayashi *et al.,* 2005) and the C-type lectin receptors including dectin 1 (Greenblatt *et al.,* 2010). Activation of the serum protein cascade, complement, promotes the deposition of complement C3b activation fragments on microbial surfaces. Complement-opsonized microbes are then recognized by PMNs through complement surface receptors, such as ClqR, CD35 (CR1), CD11b/CD18 (CR3), and CD11c/CD18 (CR4). PMNs also express Fc receptors that recognize antibody-coated microbes specifically CD23 (FcεRII, IgE receptor), CD89 (FcαR, IgA receptor), CD64 (FcγRI, IgG receptor), CD32 (FcγRIIa, low affinity IgG receptor) and CD16 (FcγRIIIb, low-affinity IgG receptor; Frank *et al.,* 2009).

Binding of antibody and complement receptors at the PMN surface triggers phagocytosis of microorganisms (Kobayashi et al. 2005). Neutrophils express receptors for pro-inflammatory mediators (e.g. the anaphylotoxin complement component C5a, platelet-activating factor [PAF] and Formyl-Methionyl-Leucyl-Phenylalanine [fMLP]), receptors for chemokines (including CXCR1 and CXCR2) and receptors for matrix proteins (Paul 2013). Moreover, neutrophil adhesion and migration into inflamed tissues is facilitated by the interaction between adhesion molecules expressed on the surfaces of neutrophils and endothelial cells (discussed in depth below). Neutrophil granules also contain membrane-surface receptors (as discussed below).

1.4 Neutrophil granules

Neutrophil granules are the most important component in neutrophil activation, migration, phagocytosis, and killing of microbes. Granules can be classified on the basis of their size or morphology or timing of biosynthesis, but the traditional classification of neutrophil granules into two major types of granules was based on the content of MPO as peroxidase-positive (azurophil, or primary) and peroxidase-negative (specific or secondary). Neutrophils contain at least four types of granules: (1) primary granules, also known as azurophilic granules; (2) secondary granules, also known as specific granules; (3) tertiary granules; and (4) secretory vesicles. These are formed sequentially during granulocytic differentiation in the bone marrow i.e., at the promyelocyte stage, the myelocyte-metamyelocyte stage, and the band cell stage. The azurophilic or primary granules are formed during the first stage while specific or secondary granules are formed as the cell enters the myelocytic stage. During the final stage, the tertiary granules are produced whereas secretory vesicles are likely to be formed by endocytosis when the PMNs are circulating in the blood (fig1.3) (Borregaard *et al.,* 1995; Borregaard and Cowland, 1997).

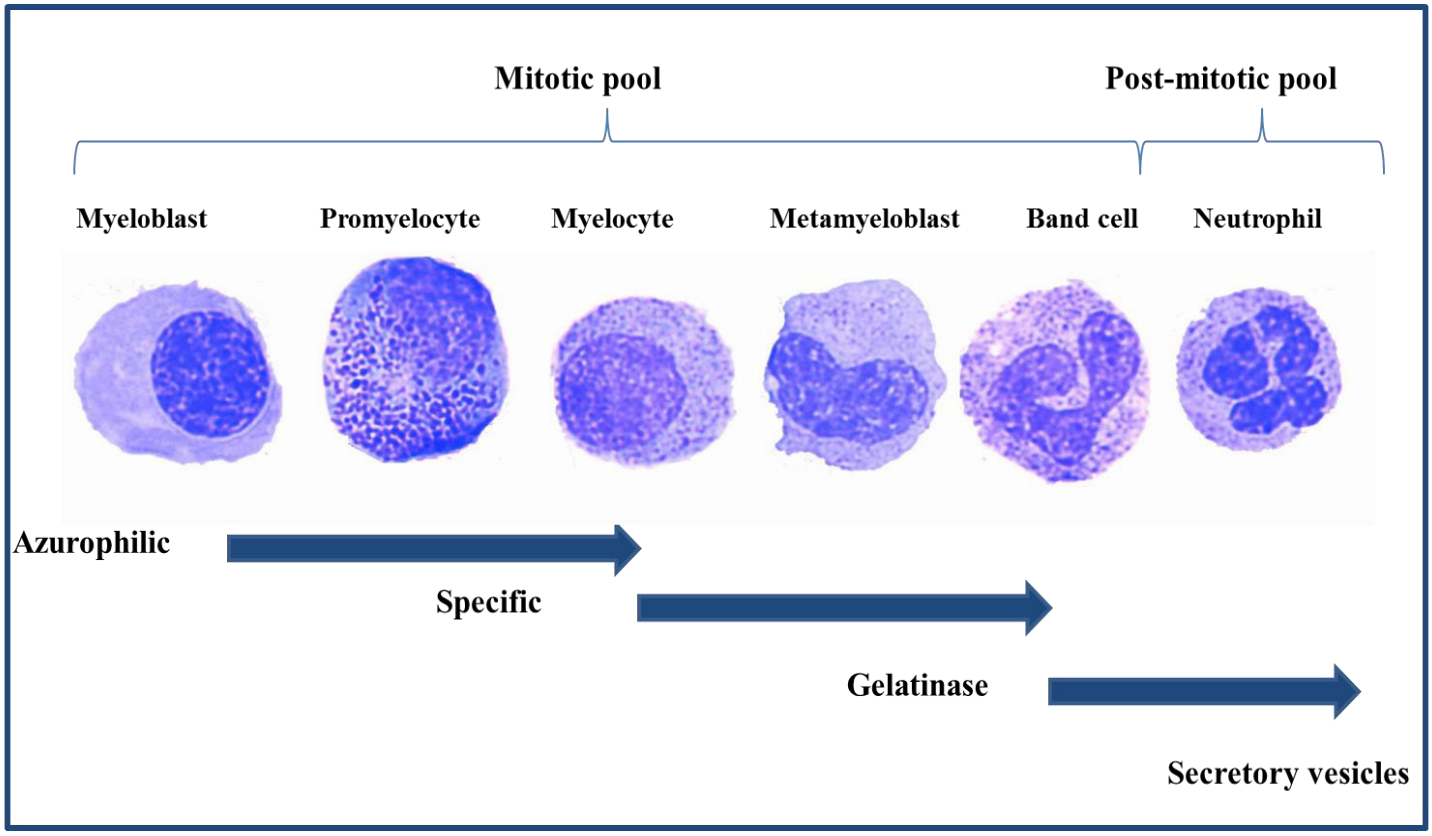


Figure 1.3 The developmental stages of neutrophils and granules. Protein components of different granules are defined by the timing of their biosynthesis during neutrophil differentiation.

The protein contents of each granule type are determined by the timing of development during neutrophil maturation. Thus, granule proteins that are synthesized at a given stage of cellular differentiation will be localized to the same type of granule (Le Cabec *et al.,* 1996). About 300 different proteins are stored in PMN granules (selected granule content shown in table 1.1) which will be released into the surrounding, incorporated into the cell membrane or remain attached to the membrane upon granule mobilization (Lominadze *et al.,* 2005). The mobilization and release (degranulation) of these granules are dependent on the function of PMNs in response to different stimuli.

1.4.1 Primary (Azurophilic) granules

Azurophilic granules are the first type of granules to be formed and recognized during neutrophil maturation and they are essential for the killing of ingested microorganisms following the fusion of granules with microorganism-loaded phagosomes. Moreover they are released extracellularly during exocytosis and have cytotoxic effects on surrounding tissues if released in excess (Lacy 2005). Primary granules make up around 30% of the total granule population, and are characterized by the presence of myeloperoxidase (MPO), bactericidal permeability-increasing protein (BPI), defensins and a family of structurally related serine proteases [NSPs; cathepsin G (Cat G), neutrophil elastase (NE) and proteinase 3 (Pr3)]. Moreover, azurophilic granules contain granulophysin (CD63) in their membrane and CD68 (Cham, *et al.,* 1994; Saito *et al.,* 1991).

CD63 has been described as a suitable marker for azurophilic granules, because it is present only in azurophilic granules (Kuijpers *et al.,* 1991). CD63, a member of tetraspanin superfamily is an activation marker in neutrophils, which has been shown *in vitro* to be expressed on the cell surface following neutrophil activation in the presence of fMLP following cytochalasin B priming.

Table 1.1 Selected contents of neutrophil granules and secretory vesicle

	Primary granules	**Specific granules**	**Gelatinase granules**	**Secretory vesicles**
Membrane components	CD63 CD68 Stomatin Presenilin vacuolar-type H+-ATPase	CD66b CD11b/CD18 fMLP receptor Cytochrome b558	Cytochrome b558 CD11b/CD18	β2-integrin CR1 fMLP receptor CD14 CD16 Leukolysin
Matrix components	Myeloperoxidase Cathepsin G Elastase Pr 3 Defensins BPI	Lactoferrin Vit. B12-bp MMP-8 Lysozyme Lactoferrin hCAP-18 NGAL Collagenase Pr3 CD177	MMP-9 Leukolysin Heparanase	HBP Albumin

PMA and fMLP alone are known to induce minimal translocation of CD63 (Cham *et al.,* 1994). Azurophilic granules also contain stomatin (Feuk-Lagerstedt et al., 2002), presenilin 1(Mirinics *et al.,* 2002), and a vacuolar-type H+- ATPase (an ATP-driven enzyme; Nanda *et al.,* 1996). These granules undergo limited exocytosis in response to stimulation (Faurschou *et al.,* 2002; Sengelov *et al.,* 1993). The presence of MPO in azurophilic granules is of major importance for oxygen-dependent bactericidal activity of neutrophils because MPO reacts with H_2O_2, formed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, generating highly toxic products. Azurophilic granule proteins are of specific interest because Pr3 and MPO are now recognized as target antigens for anti-neutrophil cytoplasmic antibodies (ANCA), which are associated with severe autoimmune vasculitis diseases.

1.4.2 Specific (secondary) granules

Specific granules are formed during the myelocyte-metamyelocyte stages of neutrophil maturation. Specific granules are peroxidase-negative granules, and named secondary granules because they are the second type of granules to appear during neutrophil maturation. Specific granules are more readily mobilized than the azurophilic granules upon stimulation (Lacy 2005). They contain high concentrations of lactoferrin and Vitamin B12 binding proteins (Vit B12-bp), neutrophil collagenase (MMP-8) and a variety of antimicrobial compounds including lysozyme (Lacy 2005, Faurschou and Borregaard 2003). Lactoferrin has broad spectrum antimicrobial activity. It is a member of the transferrin family of iron-binding proteins and it damages bacteria and inhibits bacterial growth by sequestration of iron. Moreover it binds to bacterial cell membranes causing irreversible membrane damage and cell lysis (Chapple *et al.,* 1998).

Lysozyme is a cationic antimicrobial peptide existing in other types of granules, but specific granules have the highest concentration (Lollike *et al.,* 1995). In addition to these molecules,

specific granules also contain neutrophil gelatinase-associated lipocalin (NGAL; Kjeldsen et al., 1994), cathelicidin antimicrobial peptide hCAP-18 (the proform of LL-37; Sørensen et al., 1997), Pr3 and CD177 (Stroncek *et al.,* 1990). They express CD66b in their membrane, which is suitable as a marker for specific granules. Specific granules contain other functional membrane proteins such as adhesion molecules (CD11b/CD18), receptors for chemoattractants (formyl peptide receptors FPR) (Brown et al., 1991) and the NADPH-oxidase component cytochrome b558 (Borregaard and Cowland 1997). Following specific granule mobilization, all of these receptors are merged into the plasma membrane and also become a part of the phagosomal membrane as specific granules fuse with the phagosomal vacuole.

1.4.3 Gelatinase granules

Gelatinase granules (also known as tertiary granules and peroxidase-negative granules) are formed at the metamyelocyte and band cell stages and constitute about 25% of the peroxidase-negative granule population (Kjeldsen *et al.,* 1993). Tertiary and specific granules, share a wide number of components although at different relative amounts. Gelatinase granules are more easily exocytosed than specific granules, thus gelatinase granules are essential mainly a reservoir of enzymes and membrane receptors needed during neutrophil extravasation. However, as mentioned above, specific granules also contribute primarily in the antimicrobial activities of the neutrophil by mobilisation of their antimicrobial materials either to the phagosome or outside of the cell (Faurschou and Borregaard 2003). Tertiary granules are identified by the presence of gelatinase B (matrix metalloprotease-9 or MMP-9). These granules also contain leukolysin (MMP-25), which is also found in specific granules, secretory vesicles, and lipid rafts on the plasma membrane of resting neutrophils (Kang *et al,* 2001; Fortin *et al.,* 2010). Tertiary granules contain integrins (e.g.: CD11b/CD18), heparanase and cytochrome b_{558} (Mollinedo and Schneider 1984;

Mollinedo et al., 1991). Cytochrome b_{558} is the main component of the superoxide anion-generating NADPH oxidase, and most of the proton pump ATPase-acidification activity (Mollinedo *et al.,* 1986). Exocytosis of tertiary granules leads to the increased exposure of cell-surface adhesion proteins which enhance neutrophil attachment to endothelium and the release of extracellular matrix degradative enzymes that facilitate the neutrophil passage through capillary walls and into the tissue (diapedesis).

1.4.4 Secretory vesicles

Secretory vesicles are created by endocytosis, during the late maturation of neutrophils in the bone marrow. The membrane of secretory vesicles is rich in receptors, signalling proteins, and adhesion molecules, whereas plasma proteins dominate their intra-vesicular content (Borregaard and Cowland, 1997). Secretory vesicles are the most easily mobilized subcellular neutrophil compartment in response to a wide variety of inflammatory stimuli.

The membranes of secretory vesicles are abundant in the β2-integrin, complement receptor 1 (CR1), FPRs, the LPS/lipoteichoicacid-receptor CD14, the FcγIII receptor CD16 and MMP-25. All of these receptors merge with the plasma membrane of neutrophils after exocytosis (Faurschou and Borregaard 2003). Secretory vesicles in addition contain albumin and Heparin-binding protein (HBP) which is also known as azurocidin or CAP37. HBP is a multifunctional protein and the data suggest that HBP not only has antimicrobial activity but is also able to recruit and activate monocytes and macrophages (Di Gennaro *et al.,* 2009).

1.5. Neutrophil proteases

Protease enzymes are one of the largest and most essential groups of enzymes that are capable of digesting protein into smaller-sized proteins or small peptide fragments. Proteases are implicated in many important physiological processes such as protein turnover, digestion, blood coagulation and wound healing. Moreover, they are implicated in antimicrobial defence and they can degrade components of the adherence junction during neutrophil-endothelial adhesion and diapedesis. These proteins are stored in granules until exocytosed, by regulated secretion, in response to various stimuli. They are divided into five major classes: aspartic, serine, cysteine, threonine proteases and metalloproteases (Korkmaz *et al.,* 2010).

Neutrophils express different classes of proteases, both cytosolic (such as cysteine proteases such as caspases or calpain) and granular including matrix metalloproteinases (such as MMP-8 and -9), and neutrophil serine proteases (NSPs); such as Pr3. The granular proteases can be released from neutrophil granules upon activation by chemoattractants or other stimuli. Proteases often have specific anti-protease counter-parts which are involved in the regulation of the enzyme's proteolytic activity. Uncontrolled or unregulated proteolysis can cause or contribute to the severity of several disease states such as emphysema, cancer, Alzheimer's disease, inflammation, and arthritis. Protease inhibitors thus have considerable potential utility for therapeutic intervention in a variety of disease states (Powers *et al.,* 2002).

1.5.1 Neutrophil Serine proteases (NSPs)

Serine proteases are a class of proteases that have an amino acid serine residue in the active site of the enzyme. Pr3, NE and Cat G are the major serine proteases stored in the neutrophil granules, mainly the azurophilic granules, as active enzymes. They are stored in their active

form until they are released following neutrophil exposure to inflammatory stimuli. NSPs perform many important functions in inflammatory and immune responses (fig1.4).

Active serine proteases modulate the inflammatory response by processing cytokines, growth factors, surface receptors, and signalling molecules. NSPs also act intracellularly within phagolysosomes to digest phagocytized microorganisms in combination with microbicidal peptides and the membrane-associated NADPH oxidase system, which produces reactive oxygen metabolites (Segal, 2005). They may participate also in pathogen killing associated with NETs by degrading bacterial virulence factors extracellularly (Korkmaz *et al.,* 2010).

These proteases have the capacity to degrade components that make up the extracellular matrix. For instance, Pr3 has been demonstrated to degrade elastin *in vitro*, resulting in tissue damage to the lungs when administered to hamsters via inhalation (Kao *et al.,* 1988). Elastase is able to digest almost all components of the extracellular matrix including collagen I-IV, fibronectin, laminin and proteoglycans (Delacourt *et al.,* 2002).

Cat G is essential for activation of neutrophil collagenase (Capodici and Berg 1989; Capodici *et al.,* 1989). Cat G has been suggested to have anticoagulant activity via inactivation of factor VIII (FVIII). However, this latter observation was disputed by another study that showed Cat G caused a pro-coagulant effect by activation of factor VIII (Gale and Rozenshteyn 2008). The proteolytic activity of these enzymes is tightly regulated by serine protease inhibitors such as α1-antichymotrypsin, and α2-macroglobulin. The liver constantly produces abundant amounts of these protease inhibitors which are disseminated throughout the body in the plasma and interstitial fluids and efficiently remove NSPs and other proteases after their release by complexation and delivery to lysosomes of phagocytic cells (Kessenbrock *et al.,* 2011).

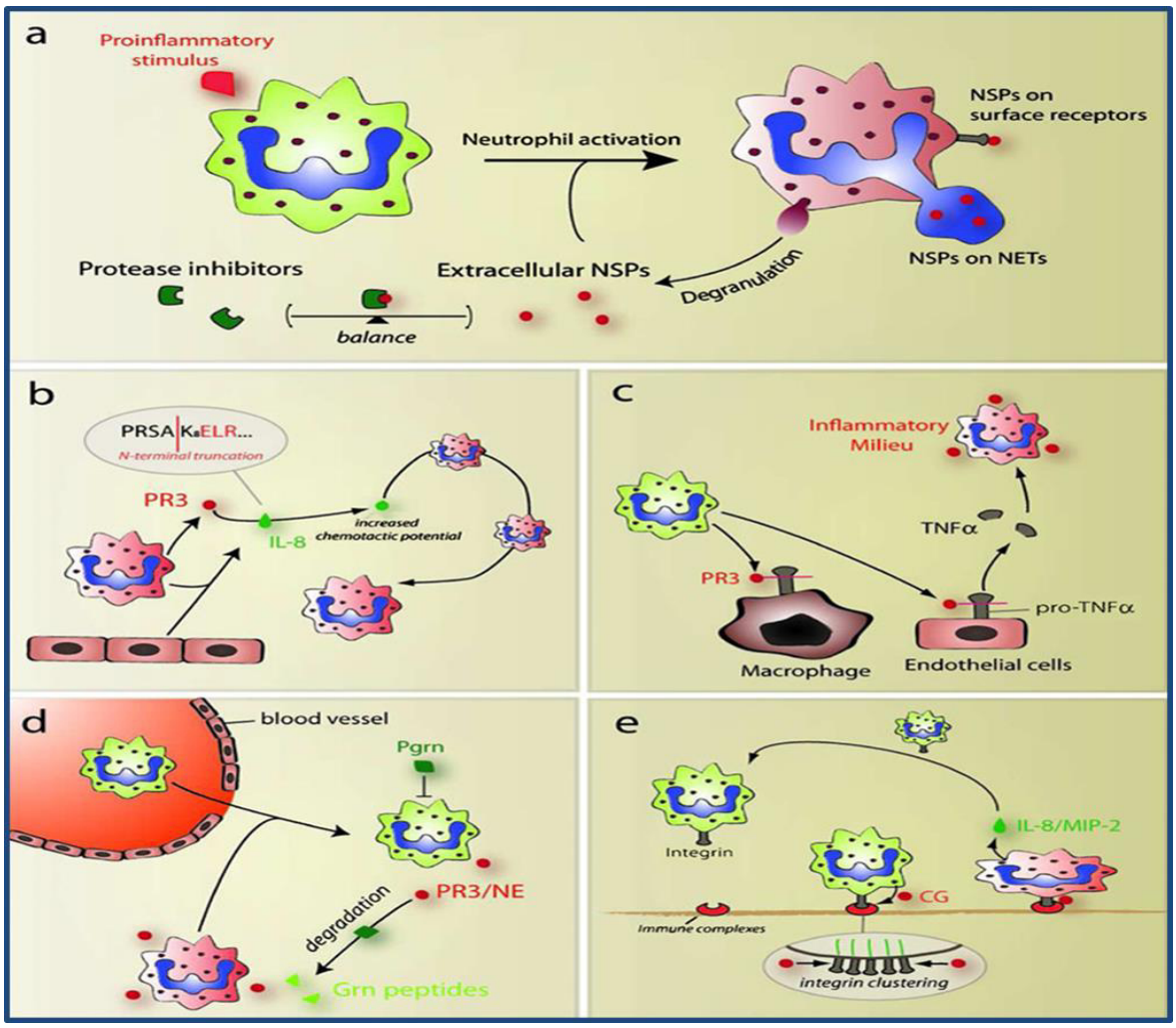


Figure 1.4 NSPs as regulators of inflammatory processes. (a) Activated neutrophils are able to externalize NSPs by degranulation, or formation of NETs. Parts of the NSPs are directed to cell surface receptors and stay in the pericellular environment. NSPs are controlled by protease inhibitors circulating in blood and interstitial fluids. (b) Pr3 converts IL-8 released from activated neutrophils and endothelial cells by N-terminal truncation into a more bioactive chemokine. This may potently enhance the recruitment of more neutrophils to the site of inflammation. (c) Pr3 is able to convert the membrane bound precursor pro-TNFα, to the potent inflammatory cytokine TNFα. (d) Neutrophils extravasating from the vasculature are initially controlled by anti-inflammatory progranulin (PGRN). PR3 and NE cooperatively enhance neutrophil activation by specifically degrading inflammation suppressing PGRN. (e) Cat G interacts with surface integrins during the adhesion of neutrophils to immobilized immune complexes, where it promotes integrin clustering, cytoskeletal rearrangements, and subsequently the release of neutrophil attracting chemokines. Reproduced from: (Kessenbrock et al., 2011), with permission from the publisher Springer.

Protease-antiprotease imbalance is involved in a variety of inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD), adult respiratory distress syndrome (ARDS), and cystic fibrosis (CF; Delacourt *et al.,* 2002).

1.5.1.1 Proteinase 3

Proteinase 3 (Pr3) is an abundant serine protease that is stored primarily in azurophilic granules along with NE and Cat G, although it has also been reported in specific granules and secretory vesicles (Witko-Sarsat *et al.,* 1999). Moreover, Pr3 is expressed at the plasma membrane of a subset of non-stimulated neutrophils from peripheral blood (Witko-Sarsat *et al.,* 1999; van der Geld *et al.,* 2001). In the neutrophil granules, Pr3 is stored as a mature and enzymatically active protein consisting of 222 amino acids (Goldschmeding *et al.,* 1989). It is unique from other neutrophil serine proteinases in many aspects. Pr3 has been identified as the major anti-neutrophil cytoplasmic auto-antigen (ANCA) which is found in patients with ANCA-associated diseases. (Van der Geld *et al.,* 2001) and is also an important factor in myeloid differentiation (Bories *et al.,* 1989; Labbaye *et al.,* 1991).

Unlike NE and Cat G, Pr3 is not released from the surface of activated neutrophils by high salt concentrations (Korkmaz *et al.,* 2005). In the general population, the percentage of membrane-bound Pr3 (mPr3) expressing neutrophils ranges from 0 to 100% and this distribution is genetically determined (Halbwachs-Mecarelli *et al.,* 1995; Schreiber *et al.,* 2003). This feature has not been found for other family members of NSPs. Interestingly, the intracellular levels of Pr3 do not correlate with mPr3. Exposure of neutrophils to multiple proinflammatory mediators such as: TNF-α, PMA, IL-18, LPS, IL-8, PAF, fMLP and GM-CSF; and by one anti-inflammatory cytokine: TGF-β, leads to the expression of Pr3 on the membrane of neutrophils (Campbell, 2000; Cseronk 1996; Hellmich, 2000; Mohamed Abdgawad, 2011). Pr3 does not contain a transmembrane domain or lipid anchor and thus

cannot be considered as a peripheral membrane protein that would have direct interaction with lipids. Several membrane binding partners of Pr3 have been identified, such as CD16/FcγRIIIb or the adhesion molecule CD11b/CD18 (β2 integrin), and glycosylphosphatidylinositol (GPI)-anchored CD177. In 2003, David and colleagues demonstrated that membrane-bound Pr3 co-localizes and co-immunoprecipitates with CD11b/CD18, therefore suggesting that both proteins are in the same complex (David *et al.,* 2003). Two years later, David *et al.,* 2005 provided evidence for the presence of Pr3, FcγRIIIb, and cytochrome b558 of the NADPH oxidase in neutrophil lipid rafts (David *et al.,* 2005).

Among Pr3 partners, CD177 is the only neutrophil protein that also has a bimodal membrane expression pattern with expression on 0% to 100% in the population (Stroncek, 2002). These Pr3 partners might be of critical importance for its functions, and its involvement in Wegener's granulomatosis (WG; Witko-Sarsat *et al.,* 2010; Hu *et al.,* 2009) (fig 1.5). Pr3 activity is controlled by a variety of natural inhibitors, such as α1- antitrypsin (AAT or α1-PI), elafin, and monocyte neutrophil elastase inhibitor (Rao *et al.,* 1991; Sugimori *et al.,* 1995). Pr3 complexed to AAT has been found in plasma of WG patients and healthy controls, although free Pr3 could also be detected (Baslund *et al.,* 1994; Perlmutter *et al.,* 1989). Pr3 is a multifunctional protein, which has several physiological and pathophysiological functions. Pr3 has microbicidal activity against gram-negative bacteria and gram-positive bacteria and fungi (van der Geld *et al.,* 2001). Pr3 contributes to intracellular killing of phagocytosed bacteria in phagolysosomes in combination with myeloperoxidase and reactive oxygen species generated by the NADPH oxidase complex. Furthermore, it has a role in extracellular killing through trapping of bacteria in NETs composed of DNA. It has a role in the degradation of a broad range of extracellular matrix proteins such as fibronectin, laminin, vitronectin, and collagen type IV (Rao *et al.,* 1991).

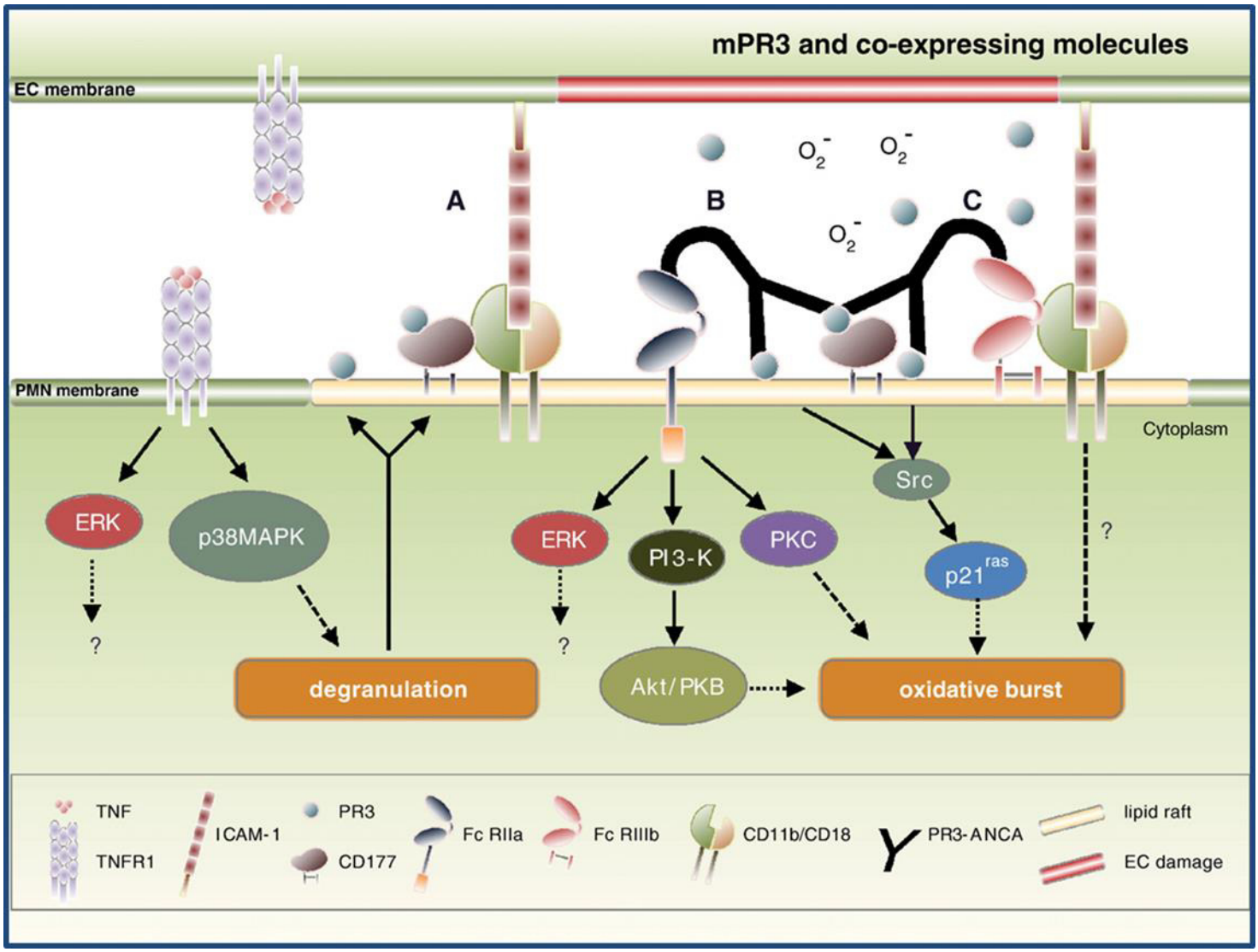


Figure 1.5 Relevance of mPr3-coexpressing molecules in the pathophysiology of Pr3-ANCA-mediated neutrophil activation. (A) Priming of neutrophils with TNF-α causes neutrophil degranulation and translocation of Pr3 to the plasma membrane. The binding of Pr3 to CD177 might encourage neutrophil firm adhesion through β2-integrin activation. (B) Pr3-ANCA cross-links mPr3 and FcγRIIa, which further induces the oxidative burst of neutrophils. Released proteolytic enzymes and reactive oxygen species cause vessel damage. (C) FcγRIIIb engagement activates β2-integrin, and the latter binds to ICAM-1 expressed on endothelial cells (EC) and, on the other hand, mediates Pr3-ANCA induced neutrophil activation. Reproduced from: (Hu et al., 2009), with permission from the publisher Elsevier.

Pr3 has been reported to be involved in the regulation and processing of inflammatory mediators and the activation of cellular receptors.

For instance, it can cleave pro-forms of TNF-α and cleave and activate IL-1β (Robache-Gallea *et al.,* 1995; Coeshottet *al.,* 1999; Korkmaz *et al.,* 2007). Pr3 and its homologous serine proteases can process the N-terminal extracellular domains of protease-activated receptors (PARs; Vergnolle, 2009). Pr3 exhibits activity in the regulation of myeloid differentiation (Bories *et al.,* 1989 and Sköld *et al.,* 1999). Internalization of proteolytic-inactivated Pr3 can cause endothelial cell apoptosis after a prolonged period (24 h) (Yang *et al.,* 2001).

1.5.1.1.1 CD 177

CD177 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein which belongs to the Leukocyte Antigen 6 (Ly-6) supergene family located on chromosome 19q13.2. CD177 has two alleles, NB1 (neutrophil antigen B-1 or Human Neutrophil Antigen-2) and PRV-1 (polycytemia rubra vera-1) which is the most common allele of CD177 (Termeniac *et al,* 2000). CD177 is only expressed on neutrophils, neutrophilic metamyelocytes, and myelocytes but no other blood cells and is first expressed at the myelocyte stage during granulopoiesis (Stroncek *et al.,* 1998 (1); Verheugt *et al.,* 1977).

The molecular mass of CD177 is 58 to 64 KDa and it was first discovered in patients with alloimmune neonatal neutropenia, where CD177-negative mothers generate antibodies that clear the CD177-positive neutrophils in their newborn babies (Kissel *et al.,* 2001; Lalezari *et al.,* 1971). CD177 is found on neutrophil plasma membranes and in secondary granules (Stroncek *et al.,* 1990; Goldschmeding *et al.,* 1992). CD177 has an identical biomodal expression pattern to surface Pr3 with a variable expression on 0-100% of neutrophils in the population (Stroncek 2002). Ninety-seven percent of Caucasians, 95% of African Americans

and 88% of Japanese express CD177 on their neutrophils surface (Matsuo *et al,* 2000; Taniguchi *et al.,* 2002: Bierling *et al.,* 1990). CD177 deficiency is the result of a gene expression defect (Kiessel et al, 2002). CD177 expression is also absent in persons with the disease paroxysmal nocturnal hemoglobinuria (PNH) since it is a GPI-anchored protein and PNH patients lose the ability to add GPI anchors to their blood cells through defects in the related protein processing pathway. Also the expression of CD177 is absent in some patients with chronic myelogenous leukaemia (CML) and the reason for that has not yet been identified (Goldschmeding *et al.,* 1992; Stroncek *et al.,* 1998b).

The expression of CD177 is increased during early and late gestation period in pregnant women (Carrucio *et al,* 2003). In women, the size of the CD177-positive subpopulation of neutrophils is approximately 49- 59% compared to approximately 42%-43% for men and the expression drops in older women but remains constant in men (Matsuo *et al.,* 2000). The surface expression (density of expression on positive cells, not the percentage of cells expressing CD177) of CD177 is up-regulated by fMLP, granulocyte-colony-stimulating factor treatment and during bacterial infections (Gohring *et al.,* 2004; Stroncek, *et al.,* 1998a). CD177 has been reported to have high-affinity binding to the adhesion molecule platelet endothelial cell adhesion molecule-1 (PECAM-1) and the binding interaction between CD177 and PECAM-1 is ∼15 times stronger than PECAM-1 homophilic interactions thus CD177 supports neutrophil transendothoelial migration (Sachs *et al.,* 2007).

1.5.2 Matrix metalloproteinases (MMP)

Matrix metalloproteinases (MMPs) are the predominant family of enzymes that are characterized by their capability to degrade the extracellular matrix (ECM) under physiological and pathological conditions, and their dependence upon Zn^{2+} binding for proteolytic activity. They degrade the basement membrane and ECM to facilitate embryo

development, morphogenesis, and angiogenesis and also play critical roles in wound healing, inflammatory diseases, and tumour metastasis (Westermarck and Kahari 1999; Yoon *et al.,* 2003). The majority of MMPs are synthesized and secreted as inactive proenzymes or zymogens (pro-MMP) and require a proteolytic process to become active. Based on their substrate specificity, amino acid similarity and identifiable sequence modules, the MMP family can be subdivided into distinct subclasses: collagenases (MMP-l, -8, -13, -15, -18, -21), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -19, -20), membrane-type MMPs (MMP-14, -15,-16, -17, -24, -25), elastases (MMP-7, -12, -26) and cAMP metalloproteinases (MMP-23, -28) (Lagente *et al.,* 2005). MMPs control cell-cell and cell-matrix interactions by various roles, including affecting cell behaviour through the activation, inactivation, or release of adhesion molecules, growth factors and receptors, cytokines, and extracellular matrix proteolysis (Lin *et al.,* 2008; Vu TH and Werb 2000). These proteinases are expressed by various cells such as fibroblasts, epithelial cells, and inflammatory cells (such as macrophages, eosinophils and neutrophils; Kumagai *et al.,* 1999).

The MMPs are regulated by endogenous MMP inhibitors and imbalance between MMPs and naturally occurring MMP inhibitors may cause an excess of ECM destruction. Neutrophil granules contain three metalloproteases with great physiological and pathophysiological significance, namely MMP-8, MMP-9 and MMP-25. The first MMP recognized in PMNs was MMP-8 that is stored in the secondary granules and is capable of cleaving type I collagen into typical ¼ and ¾ fragments (Hasty *et al.,* 1990).

1.5.2.1 Matrix Metalloproteinase-8

MMP-8 is also known as collagenase-2 or neutrophil collagenase. Neutrophils store MMP-8 in specific granules and release it following moderate stimulation. Moreover, MMP-8 synthesis and release by endothelial cells, smooth muscle cells, and macrophages occur after

a prolonged exposure to inflammatory cytokines (such as IL-β or CD40L) and MMP-8 has also been reported to be present in atherosclerotic plaques (Herman *et al.,* 2001; Lenglet *et al.,* 2013). A high concentration of serum MMP-8 is connected with the presence of atherosclerosis and poor cardiovascular disease (CVD) prognosis (Tuomainen *et al.*, 2007). An inactive pro-enzyme of MMP-8 is activated by reactive oxygen species (ROS) released from activated neutrophils, a variety of proteases (such as Cat G), several other MMPs (such as MMP-3) and several bacterial proteases (Lenglet *et al.,* 2013). After activation, MMP-8 can cleave a wide range of collagenous substrates (such as collagen type I > type III > type II), non-collagenous ECM substrates (such as fibrinogen) and non-structural substrates (such as serine protease inhibitors) (Hasty *et al.,* 1987; Hiller *et al.,* 2000; Van Lint and Libert 2006). MMP-8 has been reported to suppress tumour formation or metastasis depending on the model system (Korpi *et al.,* 2008; Balbín *et al.,* 2003).

1.5.2.2 MMP-9

MMP-9 (92 kDa protein; also known as type IV collagenase or gelatinase B) degrades native type IV and V collagens, fibronectin, ectactin, and elastin and, therefore plays a main role in invasion, tumour growth, and metastasis (Scorilas *et al.,* 2001). Neutrophils synthesize MMP-9 during maturation in the bone marrow which then is stored in tertiary granules, and is secreted upon stimulation. Different mediators, including fMLP, TNF-α, LPS and IL-8 have been shown to induce MMP-9 release from neutrophils (Pugin *et al.,* 1999). MMP-9 is also produced, by monocytes/macrophages, eosinophils, bronchial epithelial cells, Clara cells, alveolar type II cells, smooth muscle cells, endothelial cells, and fibroblasts in response to various forms of stimulation (Atkinson and Senior 2003; Chakrabarti and Patel 2005a). Inactive MMP-9 pro-enzyme is activated by reactive oxygen species (ROS) and by proteases (such as MMP-3) and inhibited by α2-microglobulin and by Tissue Inhibitors of Metallo-Proteinases (TIMP; particularly by TIMP-1) (Parks *et al.,* 2004; Page-McCaw *et al.,* 2007;

Atkinson and Senior 2003; Peppin and Weiss 1986). MMP-9 has been reported to play a role in some inflammatory diseases, such as rheumatoid arthritis, asthma and sepsis. Furthermore, increased levels of plasma MMP-9 are detected in acute coronary syndrome and myocardial ischemia-reperfusion (Lin *et al.,* 2005). MMP-9 facilitates the recruitment of inflammatory cells such as macrophages, neutrophils, and lymphocytes (Standiford *et al.,* 1993; Opdenakker *et al.,* 2001). Several studies have shown normal PMN emigration despite MMP-9 deficiency (Castaneda et al., 2005; Delclaux *et al.,* 1996; Opdenakker *et al.,* 1991).

1.5.3 Protease inhibitors

The proteolytic activity of neutrophil proteases seems to be tightly regulated in the extracellular and pericellular space to avoid unwarranted degradation of connective tissue proteins such as elastin and collagen. Neutrophil protease activity is controlled by a variety of natural inhibitors (Table 1.2). Serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors. Serine protease inhibitors regulate a number of proteases that participate in the inflammatory process. AAT is a natural inhibitor for the neutrophil serine protease produced mainly by hepatocytes and released into the blood circulation by the liver (Pierce 1988). It is present in all body tissues but appears to have its primary physiologic significance in the lungs where it protects alveolar tissues from destruction by NSPs. It is the most abundant serpin present in human blood with normal serum concentration ranging between 1.5 to 3.5 g/L (or 20 to 48 Mm) but it may increase during acute phases of inflammation and after infection (Fregonese and Stolk 2008). AAT is an excellent irreversible inhibitor of NE, Pr3 and Cat G. AAT deficiency increases the risk of developing a variety of diseases including pulmonary emphysema, cirrhosis of the liver and gut disease. An increased incidence of AAT phenotypes associated with dysfunctional AAT or low serum levels has been reported in patients with anti-Pr3 antibodies (Savage *et al.,* 1995).

Table 1.2 Inhibitors of serine and matrix metalloproteinases.

Protease inhibitors	Source	Target proteinase	Type of inhibition
I-Serpins			
AAT	Produced in the liver Found in serum	NE, Cat G, Pr3	Irreversible
Antichymotrypsin	Liver and macrophages	Chymotrypsin	Reversible
Serpin B1	Neutrophils, Macrophages	Cat G, NE, Pr3	
II-Chelonianin			
SLPI	Mucosa	NE, Cat G	Reversible
Elafin	Mucosa	NE, Pr3	Reversible
III- Alpha 2 macroglobulin	Liver and macrophages	NE, Cat G, Pr3, MMP 8, MMP 9	Irreversible
IV-TIMPs	Many cell types including epithelial cells, fibroblasts, neutrophils and monocytes	Matrix metalloproteinases MMP-2/MMP-9 MMP-1/MMP-8	Irreversible

A1-antichymotrypsin (ACT) inhibits serine proteases of the chymotrypsin type. It does not inhibit NE and Pr3, but is proteolytically degraded by NE (Rubin et al., 1994). Neutrophil MMP-8 but not MMP-9 inactivates ACT by cleaving the inhibitory loop (Desrochers et al., 1992; Korkmaz et al., 2010). Secretory Leukocyte Protease Inhibitor (SLPI) also known as anti-LeukoProtease, is a reversible inhibitor of NE and Cat G but not Pr3. SLPI is degraded by Pr3 (Rao *et al.,* 1993). It is present in a number of bodily secretions, with the highest concentration in the upper airways where it plays a more important role (Vogelmeier *et al.,* 1991). In the lower airways, SLPI concentration is low but it could play a physiological role, because it has been demonstrated by immunohistochemistry to be in contact with elastin fibres (Kramps *et al.,* 1989; Korkmaz *et al.,* 2010).

Serpin B1 (also known as monocyte neutrophil elastase inhibitor) is a highly potent inhibitor of neutrophil serine proteases, and has comparable kinetics of inhibition against NE, Pr3, and cathepsin G (Cat G). Serpin B1 is expressed in bronchial and glandular epithelial cells in addition to neutrophils, macrophages, and mast cells. (Yasumatsu *et al.,* 2006). Elafin is a 6 kDa protein that is a potent inhibitor of both NE and Pr3 but not cathepsin G. Elafin is expressed by many cell types and is present in the lung but is also expressed by endothelium and alveolar macrophages (Moreau *et al.,* 2008; Sumi *et al.,* 2002; Mihaila and Tremblay 2001).

MMPs are inhibited by both endogenous and exogenous inhibitors. The endogenous metalloproteinases inhibitors are tissue inhibitors of metalloproteinases (TIMPs). TIMPs are a family (TIMPs 1–4) of natural tissue inhibitors of MMPs which are found in most tissues and body fluids. They inhibit MMP activity by binding to the catalytic site of MMPs in 1:1 stoichiometric complexes (Brew *et al.* 2000). In addition to their functions as MMP inhibitors, TIMPs also regulate a number of cellular processes including cell growth, migration, and apoptosis (Stetler-Stevenson 2008).

1.6 Neutrophil Transendothelial Migration (TEM)

The transendothelial migration of neutrophils from the vascular lumen through vascular walls and to the site of infection or tissue damage is crucial for immune and inflammatory responses, and also is a key cause of various inflammatory disorders. The process is facilitated and mediated by a complex of mechanical, chemical, cellular and molecular responses. These are distinct events that are connected in a temporal sequence and are initiated by signals produced by tissue-resident macrophages (e.g. IL-1β and TNF-α), that receive signals from infected or damaged tissues and invading organisms (e.g. LPS), which activate endothelial cells (EC) responsible for neutrophil recruitment. The cellular responses include tethering, rolling, adhesion, crawling and, finally transmigration, (which are the classical steps of neutrophil transmigration). A number of additional steps have been added to this process such as "slow rolling" and "intravascular crawling" (Kolaczkowska *et al.,* 2013 fig1.6).

The molecular events which occur at each step involve interaction between specific neutrophil and EC adhesion molecules (e.g selectins, integrins and Ig superfamily molecules) and their counter receptors, regulated by neutrophil stimulatory molecules (e.g. chemokines). TEM (transendothelial cell migration) occurs through endothelial cells (transcellular route) or via junctions between adjacent ECs (paracellular route; Carman and Springer 2008; Carman, 2009). The venular wall is composed of two cellular components, ECs and pericytes, and a non-cellular matrix protein structure called the vascular basement membrane (BM), which is generated by both ECs and pericytes (Nourshargh *et al.,* 2010). The endothelial cells show distinct phenotypic and morphological characteristics, according to their position on the vascular tree. For instance, EC in the blood brain barrier (*BBB*) have well organised tight junctions securing the homeostasis of the cerebral environment.

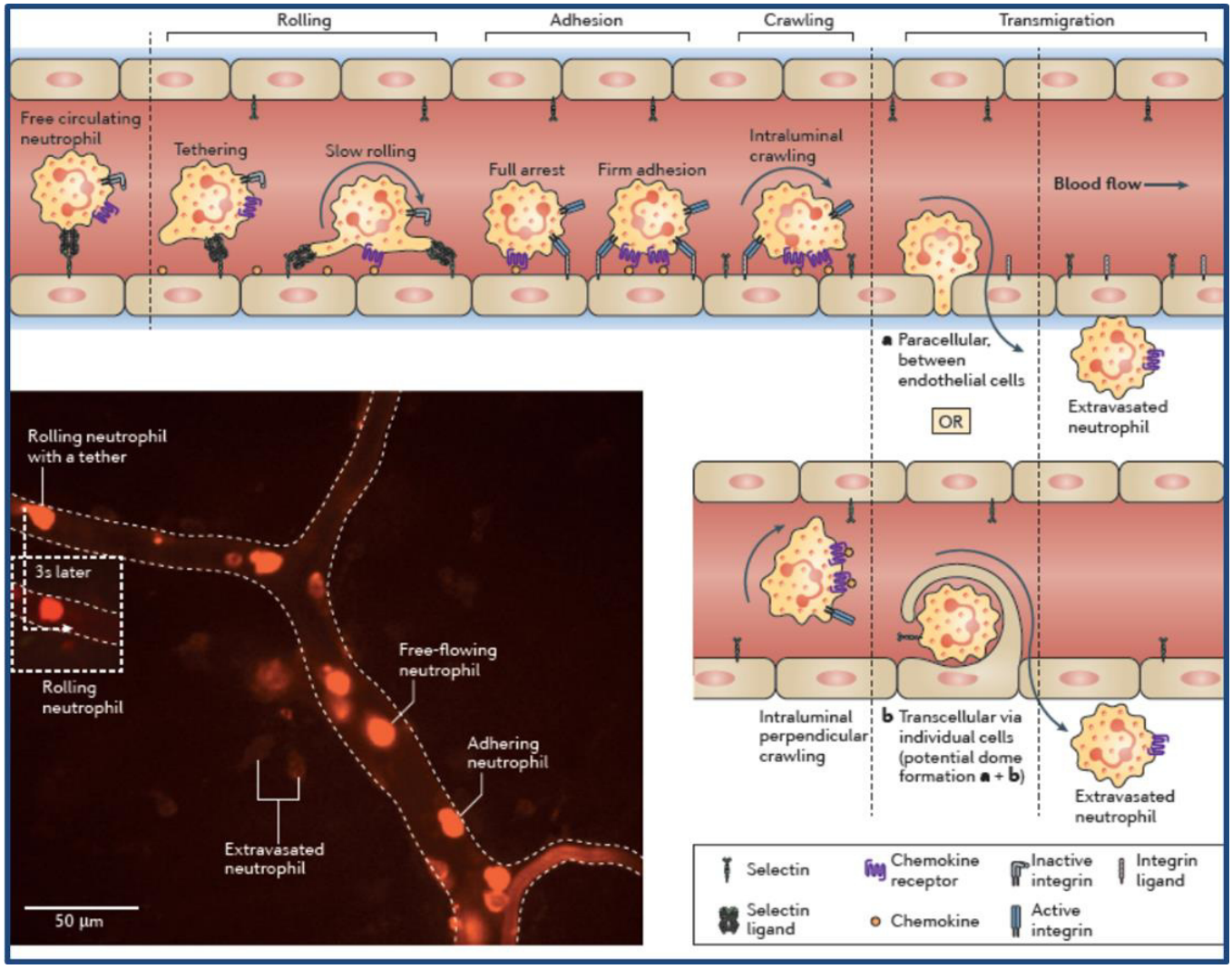


Figure 1.6 The updated classical neutrophil transendothelial Migration (TEM) cascade. A number of additional steps have been added to the cascade including "slow rolling" and "intravascular crawling" and a more multifaceted transmigration response. Rolling is mostly selectin-dependent, whereas adhesion, crawling and transmigration depend on integrin interactions. Chemokines lining the luminal part of endothelium activate rolling neutrophils, thus inducing conformational changes of neutrophil surface integrins and allowing for subsequent events. Crawling neutrophils follow the chemokine gradient along endothelium, which guides them to the preferential sites of transmigration. The intravital microscopy image shows a skin postcapillary venule from skin infected mouse with Staphylococcus aureus. The neutrophils (labelled in red) captured at different stages of migration: freely circulating cells, rolling cells extending tethers, adhering neutrophils and the cells that migrated out of the blood vessel. Reproduced from: (Kolaczkowska and Kubes 2013), with permission from the publisher Nature Publishing Group.

Whereas EC in the sinusoid capillaries of the liver lack junctions and basement membranes, as well as a highly fenestrated surface, enabling the direct movement of macromolecules from the blood to the liver parenchymal cells (Enomoto and Nishiwaka, 2004).

Neutrophil transmigration mostly occurs in the post capillary venules within the systemic circulation and in the capillaries of the pulmonary circulation. The movement of neutrophils from the central stream of blood to the periphery of a vessel (called neutrophil margination) allows a molecular interaction between the cell surfaces of the neutrophil and endothelial cell to occur, resulting in neutrophil rolling on the vessel wall (Seely *et al.,* 2003).

1.6.1 Tethering (capturing) and rolling

The initial contact between neutrophils and the endothelium is mediated by the tethering and rolling of neutrophils on the endothelial cell surface. This begins with the capture of marginating neutrophils via receptors highly expressed on the activated EC. The slow flow rate at sites of inflammation, allows a loose and somewhat transient adhesion, referred to as tethering. Throughout the tethering step, neutrophils respond to ligands, mainly chemokines, on the luminal membrane of endothelial cells (Middleton *et al.,* 2002; Wang L *et al.,* 2005). The movement of neutrophils on EC is termed “rolling”, which includes both physical and molecular forces.

The neutrophil’s capability to roll and adhere to EC is inversely proportional to the vessel shear rate (i.e. the slow moving of blood increases the ability of leukocytes to adhere). Neutrophil rolling rapidity is also directly proportional to luminal red blood cell rapidity. When in the proximity of the endothelial cell, a low-affinity adherence occurs and, in conjunction with the shear stress of passing plasma and erythrocytes, the neutrophil begins to roll along the endothelial lining of the vessel (Blixt 1985; Firrell and Lipowsky 1989; Perry and Granger 1991; Seely 2003).

The weak interaction involved in rolling is largely directed by selectins and their glycosylated ligands. Selectins are a large family of glycoprotein surface adhesion molecules, including L-selectin (expressed on circulating neutrophil usually on their microvilli; McEver 2002), E-selectin (expressed on endothelial cells), and P-selectin (expressed on platelets and endothelial cells). P-selectin, stored in Weibel-Palade bodies within inactive endothelial cells, and E-selectin, which is synthesized *de novo*, are translocated to the apical cell membrane where they transiently bind ligands on neutrophils. These selectin ligands include P-selectin glycoprotein ligand-1 (PSGL-1; expressed on the tips of neutrophil microvilli), E-selectin ligand-1 (ESL-1; expressed also on the tips of neutrophil microvilli), and CD44 which is expressed on the cell body of neutrophils (Buscher *et al,* 2010; Bruehl *et al.,* 1997; Steegmaier *et al.,* 1997).

Binding of PSGL-1 to P-selectin and E-selectin creates the initial communication between neutrophils and activated endothelial cells. E-selectin and ESL-1 facilitate the slower rolling, whereas E-selectin binding to CD44 facilitates a redistribution of PSGL-1 and L-selectin to form clusters concomitant with further reduction in the speed of rolling (Hidalgo et al. 2007). L-selectins can bind to PSGL-1, CD34 and glycosylated cell adhesion molecule-1 (GlyCAM-1) (Spertini *et al.,* 1996; Oxley and Sackstein, 1994; Lasky *et al.,* 1992). These selectin ligands on endothelial cells are inducible with LPS or an assortment of pro-inflammatory cytokines (Spertini *et al.* 1991). Pervious study demonstrated that blocking L-selectin and/or P-selectin with high-dose selectin-binding carbohydrate (fucoidin), decreases in both neutrophil rolling and adherence following ischemia/reperfusion (Kubes *et al.,* 1995). Selectin-mediated neutrophil and EC interaction is reversible and only continues for seconds.

1.6.2 Neutrophil activation and adhesion

Neutrophils can be activated by a number of molecules such as chemokines, fMLP, the complement fragment C5a, leukotriene B4 (LTB4), IL-8 and platelet activating factor (PAF). However, in the situation of physiological inflammation, the role of endothelial derived and presented chemokines are thought to be crucial for leukocyte activation through the TEM (Schiffmann *et al.,* 2008; Gerard and Gerard, 1994; Goldman and Goetzl, 1982; Hanahan, 1986). The binding of chemokines to their G-coupled protein receptors on neutrophils prompts neutrophil integrin activation, which induces neutrophils to adhere firmly to the EC surface.

Unlike rolling, which is a dynamic low-affinity adhesive interaction, adherence is a stationary high-affinity (strong) adhesive interaction between the neutrophil and endothelial cell (Seely *et al.,* 2003). The adhesion is mediated by a set of adhesion molecules, specifically the integrin β2 subfamily (CD11a, CD11b, CD11c/CD18) and their complementary surface molecule ligands on EC. Integrins are a family of heterodimeric proteins (formed by two linked heterodimers, α and β chain) that are expressed on the cell surface; and are integral to the process of cell adhesion. Leukocyte integrins bind to the immunoglobulin super family members such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), expressed by activated EC.

Integrins facilitate firm leukocyte adhesion, brought about by chemokines and chemoattractants that cause conformational alterations within integrin molecules on the neutrophil surface. Neutrophil-expressed lymphocyte function-associated antigen 1 (LFA-1) is also known as CD11a/CD18 or αLβ2is and macrophage 1 antigen (Mac-1) is also known as CD11b/CD18 or complement receptor 3 or αMβ2. CD11a/CD18 is also expressed on lymphocytes, NK cells, monocytes and macrophages, dendritic cells and eosinophils (Smith

et al., 1989). Its ligands are ICAM-1,-2,-3 and junctional adhesion molecule–A (JAM-A) (Ostermann *et al.,* 2002). Mac-1 participates in neutrophil adhesion by binding to ICAM-1 or -2 on the EC surface (Smith *et al.,* 1989). In a static, *in vitro* model of inflammation, blocking of β2 integrin caused inhibition of neutrophil adhesion (Luscinskas *et al.,* 1989). However, under flow situations, antibodies against β2 integrin specifically inhibited the firm adhesion step but not rolling suggesting that β2 integrins are required for firm adhesion but not for rolling (Bahra *et al.,* 1998). The β2 integrins are incapable of interacting with their functional ligands on unstimulated neutrophils, a safety mechanism that controls acute and chronic inflammatory responses. ICAM-1 (CD54) is constitutively expressed on venular endothelium and some leukocytes; however after stimulation by inflammatory cytokines (e.g. TNF-α), most of the body's cell types can express ICAM-1. ICAM-2 is expressed on platelets and on endothelial cells, where it is concentrated on the apical surface close to intercellular junctions and it is not up regulated by inflammatory cytokines. VCAM-1 is a transmembrane protein expressed on a variety of vascular and nonvascular cells in response to inflammatory cytokines (eg, TNF-α) and functions as a ligand for Very Late Antigen-4 (VLA-4; Smith, 2008; Ibbotson *et al.,* 2001).

Increased neutrophil surface expression of CD11b/CD18 is generated by mobilisation of intracellular pools by various cytokines (TNF-α), bacterial products (fMLP and LPS), growth factors (GM-CSF) and chemoattractants (PAF, IL-8, FMLP, and C5a). Integrins are not only responsible for the neutrophil adhesion to the endothelium, but they also transfer signals from the extracellular domain into the cell (outside-in signalling) (Ginsberg *et al.,* 2005). These signals support adhesion and prompt crawling after leukocytes flatten and extend pseudopods across the ECs surface (Ley *et al.,* 2007; Ginsberg *et al.,* 2005; Giagulli *et al.,* 2006). After firm adhesion, neutrophils migrate over the EC monolayer in their attempt to find the appropriate location to transmigrate in a CD11b/CD18 and ICAM-1 dependent manner

(Phillipson *et al.,* 2006). The important characteristic of venular ECs is the expression of EC adhesion molecules such as ICAM-1 and VCAM-1, integrin ligands whose expression is enhanced on activated ECs. Studies have shown that the expression of these molecules can be further regulated resulting in the formation of pro-adhesive sites termed "endothelial adhesive platforms" (EAPs) (Barreiro *et al.* 2008) or sites that promote TEM, termed "docking structures" or "transmigratory cups" (Barreiro *et al.,* 2002; Carman and Springer 2004).

1.6.3 Crawling and migration through EC

Once neutrophils have adhered, they crawl along the luminal surface of inflamed blood vessels seeking specific locations for transendothelial migration. During crawling, Mac-1 but not LFA-1 plays the principal role (Phillipson *et al.,* 2006). When crawling is deactivated (by using Mac-1−/− neutrophils), transmigration is delayed and occurs preferentially via the transcellular route as opposed to the paracellular route (Phillipson et al., 2006). The binding between ICAM-1 and Mac-1 is essential for leukocyte crawling that ultimately permits efficient emigration out of the blood vessel (Hepper *et al.,* 2012). Leukocyte crawling can cause signalling events which prompt the transient weakening of endothelial cell junctions or the formation of intracellular pores, that are important for paracellular and transcellular TEM, respectively (Nourshargh *et al.,* 2010).

1.6.4 Transmigration

The final step in neutrophil TEM is the migration through venular walls in a process known as transmigration (also called diapedesis or extravasation). Emigrating leukocytes must first cross three distinct barriers: endothelial cells, the endothelial-cell basement membrane, and pericytes. Leukocyte migration through the endothelium can be rapid (<2–5 minutes), but penetrating the endothelial-cell basement membrane can take much longer (>5–15 minutes) (Ley *et al.,* 2007). As mentioned previously, neutrophils can migrate, through the paracellular

route, where they squeeze between ECs or the transcellular route, whereby neutrophils penetrate the individual EC (fig1.7). However, most studies agree that leukocyte transmigration mainly occurs via endothelial junctions (Luscinskas *et al.,* 2002; Johnson-Léger *et al.,* 2000). There is evidence that *in vitro* transmigration can occur at tricellular junctions, where there are fewer junctional proteins and the alignment of endothelial cells is less well ordered (Burns *et al.,* 1997; Burns *et al.,* 2000; Woodfin *et al.,* 2011; Kolaczkowska and Kubes, 2013).

Transmigration is mediated by integrins (major neutrophil β2 integrins LFA-1 and Mac-1) and CAMs (ICAM1, ICAM2 and VCAM1) as well as different junctional proteins, including platelet/endothelial cell adhesion molecule 1 (PECAM1; also known as CD31), CD99, junctional adhesion molecules (JAMs), epithelial cell adhesion molecule (ECAM) and some other endothelial cell molecules such as poliovirus receptor (PVR; also known as CD155), ectoenzymes (e.g., vascular adhesion protein 1 (VAP1) and CD157) and leukocyte specific protein 1 (LSP1) (Kolaczkowska and Kubes, 2013). Some of these molecules may play a key role in guidance toward paracellular or transcellular migration (Barreiro *et al.,* 2008; Borregaard, 2010). PECAM-1 is expressed on the neutrophil surface, at the endothelial cell junction, and on platelets (Albelda *et al.,* 1991).

1.6.5 Paracellular transmigration

Paracellular transmigration is mediated by a number of junctional adhesion molecules that include JAMs, PECAM-1, vascular endothelia cadherin (VE-cadherin), and endothelial specific adhesion molecule (ESAM), and non-junctional adhesion molecules including ICAM-1, ICAM-2, and CD99. ICAM-1 and ICAM-2 mediating neutrophil contact via their β2 integrin partners (LFA-1 and Mac-1).

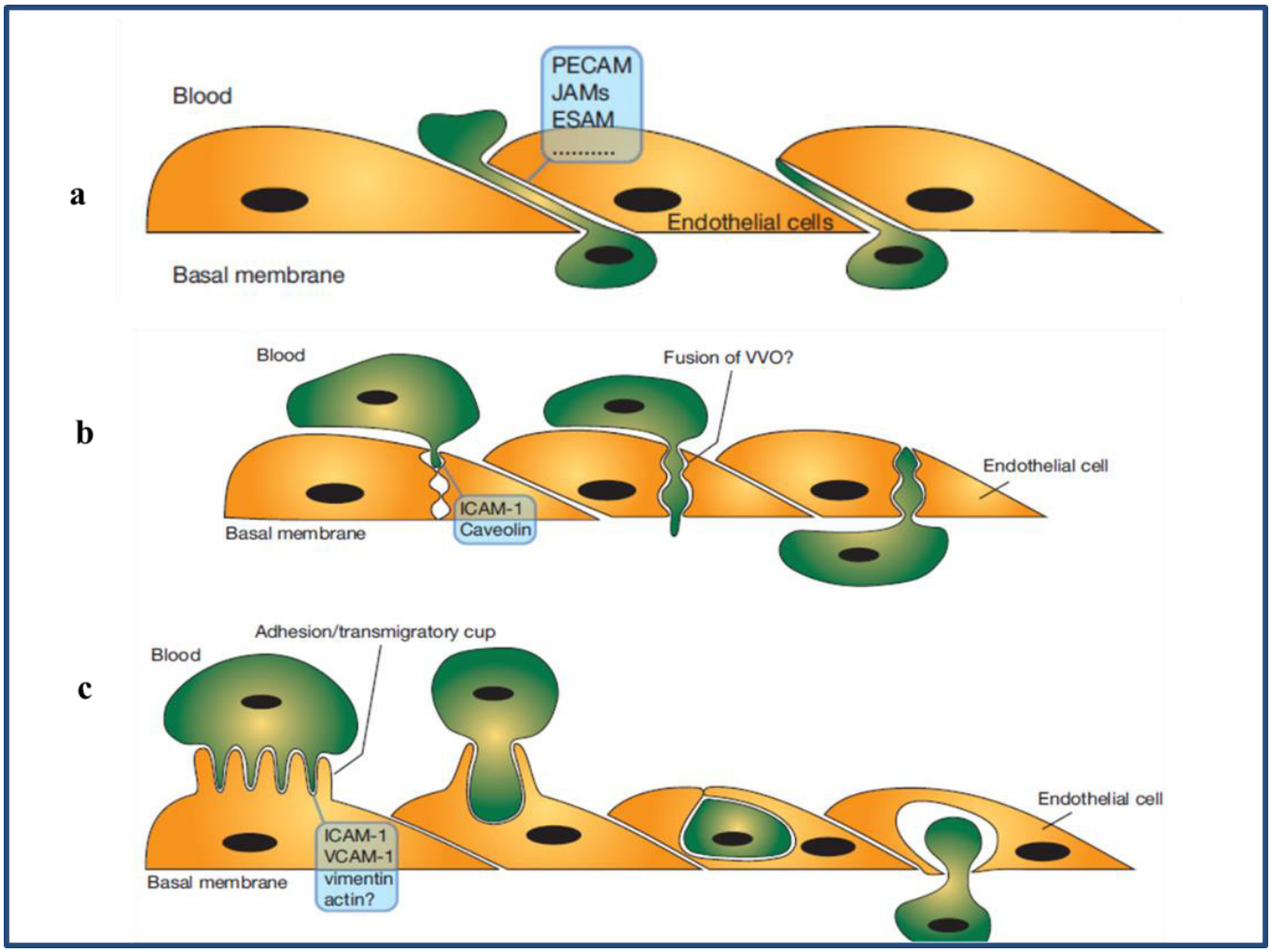


Figure 1.7 Transmigration of leukocytes through venular walls. The process can occur by two distinct routes: paracellular or transcellular. a) Paracellular route through endothelial cell junctions, where leukocytes interacting with adhesive proteins (such as PECAM and JAM) at EC junctions. In the transcellular route leukocytes can cross the endothelium by penetrating the cell cytoplasm. b) Leukocytes may actively penetrate the endothelial cell cytoplasm by elongating pseudopods inside vesicles containing caveolin and ICAM-1. These vesicles can fuse with vesiculo-vacuolar organelles (VVOs), forming a channel that allows leukocyte migration through the endothelial monolayer. c) When leukocytes adhere to the endothelial surface, an adhesion/transmigration cup is formed. This docking structure contains microvilli that elongate from both endothelial cells and leukocytes. The microvilli contain adhesion molecules (such as ICAM-1 and VCAM-1) and cytoskeletal proteins (such as vimentin and actin). (Adopted from Dejana 2006).

Signals from ICAM-1 activate Src and Pyk-2 tyrosine kinases, which phosphorylate VE-cadherin (vascular endothelial) and destabilize the VE-cadherin bonds. Blocking of ICAM-1 and ICAM-2 has shown that they are both implicated in guiding neutrophils to enter EC-junctions (Alcaide *et al.,* 2009). VE-cadherin is largely responsible for maintaining the integrity of the endothelium and regulates its barrier function to macromolecules (Crosby et al., 2005; Gavard and Gutkind 2006; Fukuhara *et al.,* 2005). The paracellular transmigration requires the transient loss of function or liberation of junctional intercellular protein bonds, such as (VE)-cadherin PECAM-1; JAM A, -B, and -C; ESAM and CD99 form homotypic contacts, thus these molecules also maintain endothelial cell-cell junctions. These adhesion molecules are also expressed on the neutrophil (PECAM-1, JAM A, CD99) and are able to bind to proteins expressed on the neutrophil surface; and therefore, facilitate the neutrophils in passage between the EC.

PECAM-1 is expressed on the neutrophil surface, at the EC junction, and on platelets (Albelda *et al.,* 1991). It mediates neutrophil extravasation via homophilic interactions (PECAM-1/PECAM-1) between PECAM-1 on leukocytes and PECAM-1 on endothelium. Blocking this interaction with a specific monoclonal antibody or with a soluble form of PECAM-1 blocks the process *in vitro* (Muller *et al.,* 1993; Liao, *et al.,* 1995). Moreover, a number of heterophilic binding partners for PECAM-1 have been described, including CD38, αvβ3, and glycosaminoglycans (Deaglio *et al.,* 1998; DeLisser *et al.,* 1993). CD177 also has been reported as a heterophilic binding partner of PECAM-1, thus the heterophilic interaction between CD177 and PECAM-1 participates in neutrophil transmigration. Blocking antibodies directed against either CD177 or Ig-domain 6 of endothelial PECAM-1 were able to significantly inhibit neutrophil transmigration toward chemotactic gradients, and CD177-positive neutrophils migrated more rapidly than CD177-negative neutrophils (Sachs *et al.,* 2007).

JAM-A is also necessary for neutrophil transmigration via homophilic interactions and heterophilic interactions with LFA-1, but the JAM-A/LFA-1 interaction was found to be stronger than the JAM-A/JAM-A homophilic interaction suggesting that during TEM, leukocyte LFA-1 binding to EC JAM-A may destabilize the JAM-A homophilic interaction thus mediating the TEM response (Wojcikiewicz *et al.,* 2009). CD99 also plays a major role in the leukocyte transmigration and blocking CD99 on both leukocytes and on ECs inhibits transmigration suggesting that it is a homophilic interaction of CD99 which facilitates TEM (Schenkel et al., 2002).

1.6.6 Transcellular transmigration

Transcellular TEM has been demonstrated in a broad range of tissues such as bone marrow, thymus and lymph nodes (Carman 2009). The transcellular route is initiated by the formation of a cup-like "docking structure" (fig1.7c) which are microvilli-like projections that move up the side of the leukocyte and *in vivo* extend all the way to the top of neutrophils to form what have been termed 'domes' (Kolaczkowska and Kubes 2013). These structures are rich in adhesion molecules (such as ICAM-1 and VCAM-1) and cytoskeletal proteins (such as vimentin and actin). This cup-like structure is important not only for migration through individual endothelial cells but also for their leukocyte arrest (Carman and Springer et al., 2004). A previous study by Feng *et al*., showed that leukocytes migrate through the endothelial cell cytoplasm *in vivo* through multivesicular structures (fig1.7b) called vesiculo-vacuolar organelles (VVO) (Feng *et al.,* 1998; Dejana 2006). Phillipson *et al*, (2008) showed that Mac-1-dependent intralumenal crawling guides neutrophils to preferentially transmigrate at junctional sites, and transcellular migration increases from 20% to 80% in the absence of Mac-1, as determined in a mouse cremaster muscle preparation (Phillipson *et al,* 2008).

1.6.7 Neutrophil migration through venular walls beyond the endothelium

Following migration through EC, migrating cells face two further barriers; the pericyte (PC) sheath and the tough endothelial basement membrane (EBM) to enter the interstitial space (Hirschi and D'Amore 1996; Rowe and Weiss 2008). Due to the complexities associated with the generation of physiologically relevant basement membranes and difficulties in isolating and studying primary pericytes *in vitro*, little is known about this step of leukocyte transmigration. Pericytes are wrapped around endothelial cells, providing an interface between the circulating blood and the interstitial space (Kolaczkowska and Kubes *et al*, 2013). Using intravital microscopy it has been shown that the EBM contains pre-formed regions with low expression of certain basement membrane components (e.g laminin-8, laminin-10 and collagen IV), termed low expression regions (LERs), that are preferentially used by transmigrating neutrophils and monocytes (Voisin *et al.,* 2009 ; Wang *et al.,* 2006). This step is reported to be supported by β1 integrins (such as α2β1 and α6β1) receptors for collagen IV and laminins, respectively) and leukocyte proteases (e.g. NE) (Dangerfield *et al.,* 2002; Hallmann, 2005; Wang *et al.,* 2006).

Furthermore, such regions overlapped with gaps between pericyte regions, suggesting that they represent a path of least resistance for emigrating neutrophils (Proebstl *et al.,* 2012; Kolaczkowska and Kubes, 2013). The neutrophil is equipped with proteases such as NE, MMP-8, MMP-9, and the membrane-attached matrix metalloproteinase MT6-MMP capable of breaking down the basal membrane collagens and laminins (Kang *et al.,* 2001). Additionally, it has recently been shown that CD177, an atypical PECAM1 ligand that is expressed by neutrophils, bound neutrophil-derived Pr3 and localized this enzyme to endothelial junctions via heterophilic interaction with PECAM1. This CD177 mediated transfer of Pr3 to endothelial junctions facilitated neutrophil transmigration (Kuckleburg *et al.,* 2012).

1.7 Neutrophil apoptosis

Neutrophil apoptosis has an essential regulatory role in many biological processes, including the inflammatory response. It is vital for resolution of inflammation and maintaining homeostasis of the immune system. Most inflammation-related processes modify neutrophil apoptosis. For instance, phagocytosis of bacteria accelerates apoptosis significantly, presumably to facilitate clearance of effete or "spent" PMNs containing dead bacteria. On the contrary, some bacteria-derived products, such as LPS, extend neutrophil survival (Kobayashi *et al.,* 2005; Kobayashi *et al.,* 2003). Neutrophils have a constitutive apoptotic programme which is accelerated during activation of the cell to ensure clearance from sites of inflammation or infection before they become necrotic and release their toxic components to surrounding tissue (Cheah *et al.,* 2005). Neutrophil apoptosis induces characteristic morphological changes which are not seen in circulating neutrophils: cell shrinkage (decrease in cell volume), nuclear condensation, and cytoplasmic vacuolations, as well as biochemical changes such as DNA fragmentation, mitochondrial depolarization, and exposure of phosphatidylserine on the cell surface (Savill *et al.,* 1989).

In addition, neutrophils lose the ability to perform chemotaxis, phagocytosis, respiratory burst, and degranulation (Whyte *et al.,* 1993). There are two major apoptotic pathways involved in neutrophil apoptosis; (i) the extrinsic (death receptor) apoptotic pathway which directly activates the caspase cascade via caspase 8; and (ii) the intrinsic (mithochondrial) apoptotic pathway which involves mitochondrial and the Bc12 family of genes and activates the caspase cascade via caspase 9 (Akgul and Edwards, 2003).

1.8 Hypothesis and Aims of thesis

Hypothesis: Neutrophils release a majority of their MMP-9, but not Pr3 and MMP-8, through the extravasation process *in vitro* and *in vivo* and accumulated levels associated with diseases (such as chronic lung disease of prematurity and severe asthma) are related to the number of neutrophils recruited to the site of inflammation and the duration of the inflammatory event. Cell surface Pr3 is also induced during neutrophil stimulation and is maintained despite the presence of serum AAT and serpins, and the surface expressing Pr3 subset of neutrophils more readily transmigrate than CD177-negative neutrophil subsets. Neutrophil stimulation induces reorganisation of granule subsets so that the association of granule-specific markers with granule-specific proteinases becomes non-specific.

Aims

1. To further investigate the hypothesis that serum serine-proteinase inhibitors (alpha-1-antitrypsin) can remove proteinase 3 (Pr3) from the surface high affinity receptor using whole blood *ex vivo* stimulation models and *in vitro* Pr3 capture assays using recombinant soluble CD177 and monoclonal antibodies.

2. To measure intracellular and released Pr3, MMP-8 and MMP-9 during *in vitro* assays mimicking infection (co-incubation with microbes or microbial extracts) and extravasation (transwell assays), comparing pre- and post-migration levels.

3. To compare intracellular and surface proteinase levels between circulating neutrophils and salivary neutrophils (or other migrated neutrophil populations) as *in vivo* human models for diapedesis, for comparison to the *in vitro* transwell assays.

4. To examine redistribution of proteinases between separate granule subsets or into new merged intracellular compartments.

CHAPTER 2
MATERIALS AND METHODS

2.1 Neutrophil Isolation

2.1.1 Blood neutrophils isolation

2.1.1.1 Percoll gradient

Human blood (20 ml) was collected from healthy adult donors by venipuncture and anti-coagulated with sodium citrate (3.8%). Blood samples were mixed gently after collection and centrifuged at 450 xg for 20 minutes at room temperature. Platelet Rich Plasma (PRP; top layer) was transferred to a 50 ml conical tube, and then centrifuged for a further 20 minutes at 1300 xg at room temperature to remove platelets and the supernatant, Platelet Poor Plasma (PPP), was kept in a 20 ml universal tube for use in the percoll gradient creation later.

6mls of dextran (Sigma) at 6% (diluted in sterile saline) were added to the leukocyte and erythrocyte cell pellet and, then made up to the original volume of blood with warm sterile saline. The mixture was transferred to a fresh tube, the lid placed on loosely and allowed to sediment at 37◦C for 45 min. This removed most of the erythrocytes (red blood cells or RBC). The RBCs sedimented to the bottom of the tube, while the leukocytes and lymphocytes remained suspended in solution. During that time two concentrations of Percoll gradient solutions (51% and 42%) were prepared. In a 10 ml test tube 1.02 ml of 90% Percoll and 0.98 ml of PPP were mixed (51% gradient layer). In a separate 10 ml tube, 42% gradient layer was prepared by mixed 0.84ml of Percoll with 1.16 ml PPP. After dextran sedimentation, the leukocyte rich upper layer was removed and centrifuged at 200 xg for 6 minutes and the pellet gently resuspended in 2 ml PPP. To separate the mononuclear cells and remaining RBCs from neutrophils, the prepared 51% Percoll solution was added to the bottom of a 15 ml conical centrifuge tube then the 42% Percoll solution was carefully layered on top to avoid disturbing the interface using a Pasteur pipette. The re-suspended leukocyte pellet in PPP was layered over the Percoll gradient and centrifuged at 350 xg 13 minutes at

room temperature with the centrifuge brake set to minimum. After centrifugation, the mononuclear cells remained in the upper layer and the neutrophils were found at the layer interface, while the cell pellet in the bottom of the tube contained the remaining RBCs tubes (the steps are shown in figure 2.1A). The layers were carefully aspirated from the gradient and placed into clean tubes. The neutrophil layer was aspirated and resuspended in FACS buffer (PBS containing 1% BSA, 15mM EDTA) and counted on a haemocytometer and then adjusted to a concentration of $1x10^6$ cells/ml. The cells were then washed once in HBSS (Lonza) without calcium and magnesium and washed twice in HBSS containing calcium and magnesium, then recounted and readjusted to a final concentration of $1x10^6$cells/ml in HBSS with Ca^{2+} and Mg^{2+} or autologous serum collected at the same time as the initial venepuncture.

2.1.1.2 Dextran sedimentation

Two methods of dextran sedimentation were used, and several experiments utilised dextran purified mixed leukocytes rather than continuing onto gradient separation of the neutrophils as above.

2.1.1.2.1Using citrate-anti-coagulated blood

Venous blood (10 ml) was drawn from healthy volunteers and transferred into a tube containing 1ml of 3.8% sodium citrate (1/10 dilution). Leukocytes were separated from erythrocytes by addition of 6% dextran (in HBSS) and allowing sedimentation to occur for 45 minutes in a 37ºC water bath. The supernatant was aspirated and centrifuged at 1000 xg for 2 min and the cell pellet was gently re-suspended in 5 ml FACS buffer. The leukocyte cells were then washed once in HBSS without calcium and magnesium and washed twice in HBSS with calcium and magnesium.

2.1.1.2.2 Using heparin-anti-coagulated blood

Venous blood (10 ml) was drawn from healthy volunteers and transferred into tube containing 100 µl of heparin (100µl/10ml blood; final concentration of 50IU per ml of blood; WOCKHARDT). Leukocytes were separated by addition of 2.5ml of 6% dextran (in BSS; Balanced Salt Solution BSS; 0.13 M NaCl, 2.6 mM KCl, 0.8 mM Na2 HPO4, 1.83 mM KH2PO4 / pH 7.5) and sedimentation was allowed to proceed for 20 min or until a clear supernatant could be observed. The layer that contained the neutrophils (supernatant) was carefully aspirated using a small Pasteur pipette (Alpha Laboratories) (figure2.1B). The layer was then centrifuged for 1 min at 576 xg. at room temperature and the supernatant carefully removed. The pellet was re-suspended gently for 15 sec in 1 ml of distilled sterile water (for hypotonic lysis of RBCs). The iso-osmolarity was restored by addition of 25ml BSS. The suspension was centrifuged for 1 min at 576 xg. at room temperature and after the supernatant was removed, the cell pellet was re-suspended in DMEM (1 ml supplemented with 10% Foetal Calf Serum, 1% Penicillin-Streptomycin and 1% L -glutamine) then the cells were counted using Cellometer cell counting chamber slides (Nexcelom Bioscience, Lawrence, USA).

2.1.2 Isolation of salivary neutrophils

Salivary neutrophils were isolated from healthy volunteers with no history of oral inflammatory conditions. The volunteers were asked to rinse their oral cavity for 1 minute with 10 ml of sterile 1X PBS for one minute. The first washing contained unwanted material and necrotic neutrophils which had extravasated into the crevicular fluid for some time in advance of the washing. This was therefore discarded. Subsequent washings contained neutrophils which had newly arrived in the oral cavity. Four consecutive oral rinses of

approximately 10 ml were then collected in a fresh 50 ml falcon tube (1 min rinse each). Pooled rinses were centrifuged at 576 xg for 3 minutes. The supernatant was discarded and the pellet suspended in 2 ml of FACS buffer and then the cells were counted using Cellometer cell counting chamber slides.

2.2 Serum Preparation

In order to separate serum from blood, the whole blood was collected into 8 ml glass universal bottles (BD Vacutainer® SST™ II Advance Tubes) and incubated in an upright position at room temperature for 30-45 min (no longer than 60 min) to allow clotting. After centrifugation (1000 xg for 10 min) the supernatant (serum) was carefully transferred to clean universal bottles and kept at room temperature until used.

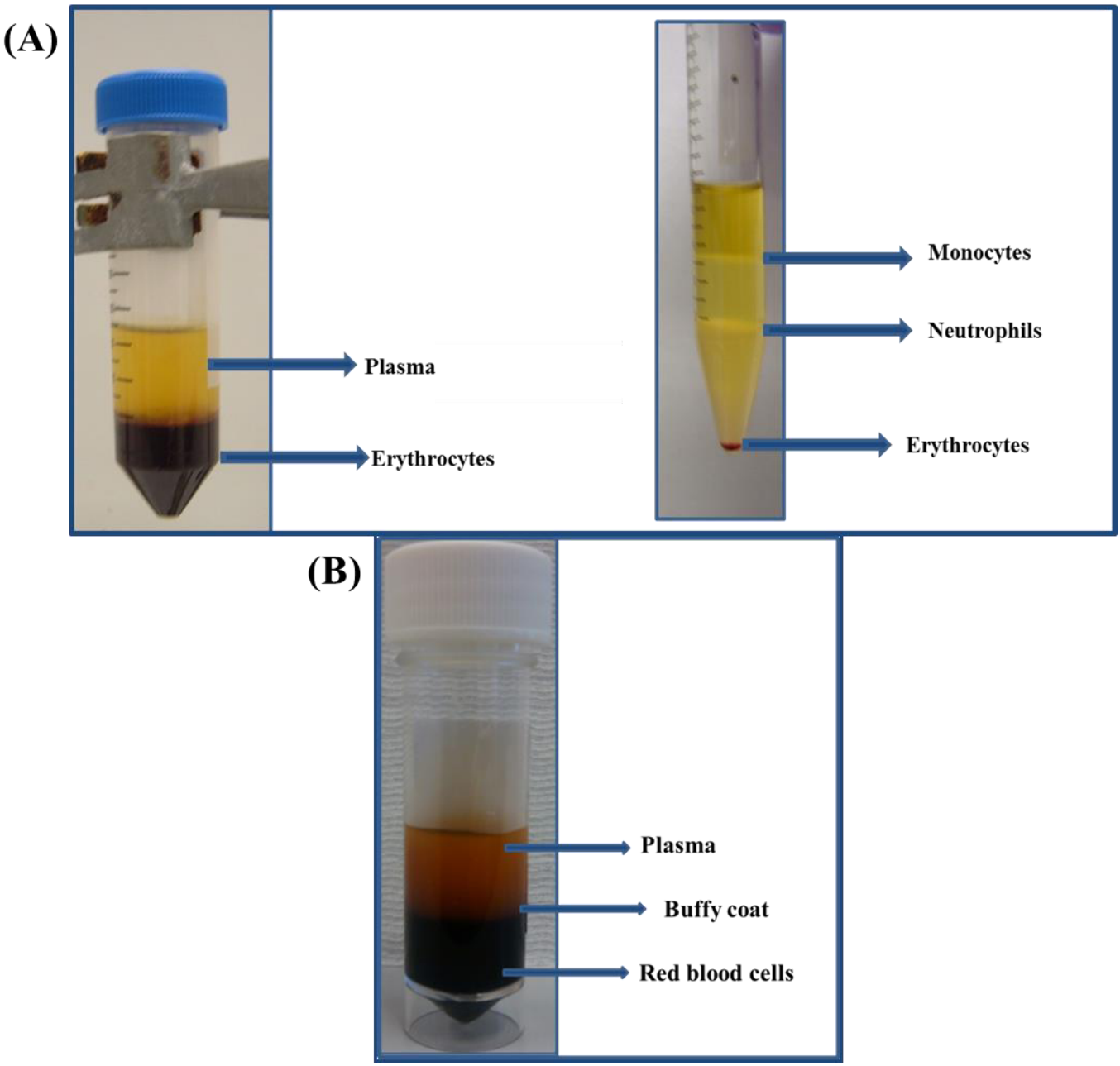


Figure 2.1 Blood neutrophil isolation by Percoll density gradient centrifugation (A), and separated by Dextran sedimentation (B).

2.3 Neutrophil stimulation

Following purification, neutrophils (10^6 cells) were placed in test-tubes so that the effect of adding formyl-met-leu-phe (fMLP; Sigma-Aldrich: final concentration 1μM) or a combination of fMLP (1μM) and cytochalasin B (5μg/ml Sigma-Aldrich) could be compared to unstimulated control cells. All tubes were mixed using a vortex mixer and incubated in 37°C for 15 minutes before vortexing again and removing part of sample for flow cytometric analysis. fMLP is bacterial peptide that plays a major role as a potent chemoattractant. The N-formyl peptide receptor is a G-protein coupled, widely expressed on the neutrophil's surface and other tissues (Wittmann *et al.,* 2002). Activation of this receptor leads to directed migration, granule mobilization and activation of the neutrophil NADPH-oxidase. Cytochalasin B is a cell-permeable fungal toxin, which enhances several fMLP-stimulated neutrophil responses, including aggregation, superoxide production, and degranulation (Honeycutt and Niedel 1986).

2.4 Flow cytometry

Flow cytometry is a technique used for detection and quantitation of cells and/or their components. An important advantage of flow cytometry is its ability to carry out measurements on thousands of cells in a very short time (Nunez, 2001). Flow cytometry can be used to count cells of different types in a complex mixture. In my project, flow cytometry was used to investigate the cell surface expression of Pr3, CD177, CD16, CD63, MMP-8 and MMP-9 in non-permeabilized (cell surface) and intracellular content in fixed/permeabilized cells (intracellular). Unconjugated isotype IgG controls as well as Allophycocyanin (APC)-conjugated or Fluorescein Isothiocyanate (FITC)-conjugated controls were used to detect background fluorescence cells.

2.4.1 Cell surface staining

Aliquots (100 µl; 10^5 cells) of cell suspension (in HBSS or serum; stimulated or unstimulated) were added to a round bottomed 96 well plate and centrifuged for 2 minutes at 1000xg at 4°C then the supernatant was discarded and the pellets were re-suspended in a appropriated antibody (Ab). The primary or conjugated Abs (table 2.1) were prepared in advance in FACS buffer according to their concentration (final working concentration 1-10 µg/ml). The plates were incubated at 4 ºC for 25 minutes, then 150 µl of FACS buffer was added to all wells and centrifuged for 2 minutes at 1000xg at 4°C, followed by two further washes with 200 µl of FACS buffer (pelleting by centrifugation and resuspension). If antibodies were un-conjugated a second incubation for 20 min at 4 ºC with R-Phycoerythrin goat anti-mouse antibody (RPE GAM; diluted1/100 in FACS buffer) was performed and washed twice with 200 µl of FACS buffer. Neutrophils were identified in mixed leukocyte populations by a third staining step using APC-conjugated anti-CD16 mouse monoclonal for 15 minutes at 4 ºC and washed again twice in FACS buffer. Finally in all conditions, the cells were re-suspended with 200 µl of FACS buffer before being transferred to FACS tubes ready to for FACS analysis.

2.4.2 Cell permeabilization (Intracellular staining)

In order to detect intracellular expression of specific molecules, cells were fixed and then permeabilized, using Fix and Permeablisation kit (An Der Grub Bio Research GmbH Kaumberg, Austria) as per manufacturer's instructions. This allowed the antibodies to cross the cell membrane and bind to their corresponding intracellular molecules. In a round bottom 96-well plate, 100 µl of cell suspension (in HBSS or serum; stimulated or unstimulated) was added and mixed with 100 µl of FACS buffer and centrifuged at 1000xg at 4°C for 2 minutes. Supernatants were then discarded and the pellet re-suspended in 100 µl of FACS buffer then

incubated with an equal volume of fixation agent (a paraformaldehyde containing buffer; Solution A), for 15 min at RT. The wells were then topped up with 100 µl of FACS buffer and the plates centrifuged. Cells were then washed once with 200 µl FACS buffer before being re-suspended in 75 µl of diluted Abs (listed in table 2.1) in permeabilisation agent B (a saponin containing buffer) and incubated for 15 min at room temperature in a dark place. For unconjugated antibodies, cells were washed and incubated with the rPE-conjugated goat anti-mouse immunoglobulin antibody diluted 1/75 in permeabilisation agent B and incubated for a further 15 min at room temperature in the dark. The cells were then washed and re-suspended in 200µl of FACS and transferred to FACS tubes ready for FACS analysis. All samples were run on a FACScalibur (Becton Dickinson) and data for 10,000 cells were collected and analysed by sub-population gating using CellQuest software the following day.

Table 2.1 List of antibodies used in flow cytometry

ANTIBODY	DILUTION	CONCENTRATION	SOURCE
ISO-CONTROL	1/100 µl	0.5mg/ml	eBioscience
APC IgG1	2.5/100	0.1mg/ml	Invitrogen
PE IgG1	2.5/100	0.1mg/ml	Invitrogen
FITC IgG1	5/100	100/test	Hycult biotech
APC CD16	1/100	150µg/ml	Biolegend
MEM-166(Anti-CD177)	1/100	0.50mg/ml	Biolegend
GAM PE	1/100	1.0g/ml	Dako
G2(Pr3) **G2(Pr3)**	0.5/100 5/100	2mg/ml 0.1mg/ml	Hycult biotech
FITC(Pr3)	10/100	0.1mg/ml	Hycult biotech
608 (Anti-MMP-8)	1/100	1mg/ml	R&D Systems
936 (Anti-MMP-9)	1/100	2mg/ml	R&D Systems
FITC CD63	4/100 1/100	100/test 400/µg	AbD Serotec Biolegend

2.5 Cell lines and cell culture

2.5.1 HUVEC cell line

Human Umbilical Vein Endothelial Cells (HUVECs) are a primary cell line derived from normal human umbilical vein, and are a common model cell line for transmigration assays. HUVECs are commonly used for physiological and pharmacological investigations and they are easy to culture, and provide a valuable cell model for many vascular biology research applications. HUVECs used in this project obtained from Professor Hallett's neutrophil signalling group. HUVECs were cultured in Dulbecco's modified Eagle medium (DMEM; Lonza) supplemented with 10% foetal calf serum (Lonza), 100 U/ml penicillin (Lonza), 100μg/ml streptomycin (Lonza) and 2μM L-glutamine (Lonza). Cells were routinely maintained in tissue culture 25cm^2 flasks (Corning Life Sciences), and incubated at 37˚C in a humidified 5% CO_2 incubator until they reached sub-confluency (2-3 days). Once the cells reached a confluence of approximately 80-90%, the medium was aspirated and the adherent cells were then detached from the tissue culture flask using 1-2 ml of Trypsin/EDTA (Lonza). Once detached, the cell suspension was poured into a 30 ml universal container and centrifuged at 348 xg for 5 minutes, in order to pellet the cells. Following that the supernatant was aspirated and the cell pellet resuspended in an appropriate amount of culture medium. Cells for subculture were diluted 1/10 in cell medium and returned to culture flasks, and cells utilised for transmigration assays were seeded onto cell culture inserts and allowed to reach confluence prior to addition of neutrophils.

2.5.2 CHO cell line

Chinese Hamster Ovary (CHO; ATCC) cells can make excellent vectors in research and biotechnology applications. CHO cells are relatively easy to culture, grow quickly, and

produce a substantial volume of gene products stably, as compared to many other cell lines. CHO cells were grown in RPMI 1640 (Lonza) supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100µg/ml streptomycin and 2µM L-glutamine (Lonza). The cells were cultivated in 75 cm^2 cell culture flasks and incubated at 37 °C and 5% CO_2. When they reached 100% confluence they were detached by incubation with trypsin/EDTA and resuspended in media, transferred to a sterile centrifuge tube, and centrifuged at 348 xg for 5 min. Following that the supernatant was aspirated and the cell pellet resuspended in an appropriate amount of medium. Cells for subculture were diluted 1/10 in cell medium and returned to culture flasks, and cells utilised for transfection of cDNA were seeded into 6-well dishes and transfected at a cell confluence of 70-90%. For Pr3 binding to CD177-transfected CHO cells, cells were seeded into 12-well dishes and allowed to reach 80-90% confluence.

2.6 Transwell assay

2.6.1 Culturing and Treating HUVECs for Transwell assay

Cell culture inserts (Millicell®) with porous membrane size of 3µm and 24 multi-welled cell culture plates (Greiner Bio-One) were used. Each insert was placed in a 24 well plate and coated with 100 µl of a 1:200 dilution (diluted in free DMEM) of matrigel (BD Biosciences) and allowed to dry for about 2h in an incubator at 55-60°C. To reconstitute the Matrigel layer, 200 µl of DMEM tissue culture medium was added to each insert and incubated at 37°C for 30 min and then the medium was aspirated. Following that the inserts and the wells were filled with DMEM medium. One or two drops of HUVECs suspension (cell pellet were diluted in 5 of cell medium) were added to each insert and the level of medium inside the inserts was kept above the level of medium outside in the culture well. After that the plate left in the incubator for 3 to 4 days for HUVECs to adhere and become confluent. Once the cells reached a confluency of approximately 100%, TNFα (100 ng/ml; PeproTech) or IL-β (1 ng/ml; PeproTech) were added to the outer and inner compartments of the inserts/wells but

not the inserts that were used as controls. The inserts were left in the incubator overnight. On the following day the inserts were washed two or three times with fresh DMEM medium to remove any TNFα remaining in the inserts before replacing it with fresh medium (200 µl into the inserts and 800µl into the wells). This step was also repeated for the inserts and wells of the non-treated control endothelial cells. The plates were then prepared for the transwell assay.

An aliquot containing 10^5 neutrophils /ml (100 µl) in DMEM medium was added to each insert and left to adhere for 30 minutes in the incubator, to ensure that all the neutrophils settled down on the endothelial cells. The remaining isolated neutrophils were suspended in 1 ml of DMEM and kept in the incubator to use as a control. After 30 minutes the DMEM medium from the outer compartment of the insert was replaced with fresh medium mixed with 7.5µM of fMLP, prepared from a 1 mM stock solution. The level of the medium inside and outside the inserts was kept at the same level. The inserts were left in the incubator for 2 h and the cells which crossed the endothelial cell layer were collected from the lower compartment for counting or flow cytometry analysis.

2.6.2 Endothelial permeability assay

HUVECs were cultured on inserts as described in section (2.5.1) until 100% confluence was reached. The insert with confluent monolayer was removed form 24 Transwell plate and placed in cuvette tube. Endothelial permeability was then determined by measuring the passage of fluorescein isothiocyanate-dextran (FITC-labelled dextran.1 mg/ml) with average mol wt 4,000 (Sigma-Aldrich) through the HUVEC monolayer by using a fluorimeter [Dual Monochromator spectrofluorometer DM 3000 (Spex Inc, NJ.USA)] to measure the FITC signal in the lower chamber. Permeability controls consisted of transwell without HUVECs grown on the surface.

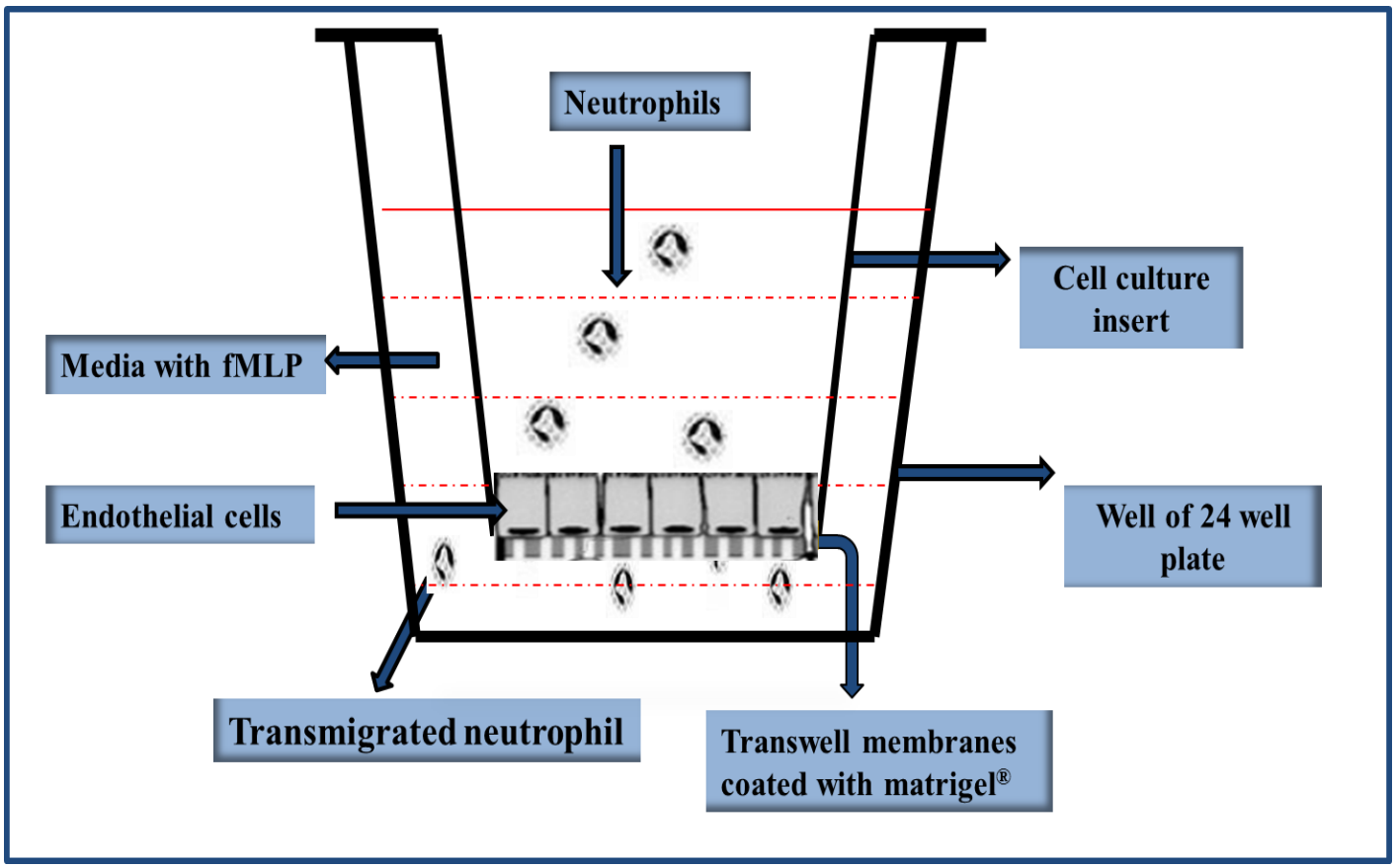


Figure 2.2 Model of neutrophil transmigration assays. Human umbilical vein endothelial cells (HUVEC) were grown in the inserts and freshly isolated neutrophils were loaded into the inserts. fMLP was used as the chemoattractant by adding into the lower chamber (wells) of a 24 well plate. Neutrophils that have successfully transmigrated were collected and analysed by flow cytometry.

2.7 Generation of recombinant CD177-Fc fusion protein and CD177 recombinant protein expression in CHO cells

2.7.1 Plasmid constructs used in this thesis

2.7.1.1 pCMV-SPORT6 (vehicle plasmid)

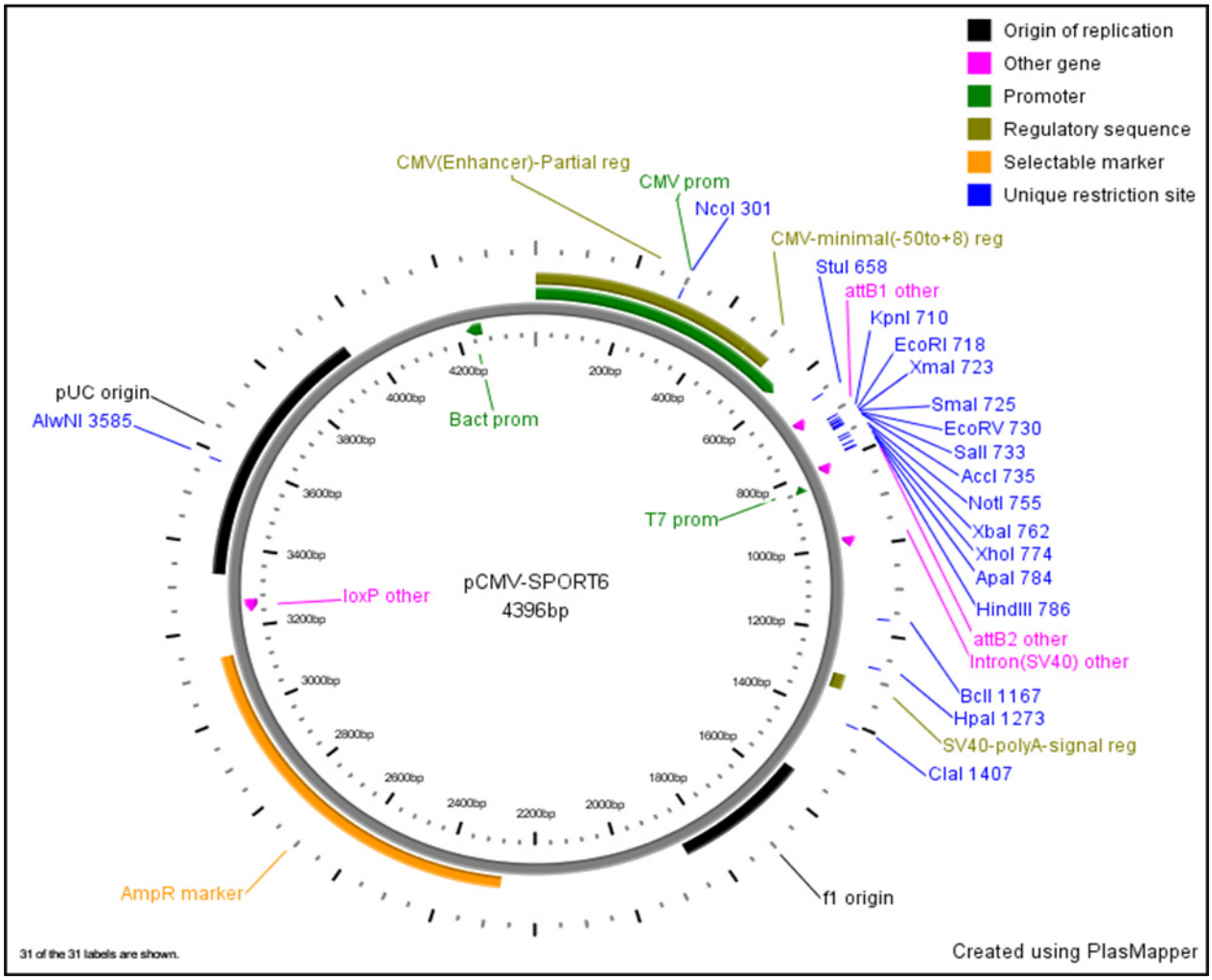


Figure 2.3 A diagram showing the pCMV-SPORT6 (vehicle plasmid) which contains a gene for ampicillin resistance. This is the vector that the commercially supplied CD177 cDNA was provided in.

2.7.1.2 pTorsten (expression vector plasmid)

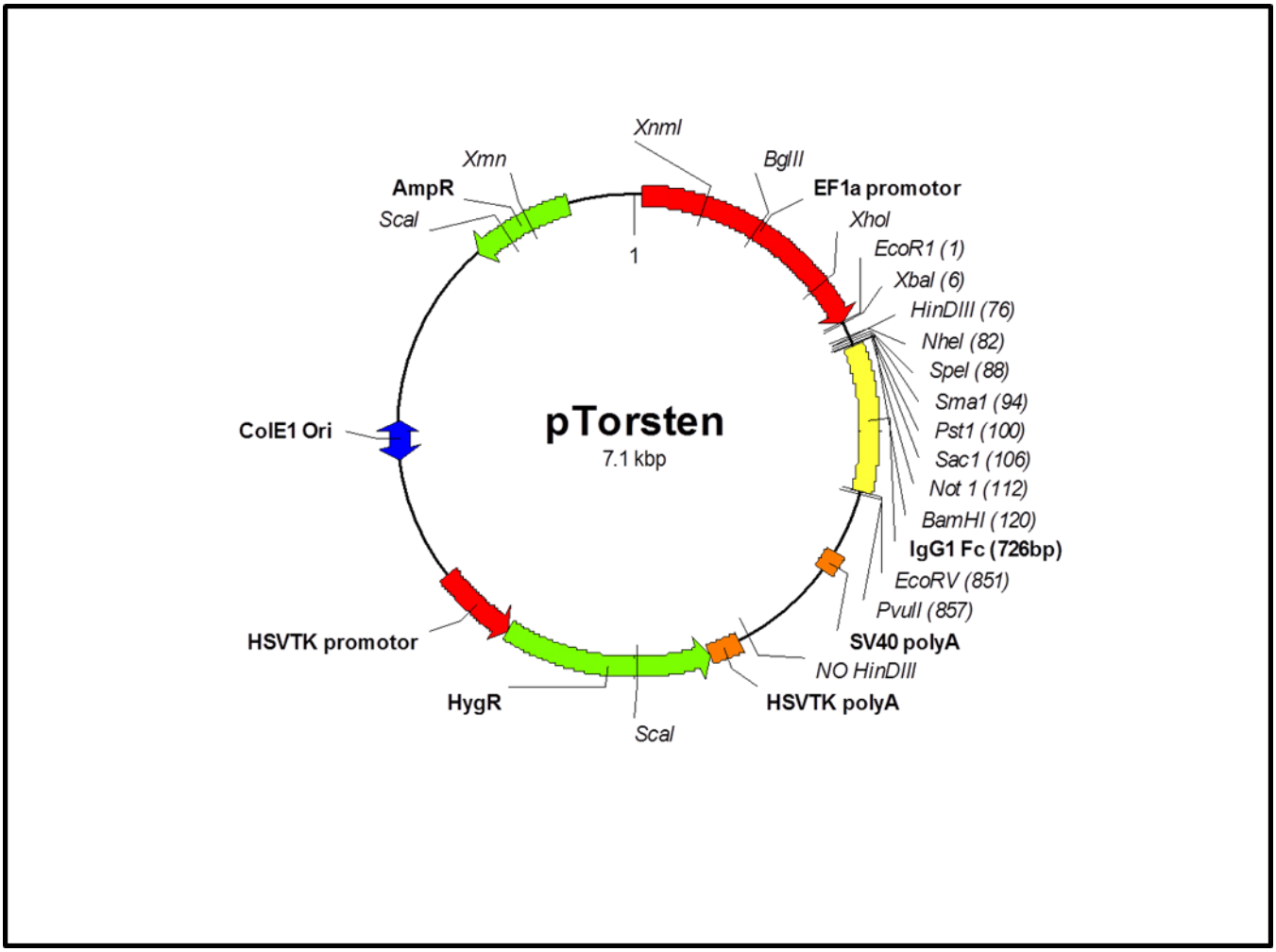


Figure 2.4 A diagram showing pTorsten (expression vector plasmid) which contains a gene for ampicillin resistance (bacterial growth selection) and hygromycin resistance (eukaryotic cell selection).

2.7.2 Plasmids purification

5 ml of Luria Broth (LB) media (Sigma-Aldrich ltd.) supplemented with appropriate selection antibiotics (Ampicillin 100 μg/ml) was inoculated with a single colony of the relevant transformed bacteria (Bacterial plasmids) and grown overnight at 37°C on a shaker. Cells were centrifuged at 3600 xg for 10 minutes. Plasmids were isolated from bacteria using the Qiagen Mini prep plasmid isolation kit according to the manufacturer's protocol which is based on alkaline lysis method (Figure 2.5).

2.7.3 Preparation of gene of interest

2.7.3.1 PCR for DNA amplification

Full-length CD177 cDNA was amplified (and the restriction enzyme sites added) by PCR using forward primer XbaI_CD177-For1 (5'-3') AAA TCT AGA GGT CAT GAG CCC GGT ATT ACT and reverse primer EcoRV_CD177-RevEND (5'-3')AAA GAT ATC TGA AGA ATC GTG GGG GTA ATA GA. Fc CD177 cDNA was amplified forward primer XbaI_CD177-For1 (5'-3') AAA TCT AGA GGT CAT GAG CCC GGT ATT ACT and reverse primer NotI_CD177-Rev1219 (5'-3') AAG GCG GCC GCA TGC TGA GAG GCA GGA GG under conditions described in Table 2.2. All primers were obtained from Invitrogen (Glasgow UK). PCR cycle reaction steps were as follows: initial denaturation of DNA at 94°C for 5 min followed by DNA denatured at 94°C for 30 sec, and primer annealing at 58.9°C for 30 sec with extension at 72°C 90 sec, final extension for 5 min, with 40 overall cycles. The PCR product (of full length 1,342 and Fc length 1,219 bp) was checked by electrophoretic separation on a 1% agarose gel electrophoresis in TBE buffer (containing 0.01% ethidium bromide for UV visualisation or 0.1% crystal violet for white light visualisation) using KAPA DNA molecular markers as standards.

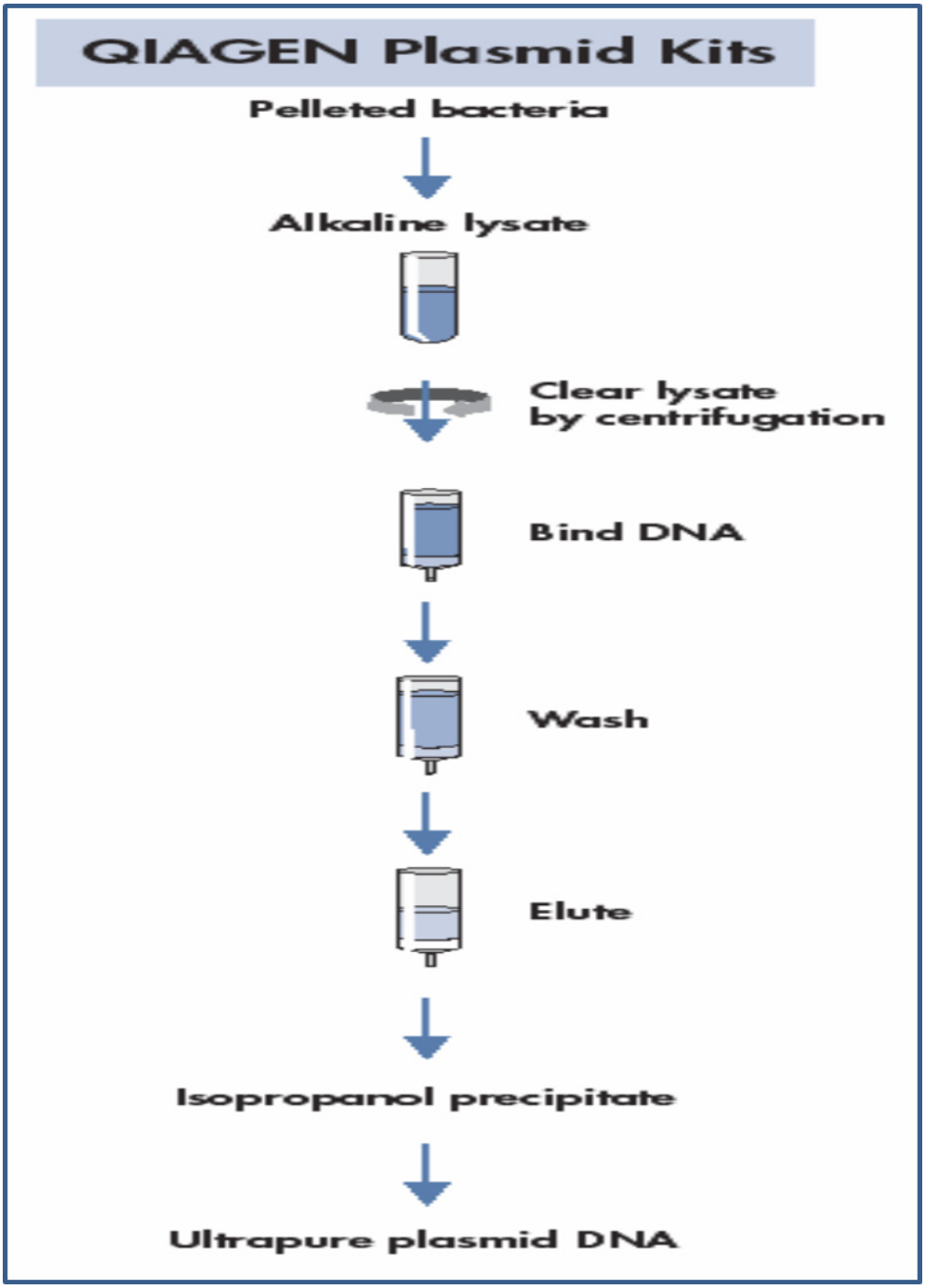


Figure 2.5 Plasmid DNA purification using the QIAprep Spin Miniprep Kit and a microcentrifuge procedure. The procedure consists of three basic steps: 1) preparation and clearing of a bacterial lysate 2) adsorption of DNA onto the QIAprep membrane 3) washing and elution of plasmid DNA. (Adapted from public.wsu.edu/~kahn_sci/Flow/E2-QIAprep_Miniprep_Handbook.pdf).

2.2 Table PCR mixture for CD177 DNA amplification

Component	50 µl reaction
PCR-grade water	37.8 µl
5 U/µl KAPA Taq DNA Polymerase	0.2 µl
10X Buffer A	5.0 µl
10 Mm dNTP	1.0 µl
10 µM Forward Primer	0.2 µl
10 µM Reverse Primer	0.2 µl
Template DNA	2 µl

Enzyme cut PCR products for use in ligation were purified with QIAquick PCR Purification Kit (Qiagen, Hilden), according to manufacturer's instructions.

2.7.3.2 Digestion of DNA with restriction enzymes

DNA restriction was performed with restriction enzymes REs; XbaI + EcoRV (Promega) for full-length and XbaI + NotI (Promega) for Fc-CD177 and carried out in separate tubes. The restriction reaction composed of 30 µl DNA (eluted from the Qiagen spin column), 4 µl of enzyme buffer, 0.4 µl of BSA and 2 µl of each REs. The empty recipient expression vector (pTorsten) was also cut with these enzymes. Restriction reactions were incubated for 2 hours in the 37°C water bath after which the restriction was terminated by separation on a preparative agarose gel, following which the bands were excised with a new scalpel blade. The DNA products were purified again using QIAquick PCR Purification Kit and kept in -20°C freezer until use.

2.7.4 Preparation of expression vector plasmid

Purified plasmid DNA (pTorsten) was cut with the same REs and the same steps used with the vehicle plasmid (full-CD177 and Fc-CD177) and prepared in separate tubes. Cutting pTorsten with NotI leaves the human IgG1 Fc cDNA sequence intact and allows in-frame addition of the Fc portion of the protein as long as the designed primer lacks a stop codon and has the NotI site added after a complete codon (in reverse). Cutting pTorsten with EcoRV removes the Fc portion of the expression vector to enable full length, unmodified protein expression. In ethidium bromide agarose gel electrophoresis the DNA is exposed to UV light which may damage the DNA and decrease the cloning efficiency. To avoid damaging the PCR products, preparative agarose gel electrophoresis using 0.1% crystal violet was selected. The DNA band of interest (relative to the KAPA DNA ladder) was excised from the gel and transferred into a clean Eppendorf tube. Extraction was performed using QIAquick Gel Extraction Kit as recommended by manufacturer.

2.7.5 Ligation

Purified insert (containing the CD177 sequence) was inserted into the linearised expression vector (containing the promoter to drive expression and hygromycin resistance gene for eukaryotic cell expression) be joined in the correct orientation (due to the use of 2 separate non-complementing enzymes). In Eppendorf tubes, the following ligation mixture was set up: 5µl insert DNA

1 µl vector DNA (pTorsten)

2 µl 10x Ligase Buffer (Promega)

1 µl T4 ligase (Promega)

The ligation mixture was set to incubate overnight at 16°C.

2.7.6 Transformation of DNA into competent cells

Transformation of E. coli One shot Top 10 chemically competent bacteria was carried out as per manufacturer's instructions (figure 2.6) using brief heat shock at 42°C. Frozen competent cells were thawed on ice and a 10 µl of ligation mixture added into the vial of cells and mixed gently, followed by incubation on ice for 30 minutes. The cells were then heat shocked for 30 sec at 42^{o}C and placed on ice for 2 min, after that 250 µl of pre-warmed S.O.C. medium (supplied with the kit) was added and incubated at 37^{o}C for 1 hour at 225 rpm in a shaking incubator. The transformed cells were then plated on LB agar plates containing 100 mg/L ampicillin.

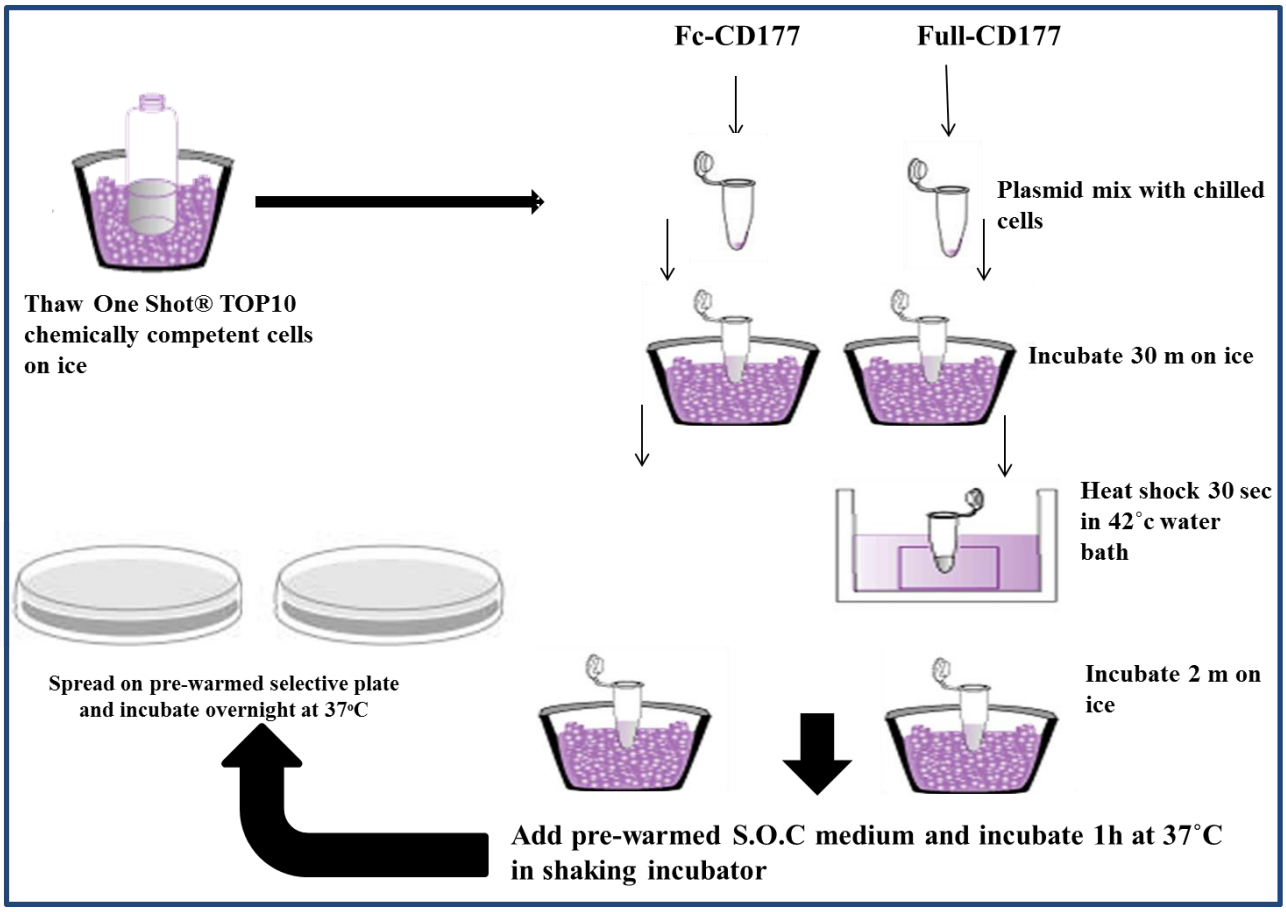


Figure 2.6 Transformation of CD177 DNA into competent cells. (Adapted from http://www.biochem.arizona.edu/classes/bioc471/pages/Lecture4.html)

2.7.6.1 Screening of transformed bacteria

In order to check positive transformed cells the plasmids from single colonies were grown up in 5 ml in LB-amp broth and purified using QIAprep Spin Miniprep KIT. Successful ligation was confirmed by cutting with the same REs and viewing on the gel (figure 2.7). All positive plasmid DNA were then submitted to MWG Eurofins (Germany) with the primers listed in section 2.6.3.1 for sequencing. Returned sequence was compared against the original cDNA CD177 gene sequence for homology using NCBI's nucleotide BLAST (comparing 2 sequences) program (freely available at www.ncbi.nlm.hih.gov/Blast). Bacteria containing error free inserts were scaled up to 50 ml LB-amp broth and plasmid DNA was purified using QIAGEN Plasmid Midi and Maxi Kits for higher plasmid DNA concentration ready for transformation. The DNA concentration was measured using a BIO-SPEC-1601 dual beam spectrophotometer (Shimadzu, Milton Keynes) using internal measurement protocols for samples diluted 1/100 in distilled water and analysed at 260 nm. Long term stocks of bacteria containing the sequenced plasmids were kept following resuspension in 50% sterile glycerol and stored at -80oC.

2.7.6.2 DNA sequencing

DNA sequencing reactions were performed by Eurofins MWG. An aliquot of 15µl of each purified plasmid (containing at least 100 ng) and 3 µl of a single primer (at 10 pmol concentration) was added to a barcoded pre-paid sequencing tube and sent by courier. Two reactions for each construct, one with forward primer and one with a reverse primer were performed.

2.7.7 Transfection of CD177-Fc and full-CD177 plasmids into CHO Cells

Transfection was carried out according to Lipofectamine® 2000 DNA Transfection Reagent Protocol (Lifetechnologies). CHO cells were seeded into two 6-well plates until 70-90% confluent and transfected with each plasmid in a separate plate as shown in figure 2.7. Briefly, Lipofectamine LTX reagent was diluted in serum-free medium in different 4 concentrations in different tubes. Following that 0.5–5 µg of plasmid DNA (Fc-CD177 or full-CD177) was diluted in 700 µl of serum-free medium then 14µl of PLUS reagent was added. The diluted DNA (150 µl) was added to each tube of diluted Lipofectamine® 2000 Reagent (1:1 ratio) and the mixture was incubated at room temperature for 5 min. 250 µl of the DNA Lipofectamine LTX complexes was added to each well of CHO cells and cells were incubated at 37^{o}C 5% CO2 for 2-4 days. Following this, the transfection medium was removed and replaced with selection cell culture medium containing 400 µg/mL of hygromycin B (Invitrogen). CHO cells were also transfected with positive (CD55-Fc in pTorsten) plasmid as positive control for transfection.

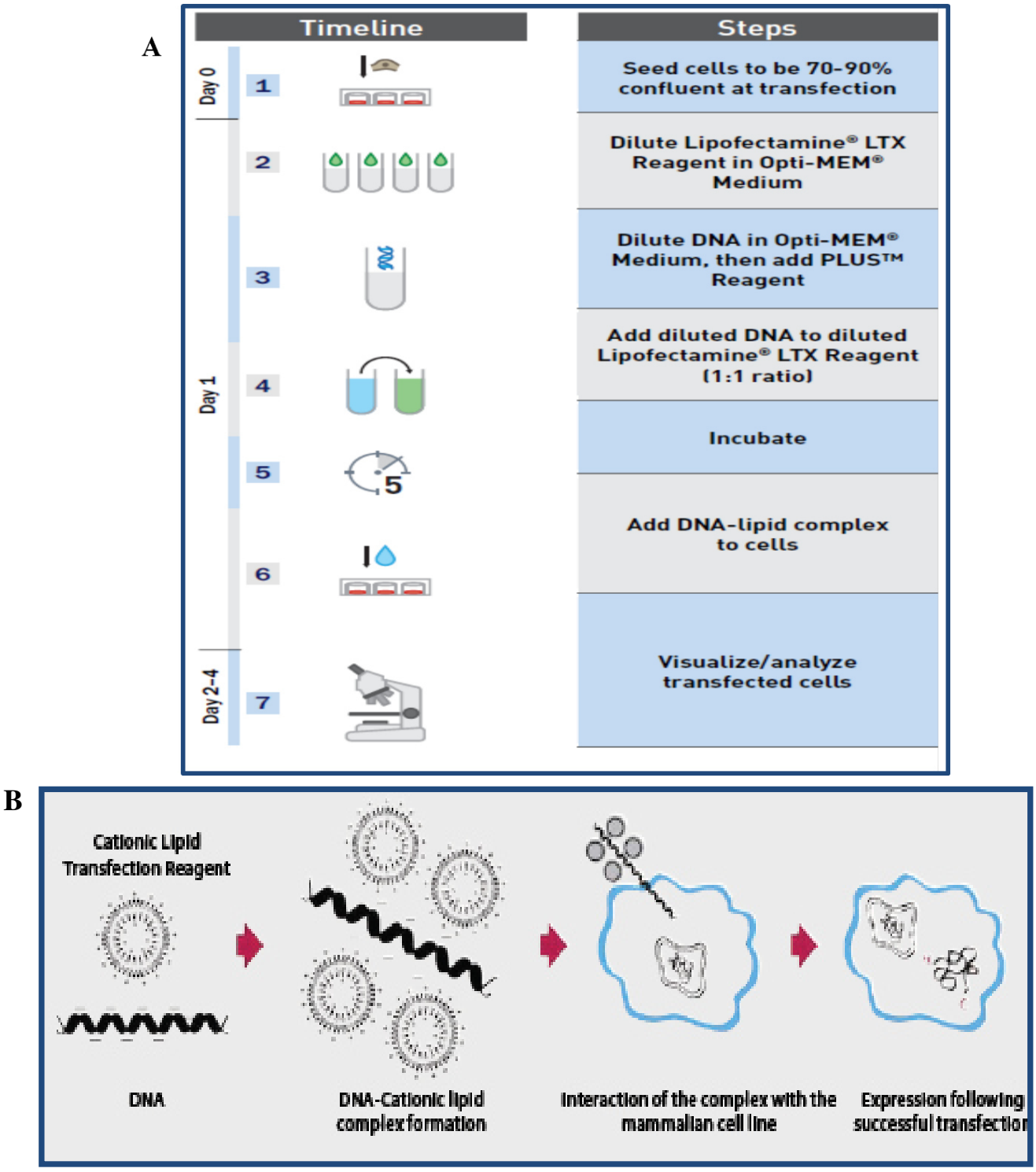


Figure 2.7 A) Diagram of Lipofectamine transfection procedure. B) Mechanism of cationic lipid-mediated transfection. Cationic lipids (positively charged) with DNA (negatively charged) forming structures called liposomes (DNA-Cationic lipid complex). The complex enters the cell through endocytosis. Once inside the cell, the complex must escape the endosomal pathway, diffuse through the cytoplasm, and enter the nucleus for gene expression. (Adapted from www. Invitrogen.com)

2.7.7.1 Screening of transfected CHO cells

After two or three passages in 175 cm^2 cell culture flasks, the concentration of Hygromycin B was reduced to 100 µg/ml. When the cells achieved 100% confluence the medium from CD177-Fc transfected CHO cells and the control cells (untransfeted cells) were collected to analyse the presence of CD177 fusion protein and the CD177-full transfected cells were cultured in 12-well cell culture plates for analysis by flow cytometery.

2.7.7.1.1 Detection of recombinant CD177-full expression in CHO cells by Flow Cytometry

The day prior to analysis, transfected cells and CHO control cells were cultured in 12 well plates. In preparation for analysis, the wells were washed two times with warm saline then the cells detached from the plate using 1-2 ml of FACS buffer (which contains 15 mM EDTA as a non-enzymatic disaggregator). Once detached, the cell suspension was poured into 30 ml universal containers and centrifuged at 1000xg for 2 minutes, in order to pellet the cells and then re-suspended in FACS buffer. Aliquots (100 µl) of cells suspension were added to a round bottomed 96 well plate and centrifuged for 2 minutes at 1000xg at 4°C then the supernatant was discarded and the pellets were re-suspended in 5 µg/ml (final concentration) isotype IgG control antibody or monoclonal anti-CD177 (MEM-166) antibody. The plate was incubated at 4ºC for 25 minutes, and washed two times using FACS buffer. Both antibodies were detected by 1/100 PE-conjugated goat anti-mouse antibody (rPE-GAM) and incubated for 20 min at 4ºC then the cells washed and re-suspended with 200µl of FACS ready for FACScalibur analysis.

2.7.7.1.2 Detection of recombinant CD177-Fc by Dot Blot

This technique is simple and offers a rapid screening method for expressing cells, but gives no information on the size of the target molecule. The samples were collected from transfected cells and clarified at 1400 rpm for 5 min and the supernatant removed and 5 µl spotted onto nitrocellulose membranes. After drying, the membranes were blocked using blocking buffer (2% w/v skimmed milk in PBS with 0.05% Tween 20 detergent) on a roller for 30 min. 10 µl of goat anti-human IgG1 (peroxidise-conjugated; Sigma) was added and incubated for 30 min followed with three washes with PBS Tween and 2 washes with PBS. 500 µl of Pierce ECL super signal (peroxide and enhancer solutions) were added to the membranes and the blot developed by exposure to photographic film in a dark room.

2.7.7.1.3 Detection of recombinant CD177-Fc by Western Blot

To detect the molecular weight of CD177-Fc protein, immunoblotting technique (Western Blot) was used. Proteins were separated by mass using SDS-polyacrylamide gel electrophoresis, and then electrophoretically transferred to nitrocellulose, and specific proteins were visualised by the binding of specific polyclonal or monoclonal antibodies. In the first step, aliquots of 1 ml from each cell culture medium (transfected cell and control cells) were transferred to 1.5 ml Eppendorf tubes and centrifuged at 16,000 xg for 5 min to remove cell debris and then the supernatant collected. 20 µl of cell-free supernatant was mixed with 5 µl LDS gel loading buffer (Life technologies) and boiled for 2 min at 95^{o}C and loaded on a 7.5% SDS-PAGE gel. The SDS-PAGE gel was cast as follows: Gel casting apparatus was set up as according to the manufacturer (BIORAD, Hertfordshire, UK) and resolving gel made up by using the volumes shown in the table 2.2 then the mixture was transferred between the gel casting plates and allowed to set leaving 1.5-5cm space at top, 400 µl of butanol was immediately overlaid to remove undesirable air bubbles from the

surface prior to polymerisation. After the gel had polymerised, the butanol was washed off with de-ionised water. The stacking gel was prepared as shown in table 2.2 and then layered on top of the set resolving gel between the gel plates, and a gel comb add to form loading wells, which was removed after the gel had polymerised. All gels were poured at 1.5mm thickness with a 15-well comb. Plates containing set gels were set up in the running tank with the central reservoir filled with running buffer composed of 25mM Tris, 192mM glycine and 0.1% SDS at pH 8.3 (Biorad, Munich, Germany). 5µl of EZ-run molecular mass marker (Fisher) was loaded alongside with 10 µl or 5µl of each sample with elongated pipette tips. Gels were electrophoresed at 150 Volts (V) for 1 hour or until the dye front reached the bottom of the gel. The proteins were electrophoretically transferred to 0.22 µm nitrocellulose membrane (Anachem) using a BioRad Laboratories Mini-Protean 3 transblot system as directed by manufacturer. The transfer was carried out in a transfer tank with the transfer cassette, using transfer buffer (14.4 g/l Glycine, 3 g/l Tris base, 20% methanol), an ice pack to keep the transfer buffer cool and a magnetic stir bar to circulate buffer. Transfers were run at 100 V for 1 hour. Nitrocellulose membranes containing transferred proteins were placed within a 50 ml flacon tube ensuring that the membrane surface that had been in contact with the gel was facing upwards and blocked for 1 hour with 10 ml blocking buffer on a roller for 1 hour. Membranes were probed with 1:100 of mouse anti-human CD177 antibody (MEM166), diluted in blocking buffer and incubated overnight in a cold room (4^{o}C) on a roller. On the following day the membranes were washed three times with 15 ml of PBS-Tween (each for 10 min), followed by two washes with PBS for 10 min on a roller. Ten microliters of a donkey anti-mouse IgG (FC specific) peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories,UK), diluted in 10 ml blocking buffer, was incubated on a roller for 1 hour and washed three times with PBS-Tween and two times with PBS as described above. Blots were developed by using a mixture of 1.5ml of peroxide

solution and 1.5 ml of luminol enhancer solution (Pierce® ECL super signal reagent, Thermo scientific, Loughborough) poured on the nitrocellulose membranes. In a photographic dark room the nitrocellulose membrane was exposed to X-ray film in a light proof cassette for varying lengths of time (usually 2-5 min) before being developed.

Table 2.3 Composition of Western Blot stacking and resolving gel

Stacking gel		**Resolving gel**	
Reagent	**4%**	**Reagent**	**7.5%**
40% bis/acrylamide 37.5:1	1.1012 ml	40%bis/acrylamide 37.5:1	3.8 ml
dH_2O	6.4 ml	dH2O	11 ml
Upper buffer pH 6.8	2.4 ml	Lower buffer pH 8.8	5 ml
10% APS[1] W/V	100 µl	10% APS W/V	200 µl
TEMED[2]	40 µl	TEMED	20 µl

1=Ammonium persulphate. 2=Tetramethylethylenediamine.

2.7.7.1.4 Detection of recombinant CD177-Fc by ELISA

ELISA is a commonly used laboratory technique for the detection and quantification of substances, such as proteins, hormones and bacterial antigens (Delves et al., 2006; Van Emon, 2007). In the most basic ELISA protocol, an antigen is fixed to microtiter well plates. The plates are then incubated with a specific Ab which is linked to an enzyme. Upon addition of a colourless substrate a coloured reaction is generated by the conjugated enzyme. The colour is then measured using a spectrophotometer and related to the concentration of the antigen by using a standard curve. To detect antigen, a "sandwich" ELISA can be used, whereby the substance to be analysed is detected by two antibodies; the capture Ab and the detection Ab (Janeway et al., 2008).

Various constructions of ELISA were tested in order to choose the most sensitive antibodies to detect the CD177-Fc protein. In the first ELISA, a flat bottom 96-well plate (Nunc-ImmunoMaxisorb, Thermo Fisher Scientific, Denmark) was pre-coated with 10 µg/ml of a monoclonal mouse anti-human IgG (BIO-RAD) diluted in bicarbonate buffer ($NaHCO_3/Na_2CO_3$, pH9.6). Aliquots of 100µl/well were dispensed and plate sealed and incubated overnight at RT and in the following day the fluid was discarded and the plate was blocked with 100 µl/well of blocking buffer [5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), containing 0.05% Tween (PBST)] for 1 hour at 37ºC then the blocking buffer was discarded. 100µl of CD177-Fc supernatant diluted in blocking buffer by a 2-fold serial dilution series was added to each well in duplicate and incubated 1 hour at 37ºC. After being washed three times with PBST, 100 µl/well of Horseradish peroxidase-conjugated (HRP) anti-human IgG antibody (Jackson ImmunoResearch Laboratories) diluted at 1:500 in blocking buffer was incubated for 1 hour at RT. Alternatively the captured antigen was detected with 100 µl/well of HRP-conjugated anti-human CD177 antibody (Biosys USA) diluted at 1:250 in blocking buffer for 1 hour at RT. Unbound detection antibodies were then

removed by washing three times with PBS-Tween and twice with PBS. The plate was developed with a substrate solution made up by adding two tablets of Ortho-Phenylenediamine, (OPD-EASY) (each contain 3.5 mg of 1,2-phenyendiamine dihydrochloride; Fisher Scientific), to 6.4 ml of sterilised water mixed with 2.5 µl of hydrogen peroxide (Aldrich, Steinheim, Germany). One hundred microliters of substrate solution was added to each well and incubated for about 10-20 min, depending on the rate of colour change. The reaction was stopped by adding 100 µl of 2N sulphuric acid (H_2SO_4; SIGMA-Aldrich) and read on a plate reader (Dynex technologies, Chantilly, VA) and the absorbance was measured at 490 nm.

In the second ELISA the same steps were performed except that an additional HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) diluted at 1:1000 in blocking buffer was added after incubation with HRP–conjugated rabbit anti-human CD177 antibody before being washed and developed with OPD-EASY.

In the third ELISA, 96-well flat bottom plates were pre-coated with anti-human CD177 (MEM166; 1:100 diluted in bicarbonate buffer) by adding 100 µl of diluted antibody into each well. The plate was then sealed with an adhesive strip and incubated at 37ºC for 1 hour. Following coating the liquid was removed by inverting and tapping the plate against clean paper towel. Non-specific binding sites were then blocked by filling the wells with 100 µl blocking buffer and the plate was incubated for 1 hour and washed three times with PBS-Tween. Aliquots of 100 µl CD177-Fc supernatant (two-fold dilution series) or purified CD177-Fc (10-fold dilution series) were added and wells were incubated for 1 h at 37°C. After washing three times with PBST, 100 µl of anti-human IgG (Fc specific peroxidase; dilution 1:500 in blocking buffer) were added and incubated for 1 h at 37°C. After washing three times with PBS-Tween and twice with PBS the plate then developed with OPD-EASY (Sigma-Aldrich), and the reaction stopped with 2N H_2SO_4 before reading at 490 nm.

2.7.8 Purification of recombinant CD177-Fc fusion protein by Protein-A Sepharose column

Stable cell lines were cultured in 175 cm^2 flasks and grown in RPMI 1640 supplemented with 10% of ultra-low IgG FBS (Gibco®), 1% L-glutamine, 1% Penicillin-Streptomycin. Once the cells had reached an adequate confluence, culture supernatants containing CD177-Fc fusion protein were harvested and the remaining cells were cultured for further production. Culture supernatants were stored at -20°C until the volume exceeded 2 litres. Supernatant was thawed and protease inhibitor (Sigma) was added to collected culture supernatants followed by filtration through 0.22 µm syringe filter (ELKAY) then the supernatants left in the fridge until purification. The CD177-Fc fusion protein was purified by affinity chromatography on Protein-A Sepharose column which binds specifically to the Fc part of CD177. Protein-A Sepharose column (HiTrap Protein A HP; GE Healthcare, Chalfont St. Giles, United Kingdom) was equilibrated with 20 ml of PBS. Supernatant containing soluble CD177-Fc fusion protein was run on to the column and washed thoroughly with 100 ml of PBS. The bovine IgG was eluted with 100 ml of 0.1M citrate (2.1 g of citric acid in 100 ml of water and NaOH to a final pH = 5.0) and the column washed again with 100 ml of PBS. Bound CD177-Fc fusion protein was eluted in 15 x 3 ml fractions of 0.1M glycine (0.75 g of glycine and 0.9 g of NaCl in 100 ml of water with lots of HCl to pH to 2.5). The pH of the eluted fractions was neutralised by immediate addition of 1/10 volume of 1 M Tris (pH 8.0).

2.7.8.1 Colloidal Coomassie blue staining

Coomasie blue is used to stain polyacrylamide gels following SDS-PAGE. This allows for visualisation of protein bands if no immunoprobing is required. Proteins in polyacrylamide gels were stained using colloidal Coomassie blue stain kit according to manufacturer's guidance (Invitrogen, UK). The staining was performed manually and solutions were

prepared as shown in Table 2.3. After the end of electrophoresis, gels (7.5% SDS gels, NuPAGE Novex 4-12% Bis-Tris mini gels, or NuPAGE 4-12% Bis-Tris Zoom mini gels) were transfered to a weighing boat and soaked in the fixing solution for 10 min at room temperature. The fixing solution was then removed and the staining solution without stainer B was added on top of gels. Gels were soaked in this solution for 10 min at room temperature before adding stainer B to the existing staining solution. Gels were soaked in the staining solution with stainer B for about 12 h at room temperature. The staining solution was then decanted and replaced with 200 ml deionised water. The gels were left to gently rock in water for at least 7 h until gels had a clear background. Gels were then scanned using an Image Scanner with MagicScan software (Amersham Biosciences) to record electronic images.

Table 2.4 Preparation of fixing and staining solutions for Coomassie blue staining

Component	Fixing Solution	Staining Solution
Deionized Water	40 ml	55 ml
Methanol	50 ml	20 ml
Acetic Acid	10 ml	-
Stainer A	-	20 ml
Stainer B	-	5 ml

2.8 Confocal Image Analysis

Using confocal microscopy, I investigated intracellular distribution of neutrophil proteinases from 2 granule subsets before and after stimulation. Primary conjugated monoclonal, and secondary conjugated monoclonal antibodies were used, as single or double staining (table 2.4). Confocal microscopy is able to test whether two fluorescently labelled molecules are associated with one another (co-localization). Within the context of a cell or sub-cellular organelle, fluorescence indicates the molecules are in very close proximity or even in contact, while in the context of analysing digital images it means the colours emitted by the fluorescent molecules occupy the same pixels in the image. The investigation of co-localization between two fluorescence (dye) channels (two proteins) broadly divides into two categories: (i) methods that simply consider the presence of both fluorophores in individual pixels, which is known as the dye-overlay method. This method is simple and widely used but has a number of drawbacks. It is no longer sufficient to merely overlay the red and green images, and say that where yellow appears is where co-localisation has occurred. User bias can play a large role in determining the amount of yellow colour present, leading to false positives as a result of increasing the background values. An imbalance of red and green pixel ratios can lead to widely varying results. (ii) The second method examines the quantitative evaluation of images with co-localization based on the calculation of a number of specialized coefficients. There are a number of software packages that provide co-localization analysis. In this study I used the software program Image-J with two different types of Plugins; intensity correlation analysis (ICA; Li Q et al. 2004) and Just Another Co-localisation Plugin (JACoP; Bolte and Cordelieres, 2006).

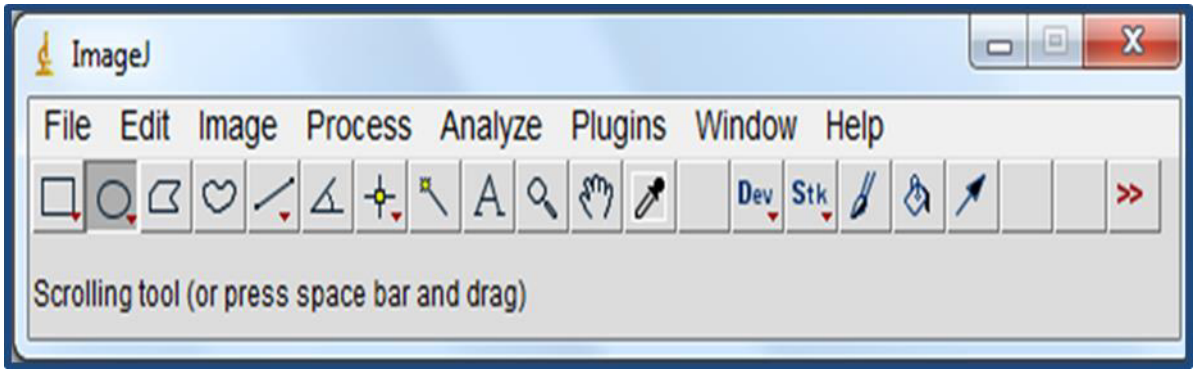
2.8.1 Sample preparation

All samples were prepared as described in section 2.3 then stained with antibodies listed in table 2.4.

2.7.2.1 ImageJ and JACoP

Steps of analysis:

1. Ensure that images are TIF files; they can then be analysed using ImageJ and JACoP.
2. Download ImageJ software [free download from; MacBiophotonics, URL, or ImageJ, URL]. JACoP (Just Another Colocalisation Plugin) [available from ImageJ Plugins, URL] then needs to be downloaded to the Plugins folder of ImageJ.
3. Open ImageJ
4. Drag and drop a TIF image onto the ImageJ bar, opening the image in ImageJ
5. The images from the confocal are 8-bit gray scale images and ready for analysis.
6. Click "Plugins – JACoP", bringing up the JACoP window and automatically selecting the Red and Green image.

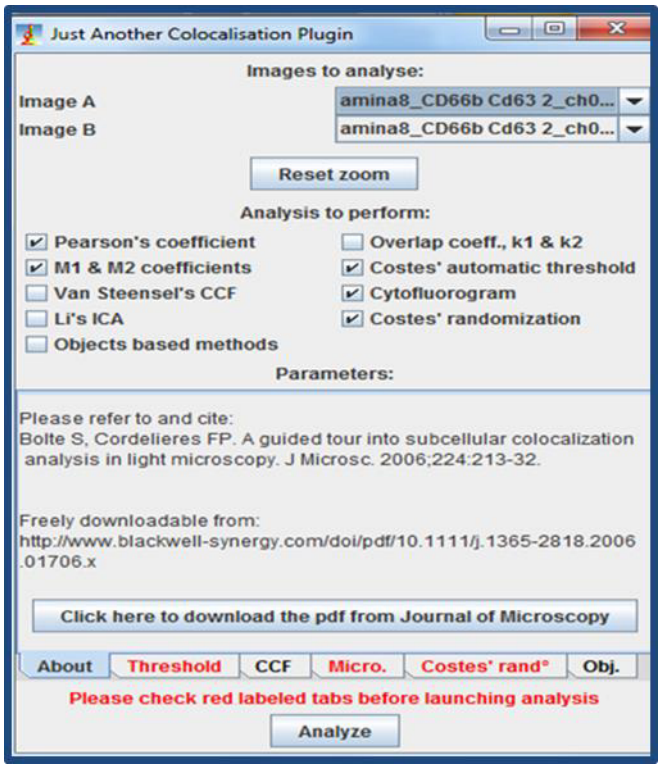


7. The images to analyse can be changed using the drop down menu for Image A and Image B.

Table 2.5 List of antibodies used in confocal microscopy.

ANTIBODY	DILUTION	CONCENTRATION	SOURCE
608(Anti-MMP-8)	1/100	1mg/ml	R&D Systems
936(Anti-MMP-9)	1/100	2mg/ml	R&D Systems
G2(PR3)	0.5/100	2mg/ml	Hycult biotech
CD177 UN (MEM-166)	1/100	0.50mg/ml	Biolegend
FITC CD63	6/100	100/test	AbD Serotec
FITC CD177			Biolegend
FITC Pr3	10/100	0.1mg/ml	Hycult Biotech
CY3	1/100	0.5mg/ml	Jackson ImmunoResearch
ELASTASE UN	1/100	0.58mg/ml	Hycult Biotechnology
CD66b UN	1/100	100/test	AbD Serotec

8. Under “Analysis to perform”, make sure Pearson’s coefficient, M1 & M2 coefficients, Costes’ automatic threshold, Cytofluorogram, and Costes’ randomization are all checked. Van Steensel’s CCF, Li’s ICA, Objects based methods, and Overlap coeff., k1 & k2 are not required
9. Click on the Micro tab, select Confocal rather than Wide-Field
10. If wanted, in the Costes’ random tab, can be changed to the Nb of random. rounds, depending on computing power (any value between 200-1000)
11. Click on Analyze to run the analysis

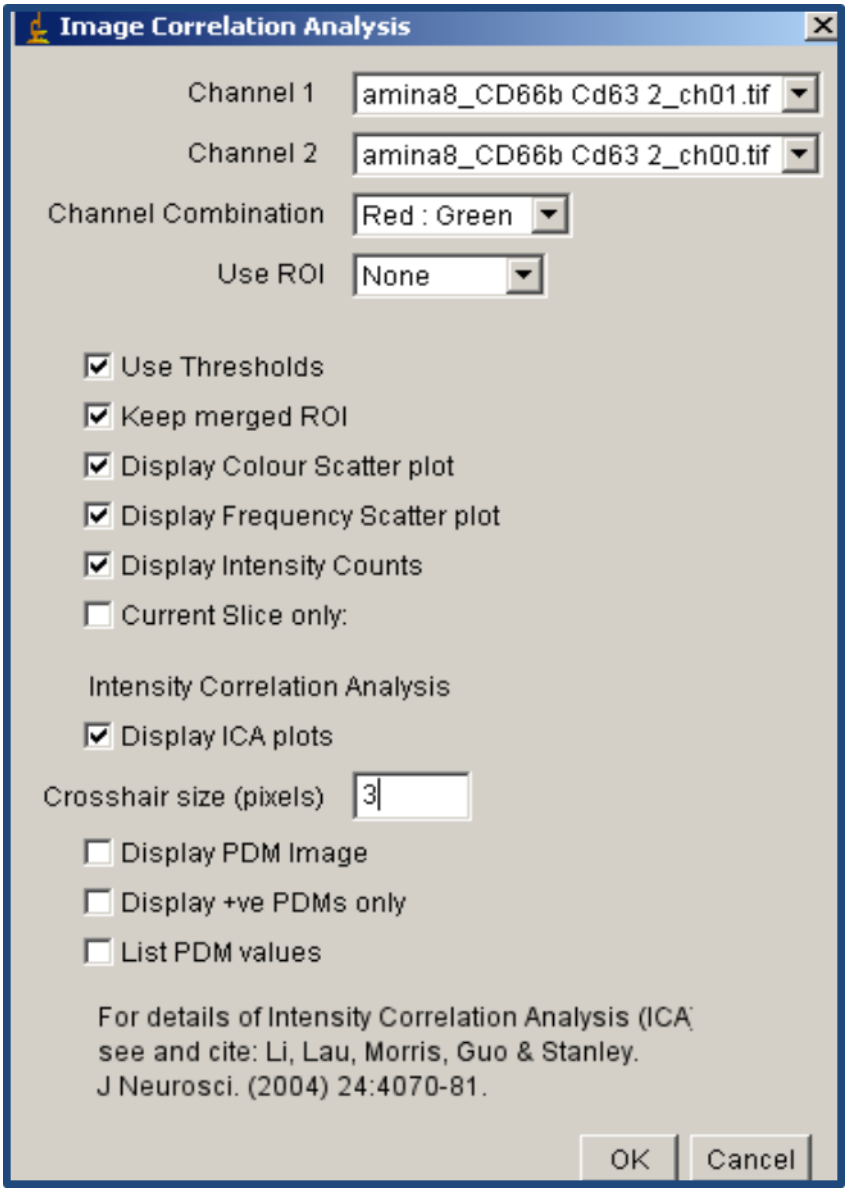


2.8.2.2 ImageJ and Intensity correlation analysis

Steps of analysis:

1. Download Intensity Correlation Analyis class to the plugins folder and restart ImageJ. Also bundled with WCIF ImageJ.
2. Open your two images (File/Open).
3. Select a region of interest (ROI): Select ROI in the first image/go to second image/Edit/Selection/Restore seletion.

Run the ICA plugin (Plugins/Colocalization Analysis/Intensity Correlation Analysis; ICA).



Once the analysis has run in both plug-ins, several windows pop up. The log is the most important window, as it provides all the statistical data. It details which two images have been compared, Pearson's coefficient, Mander's Coefficients (original and threshold values), Costes' randomisation based co-localisation, Costes' automatic threshold values, and the Cytofluorogram's parameters. The most important values to note are the r(obs), r(rand), and the p-value. These three values are required for each set of images taken, to statistically determine the extent of co-localisation between two images.

2.9 Statistical analysis

Data in this thesis were analysed using GraphPad Prism 5.01 software (GraphPad software Inc). Where possible, One-way ANOVA of variance with Bonferroni post-hoc testing (with correction for multiple tests) or t-test was performed for normally distributed data. For non-parametric data the comparisons were made using Kruskal-Wallis test/ Dunn's Multiple Comparison post-tests or Mann Whitney U tests especially when the sample size was small. Results were viewed as statistically significant when the P value < 0.05. The statistical analysis in chapter 7 was performed using Image J.

CHAPTER 3

EFFECT OF SERUM ON mPr3 AND CD177 EXPRESSION ON NEUTROPHILS

3.1 Introduction

Pr3 and other serine proteases are proteolytic enzymes involved in destruction of pathogens within the phagolysosome and in producing tissue damage when secreted extracellularly. Pr3 is stored in neutrophil azurophilic granules and also in the secretory granules thus, can be easily mobilised to the neutrophil surface following moderate and maximal stimulation (Witko-Sarsat *et al.,* 1999). It is a peripheral membrane protein, does not comprise a transmembrane domain and needs to cooperate with other membrane proteins or lipids to maintain a stable membrane anchorage. Moreover, Pr3 seems to be a protein with specific hydrophobic properties which interact with lipid bilayers by hydrophobic insertion (Goldmann 1999). I have mentioned previously that glycosylphosphatidylinositol (GPI)-anchored receptor CD177 presents Pr3 on a neutrophil surface and therefore mPr3/CD177 complex may have implications in systemic vasculitis autoimmune disorders (e.g. Wegener's granulomatosis).

Circulating neutrophils in patients with active Wegener's granulomatosis express Pr3 on their cell surface, and the binding of Pr3-ANCA to membrane-bound Pr3 is followed by neutrophil activation that results in the release of reactive oxygen species and proteolytic enzymes (Falk 1990). Like the other serine proteases, Pr3 is inhibited by serpins, predominantly α-1-antitrypsin (AAT). AAT, the major physiological proteinase inhibitor in serum, inhibits a broad variety of serine proteases (especially HNE and Pr3) and is present in serum at 1.5-3.5 g/L (Fregonese and Stolk 2008). In the presence of AAT, Pr3 remains on the neutrophil surface and can bind ANCA, resulting in neutrophil activation and release of neutrophil granule contents, with higher levels of membrane Pr3 associated with greater responsiveness to Pr3-ANCA stimulation (Muller Kobold *et al.,* 1998).

A previous study demonstrated that AAT can impair the binding and activation of neutrophils by anti-Pr3 antibodies from both healthy control and individuals with WG which suggests that mPr3 activity and the protease-antiprotease balance are implicated in neutrophil activation during WG (Rooney *et al.,* 2001). Korkmaz *et al.* (2008) has shown that addition of purified AAT to Pr3-bound NB1 (CD177) transfected CHO cells completely removed Pr3 from the surface of NB1 receptor-expressing CHO cells. Furthermore he demonstrated that the binding of CD177 possibly occurs via the single hydrophobic cluster on the surface of Pr3. The membrane-bound human neutrophil elastase (mHNE) is rapidly cleared from the surface of activated neutrophils by AAT and by EPI-hNE4, a low molecular weight recombinant inhibitor, with which it forms soluble, inactive complexes. The behaviour of mPr3 clearly differs from that of mHNE, which explains why it may be a preferential target for autoantibodies and so contributes to the pathogenicity of Wegener disease (Korkmaz *et al.,* 2005; Attucci et al., 2006; Korkmaz et al., 2009).

3.2 Aims

● In this part of my project I sought to investigate how the surface expression of Pr3 and CD177 was affected by stimulation of neutrophils (the source of Pr3 and CD177) in the presence or absence of 100 % autologous serum (the physiological compartment of AAT) as well as purified commercial AAT with two different concentrations. In chapter 6 I investigated the effect of AAT (from autologous serum and purified commercial sources) on Pr3 binding CD177 recombinant protein expression on transfected CHO cells.

● I have sought to examine the level of neutrophil marker CD16 expression in the presence of serum and HBSS.

● Moreover I examined the impact of neutrophil isolation techniques (Percoll gradient and Dextran sedimentation) on unstimulated neutrophils in terms of detecting the levels of mPr3.

3.3 Results

3.3.1 The effect of serum on mPr3 and CD177 expression on neutrophils

Neutrophils were taken from healthy donors and stimulated with fMLP only or cytochalasin B (cytoB) followed by addition of fMLP (maxima stimulation), and compared to unstimulated control cells in the presence or absence of 100% autologous serum. These natural agonists are potent inducers of neutrophil degranulation and activation. Cell surface expression of Pr3 and CD177 was detected by mouse monoclonal antibodies, relative to isotype matched controls, which were in turn detected by PE-conjugated goat anti-mouse immunoglobulins antisera. Neutrophils were identified in mixed leukocyte populations by co-staining using APC-conjugated mouse monoclonal anti-human CD16, which is highly expressed on neutrophils.

Consistent with the results from other studies, we observed that unstimulated neutrophils, isolated from healthy individuals, expressed varying levels of Pr3 on their cell surface (Halbwachs-Mecarelli and Sarsat 1995, Schreiber 2004). As mentioned before the percentage of Pr3 and CD177 expression on the neutrophil surface is variable among individuals ranging from 0-100%. However, our results showed that in some individuals (n=2) the expressions of Pr3 was bimodal (the presence of both $mPR3^{low}$ and $mPR3^{high}$ populations within one individual) where as in others expression was monomodal (one uniform population of neutrophils), which does not reflect the CD177 expression (figure 3.1). The CD177 low subset represents low or possibly negative CD177 expression, whereas the high subset expresses a substantial amount of CD177.

Following, stimulation especially with fMLP combined with cytoB, neutrophils that expressed a monomodal mPr3 on unstimulated cells, were induced to express an increased sub-population of neutrophils with low mPR3 expression (figure 3.1 donors 2&4).

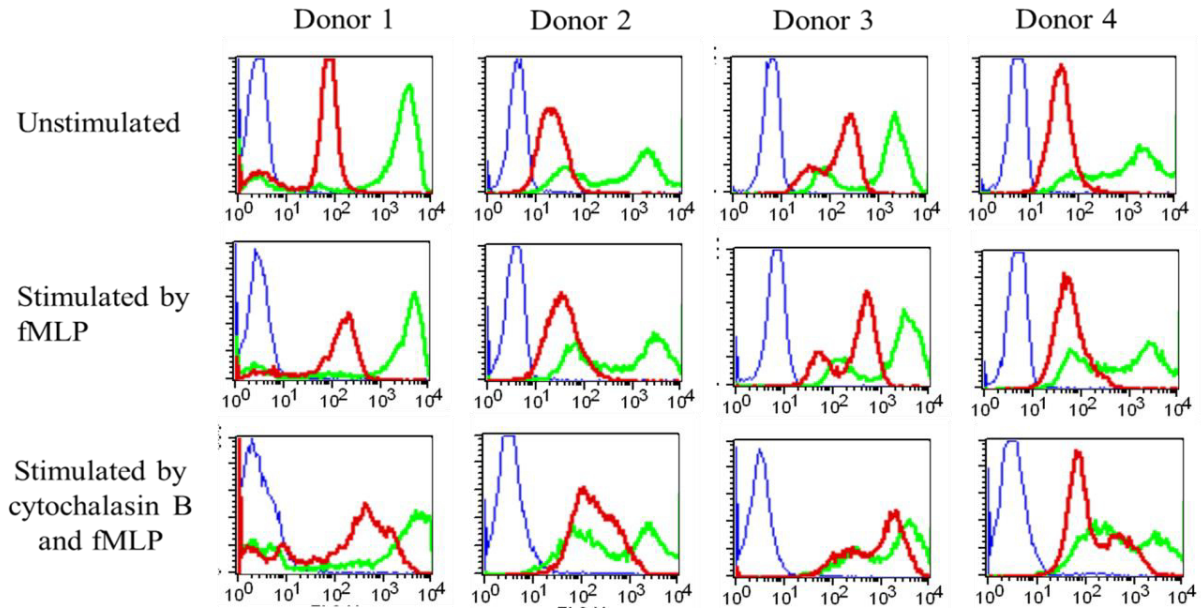


Figure 3.1 Patterns of mPr3 CD177 expression on surface neutrophils from different donors detected by flow cytometry. The blue line represents nonspecific binding of isotype-matched control. The red line shows binding of monoclonal anti-Pr3 antibody (G2) and green line shows binding of monoclonal anti-CD177 (MEM-166) both antibodies were detected by PE-conjugated goat anti-mouse antibody (rPE-GAM) in different tubes prepared in parallel, but histograms are overlaid to compare the expression distribution.

The results demonstrated that Pr3 and CD177 expression was still detectable in the presence of serum (figure 3.2A). The levels of CD177 showed no change in expression in the presence of serum, compared with HBSS (no serum) controls. While the mPr3 expression on unstimulated neutrophils in the presence of serum appeared to be less than that on cells in the absence of serum, this difference was not statistically significant. Both mPr3 populations showed reduction in mean cellular fluorescence (MCF) in the presence of serum and it reduced the level of $Pr3^{low}$ population to around that of the background isotype control staining ($Pr3^{low}$= 5.02 and control= 4.21; figure 3.2B). No significant increase was observed in $mPr3^{high}$ expression following stimulation with fMLP in either the presence or absence of serum.

In contrast, stimulation with cytochalasin B combined with fMLP resulted in a 9-fold increase ($P<0.0001$) in the $Pr3^{high}$ cells compared with unstimulated cells in the absence of serum. This increase was only 3-fold ($P< 0.002$) when stimulation occurred in the presence of serum (Figure 3.3). When the data is transformed to represent the percent increase in mPr3high expression (to remove variation in staining procedure on separate days), the effect of serum on decreasing surface Pr3 expression post-stimulation is very clear and reproducible (Figure 3.4).

No significant increase was observed in $mPr3^{low}$ expression following stimulation with fMLP in either the presence or absence of serum. However, stimulation of the cells with cytochalasin B combined with fMLP resulted in a 3.8-fold increase ($P=0.03$) in the $Pr3^{low}$ cells compared with unstimulated cells in the absence of serum. The statistical significance of this change is lost in the presence of serum, despite the average increase being 2-fold following maximal stimulation (Figure 3.5). In fact the only condition that showed a significant increase in $mPr3^{low}$ subpopulation expression was following stimulation of the cells by cytochalasin B & fMLP in the absence of serum ($P=0.03$).

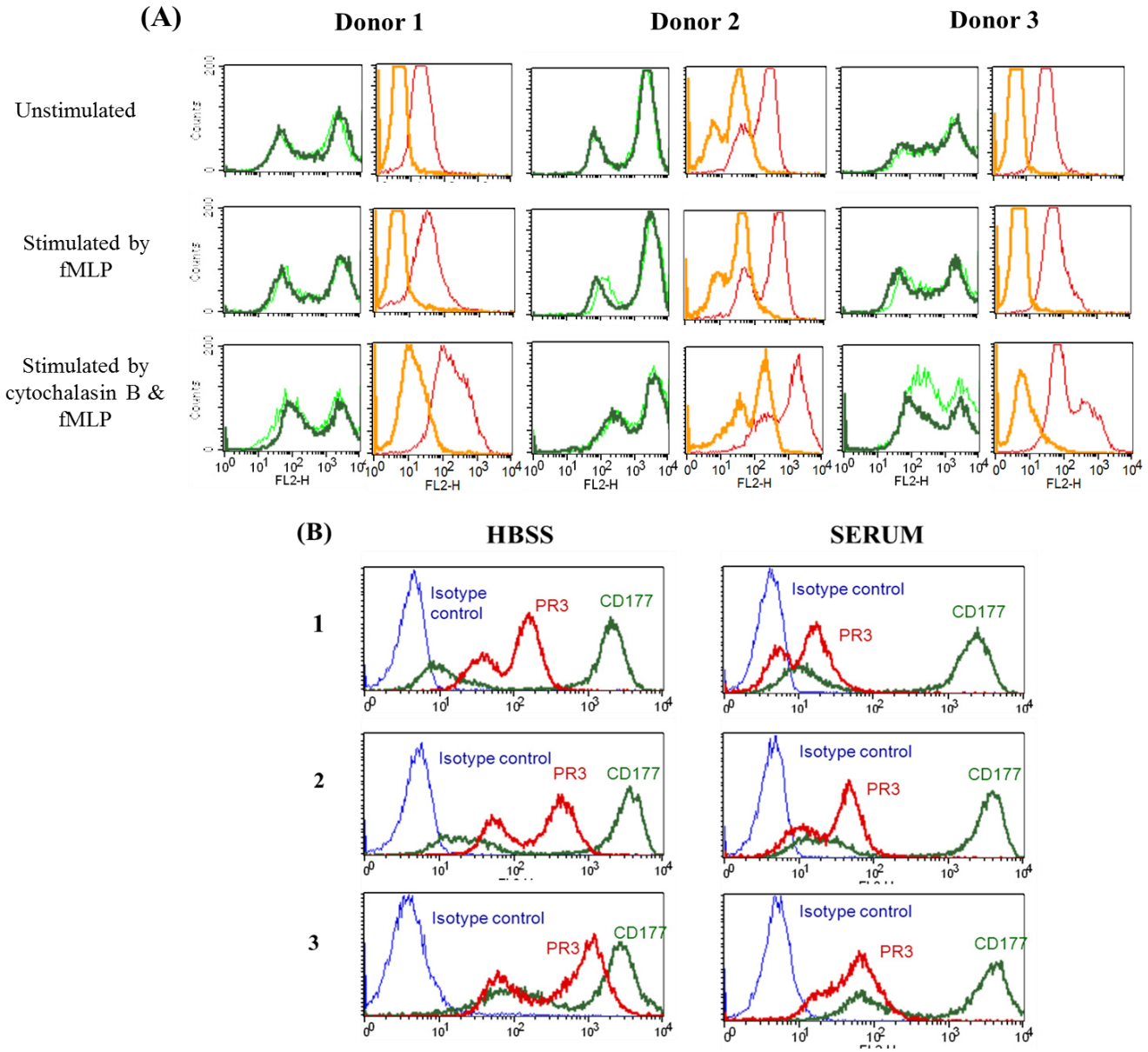


Figure 3.2 A) Surface expression of CD177 and Pr3 on neutrophils in presence or absence of serum from three different donors. The histograms show the fluorescence intensity of neutrophil surface expression of Pr3 (red line in presence of HBSS and orange line in presence of serum), CD177 (dark green line in presence of serum and light green line in presence of HBSS). The levels of CD177 showed no change in expression in the presence of serum, compared with HBSS whereas the cells showed a decrease in the levels of Pr3 expression. B) These histograms show the expression of mPr3 and CD177 from a single donor comparing CD177 (green) and Pr3 (red) expression relative to the isotype background control (blue). Neutrophils were unstimulated (1) or stimulated with fMLP (2) or stimulated with Cyto B in combination with fMLP(3).(n=7).

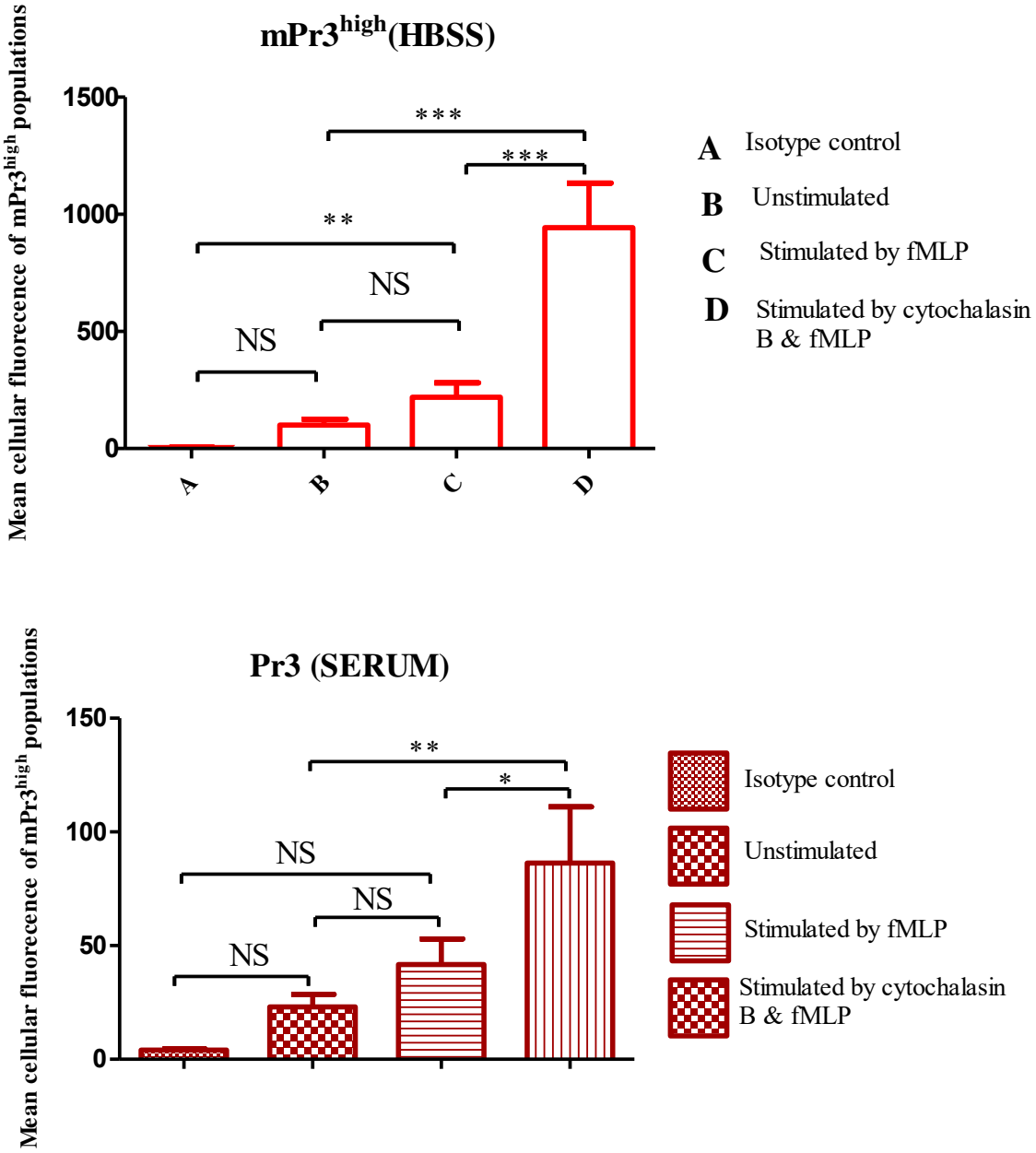


Figure 3.3 Bar graphs show the mean cellular fluorescence of mPr3^{high} populations in presence of HBSS or serum. Neutrophils were unstimulated and stimulated with fMLP only or cytoB in combination with fMLP (n=7).

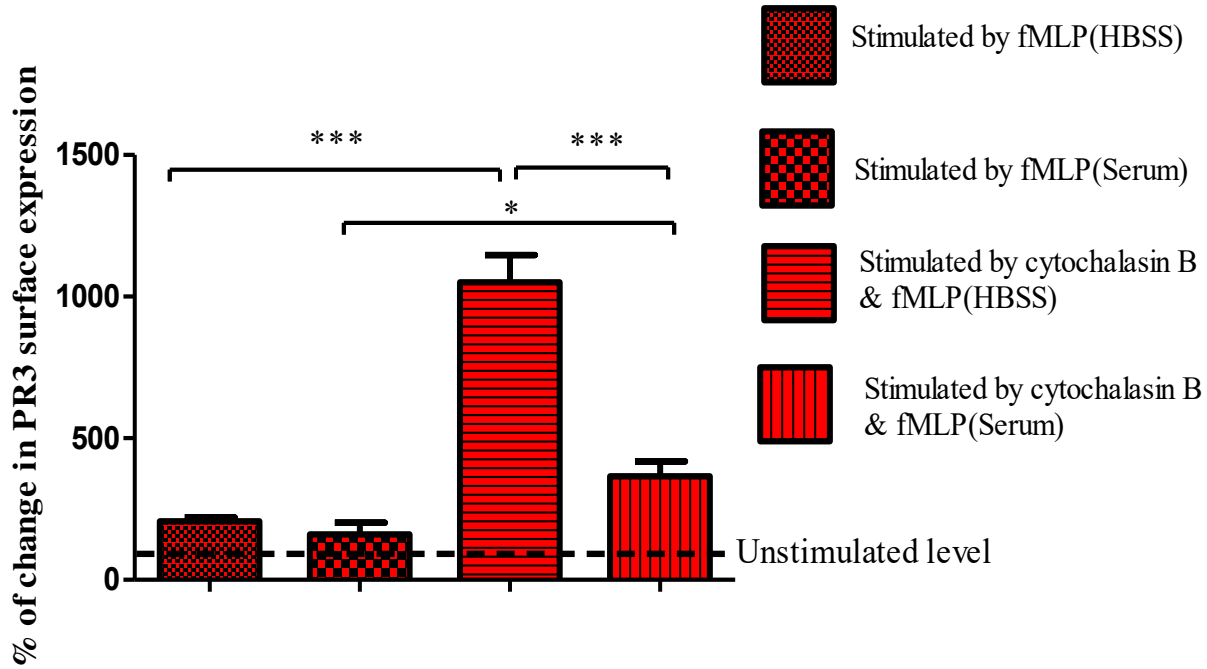


*Figure 3.4 The percentage of change in Pr3 surface expression in presence of HBSS or serum. The percentage of change is shown by dividing the level of detected mPr3 on surface of stimulated cells (with fmlp only or in combination with cytoB) by the amount of mPr3 detected on unstimulated cells. Data expressed as mean, error bars demonstrate SEM, *= p< 0.05; ***=p<0.001 (n=7).*

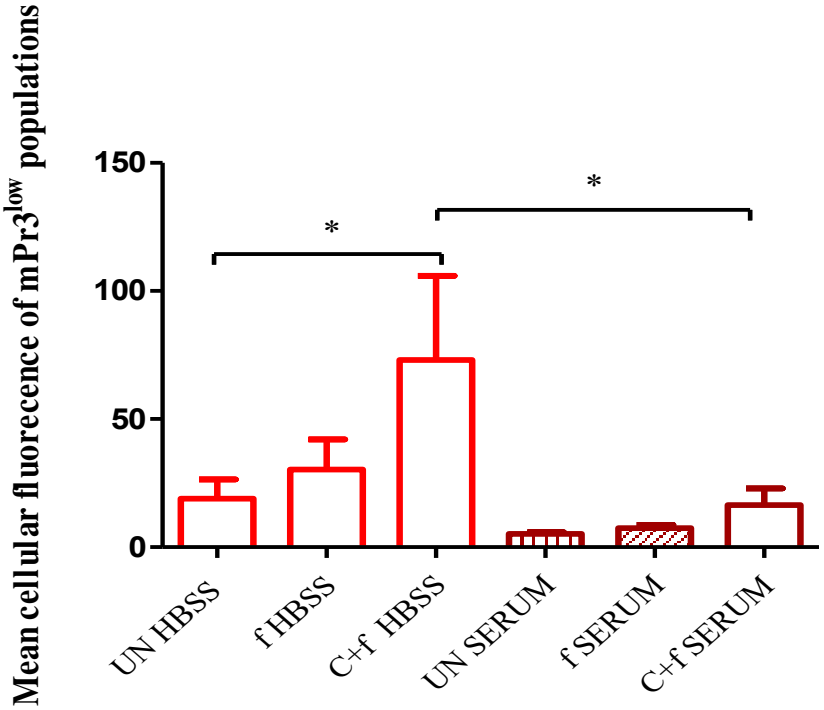


Figure 3.5 Percentage of Pr3 expressing neutrophils ($Pr3^{low}$ neutrophils) before (UN) stimulation with fMLP (f) or with cytoB & fMLP (C+f) in presence and absence of serum. The data are presented as mean cellular fluorescence ± SEM for five healthy donors (P=0.03).

These results indicate that serum (likely due to the AAT present) at physiological concentrations can reduce or remove Pr3 from the surface of neutrophils; however, for cells that express high levels of Pr3, this reduction does not result in complete loss of surface Pr3, whereas the decrease in Pr3 on the surface on $mPr3^{low}$ sub-populations result in a decrease below measurable residual amounts. Whether these surface levels of Pr3 that remain in the presence of serum retain enzymatic activity is unknown.

As mentioned previously, the levels of CD177 showed no change in expression in the presence of serum, compared with the absence of serum as seen in figure 3.6. Moreover, the statistical analysis shows no significant difference in expression of $CD177^{low}$ and $CD177^{high}$ between the two conditions, despite the significant increase in CD177 expression observed after stimulation with cytochalasin B & fMLP in presence or absence of serum (figure 3.6). These data indicate that serum (most likely AAT contained within) acts by reducing Pr3 surface expression by dissociating the Pr3/CD177complex rather than removing the Pr3/CD177 complex.

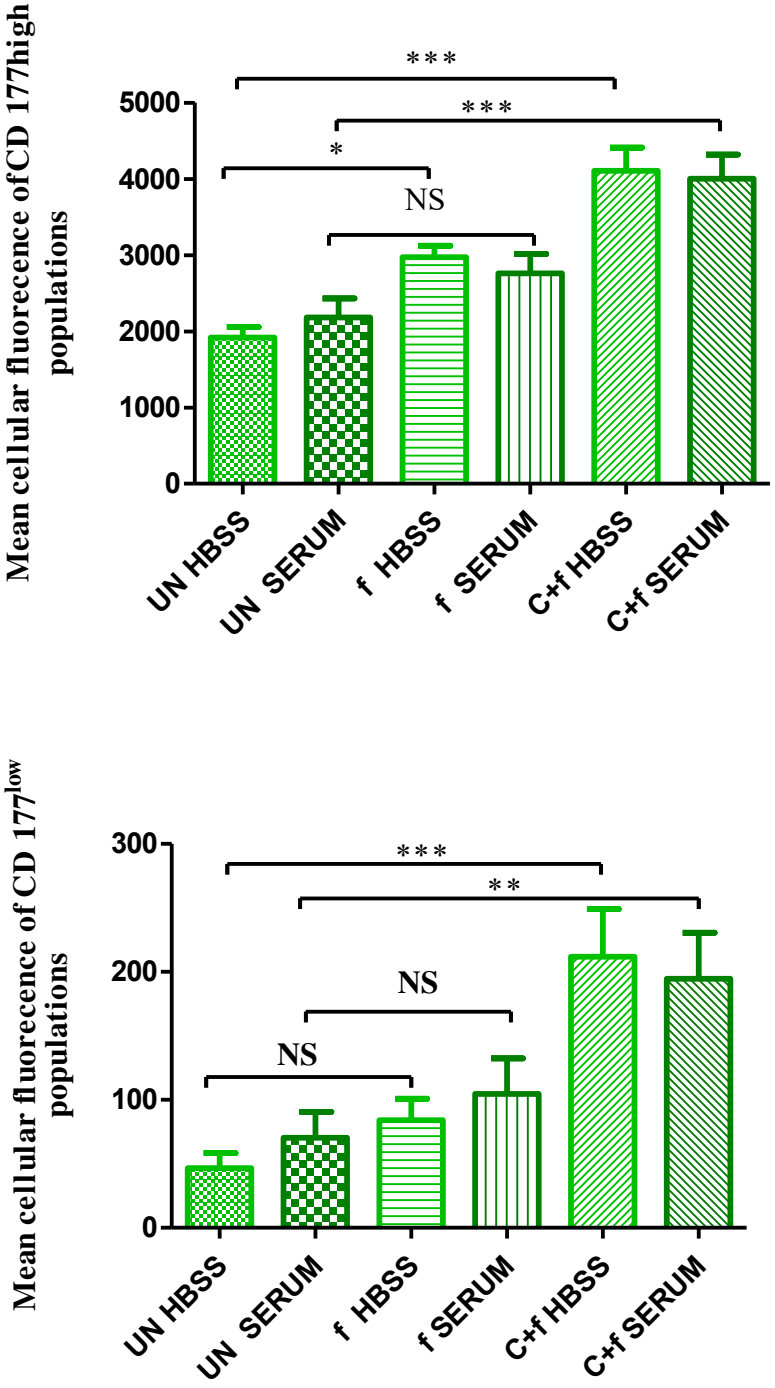


*Figure 3.6 Comparison of CD177 expression populations in presence of HBSS or serum. Mean cellular fluorescence of CD177^{low} (A) and CD177^{high} (B) expression as measured by flow cytometry. Data expressed as mean, error bars demonstrate SEM, *= p< 0.05; **=p<0.01; ***=p<0.001 (n=7). UN= unstimulated; f= fMLP stimulated; C+f= cytochalasin B and fMLP stimulated.*

3.3.2 The effect of purified AAT on the expression of Pr3 and CD177 on unstimulated and stimulated neutrophils

Neutrophils from healthy individuals were analysed for the expression of mPr3 and CD177 in presence and absence of AAT. As mentioned above Pr3 is controlled by a variety of inhibitors, including AAT, which is present in serum at 1.5-3.5 g/L. Therefore following purification neutrophils were resuspended in AAT (2µg/ml of $1x10^6$ cells) and incubated in the absence or presence of cytochalasin B and fMLP (representing maximal stimulation conditions).

Figure 3.7 shows that the expression of Pr3 was still detected on the surface of unstimulated cells in presence of AAT (MCF=10.57) compared to isotype matched control staining (MCF=2.49). Moreover, stimulation of neutrophils in the presence of AAT caused a reduction in mPr3 expression, but did not completely remove Pr3 from the surface of neutrophils. The MCF for Pr3 expression on unstimulated cells in presence of AAT showed no significant difference compared to unstimulated cells in absence of AAT as seen in figure 3.8. However, the expression of mPr3 on stimulated cells in the presence of AAT was significantly lower than that on stimulated cell in the absence of AAT. Stimulation of neutrophils in the absence of AAT resulted in a 13.9-fold increase in mPr3 expression, whereas stimulation of neutrophils in the presence of AAT caused a 5.6-fold increase (figure 3.8). Stimulation in the presence of AAT still resulted in a significant increase in mPr3 compared to unstimulated neutrophils ($p<0.05$).

Figure 3.9 shows that comparing mPr3 levels on neutrophils following stimulation in the presence of a physiological concentration of purified AAT (2 mg/L = 2 µg/ml) resulted in a significant reduction of mPr3 on the surface ($p<0.01$), and that while stimulation in the presence of a 1000-fold excess of AAT (2 mg/ml) appeared to reduce the mPr3 levels further ($p<0.001$), no significant difference was observed in mPr3 between stimulation in the

presence of 2 μg/ml and 2 mg/ml AAT. However, the MCF for mPr3 for the 2 mg/ml AAT conditions were still significantly greater than the MCF for isotype control, confirming that even this excess concentration of AAT could not remove all of the mPr3.

The surface expression of CD177 was unaffected by any concentration of AAT in all experiments (representative histogram in figure 3.7).

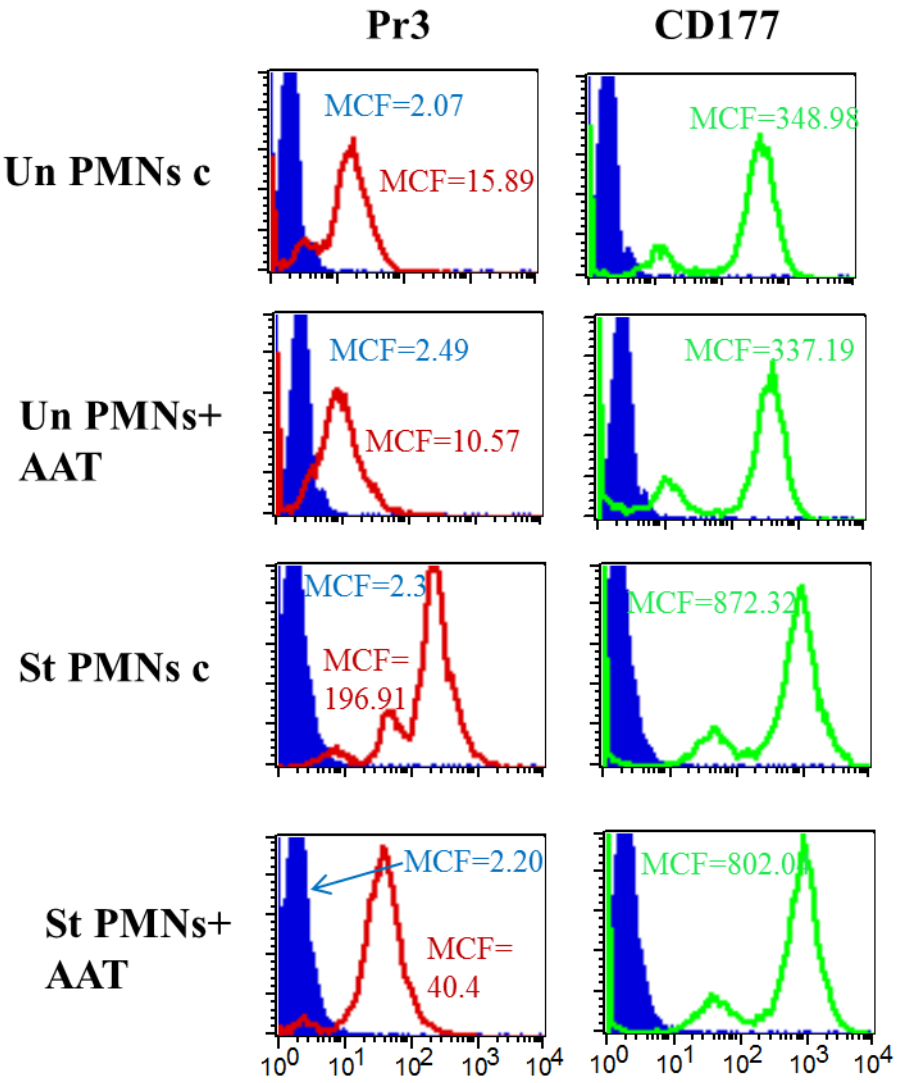


Figure 3.7 Overlay histograms of MCF showing the expression of Pr3 (red), CD177 (green) on unstimulated and stimulated neutrophil compared to the isotype control (blue). Purified neutrophils were either unstimulated (Un) or incubated for 15 min at 37°C with fMLP plus cytoB (St) in presence and absence of AAT (2 µg/ml).

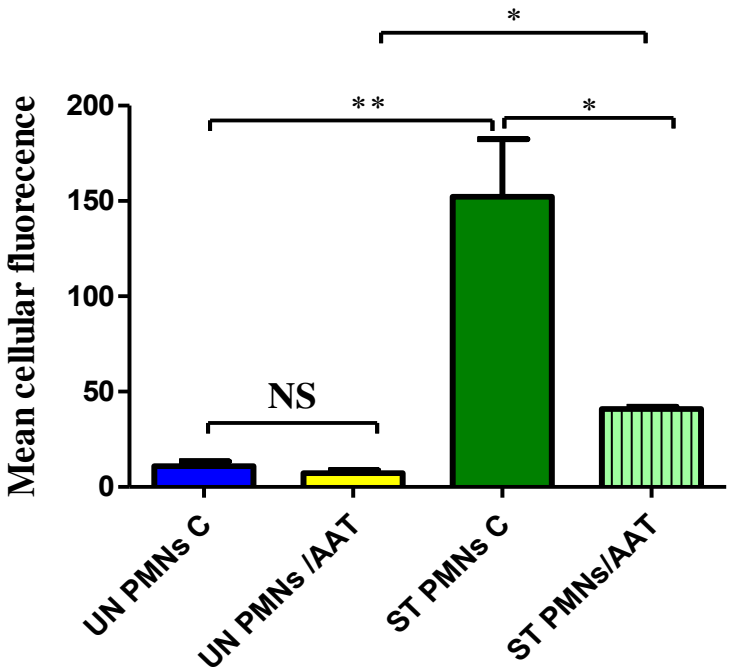


*Figure 3.8 Effect of AAT on membrane expression of Pr3 on unstimulated and stimulated neutrophil as measured by flow cytometry. Note that the maximum stimulation of neutrophils induced a significant increase in cell surface expression of mPr3 in the presence and absence of AAT when compared with unstimulated cells in both conditions. Data are shown as mean ± SEM. * $p<0.05$, ** $p<0.01$; n =4. UN = unstimulated; ST= cytochalasin B and fMLP stimulated; C= control (absence of AAT).*

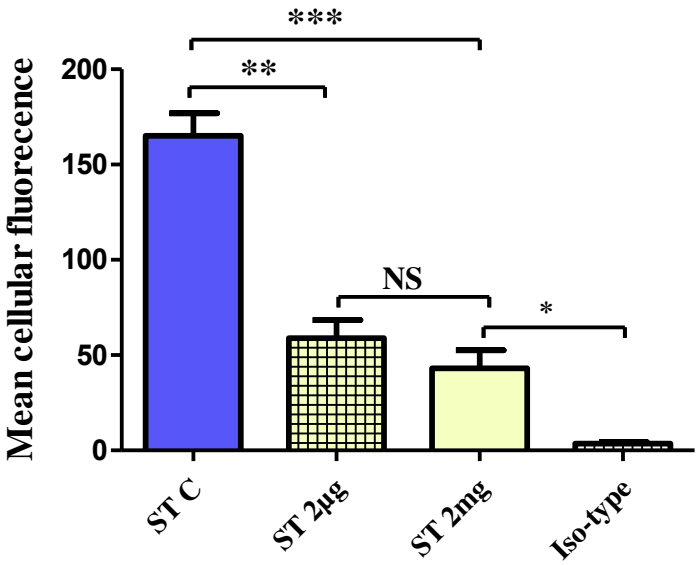


*Figure 3.9 The effect of different AAT concentrations on mPr3 expression on stimulated neutrophils. One million neutrophils in a 1 ml suspension were stimulated with cytochalasin B followed by fMLP in the presence of 2µg/ml or 2mg/ml of human purified AAT (n=3). *** = p<0.001, ** = p<0.01, NS= not significant. [ST= cytochalasin B and fMLP stimulated; C= control (absence of AAT)].*

3.3.3 The expression of CD16 on stimulated cells in the presence and absence of serum

In this study, I used $CD16^{high}$ as the neutrophil marker, but I noticed that in the presence of HBSS the level of CD16 expression was reduced significantly after stimulation with fMLP ($P < 0.05$) and highly significant following maximal neutrophil stimulation compared to unstimulated cells ($P< 0.0001$), but in presence of serum significant change was only seen following maximal stimulation ($P< 0.0001$).

No statistically significant difference was observed comparing CD16 expression on unstimulated cells exposed to serum or resuspended in HBSS, nor was there any difference between CD16 expressions following fMLP stimulation in the presence of absence of serum. There was a significant difference ($P<0.01$; figure 3.7) in CD16 expression following maximal stimulation: serum inhibited the decrease in CD16 caused by shedding from the surface of neutrophils.

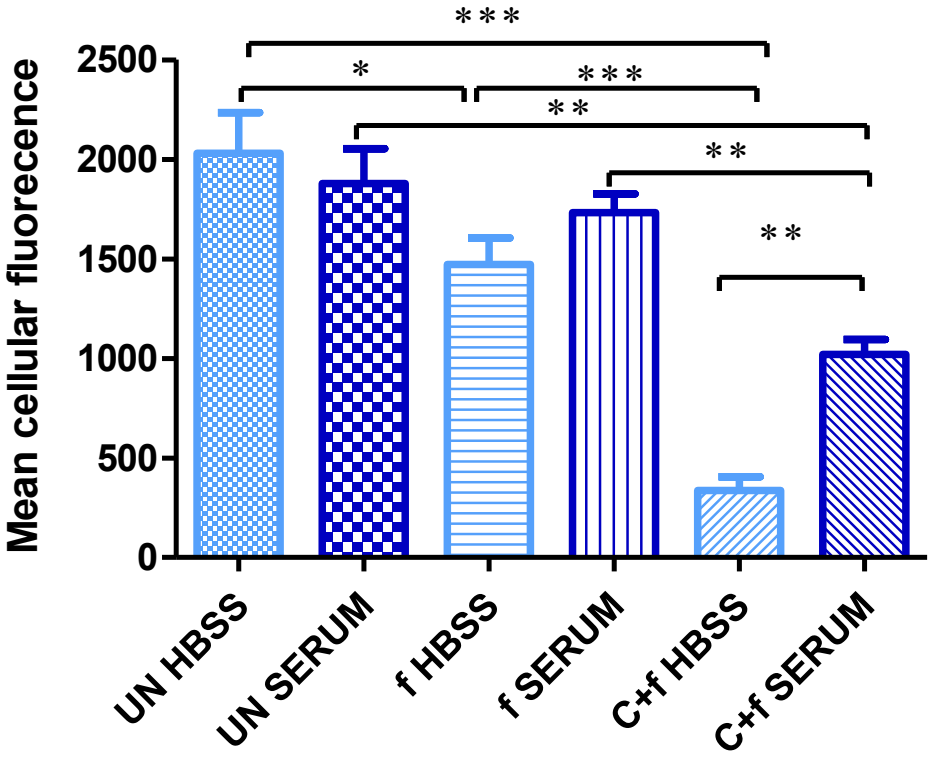


*Figure 3.7 CD16 expression on neutrophils after 15 minutes stimulation (in absence or presence of serum) with fMLP alone or fMLP with cytoB compared to unstimulated cells, statistical significance is shown in the figure: * p>0.05, ** p>0.01, *** p>0.001. UN= unstimulated; f= fMLP stimulated; C+f= cytochalasin B and fMLP stimulated (n=4).*

3.3.4 Comparison of surface marker expression following Percoll and Dextran neutrophil isolation methods

Neutrophils are usually isolated from peripheral venous blood to study their function and behaviour. Isolation demands an efficient method to obtain non-activated and viable cells, and technique should not influence neutrophil behaviour, specifically initiation of inappropriate activation. Characterising the levels of mPr3 on stimulated neutrophils requires a method of neutrophil isolation that does not stimulate the cells. The majority of previous experiments used Percoll gradient for neutrophil separation, allowing study of neutrophils in isolation.

However, there was a possibility that Percoll itself might cause neutrophil stimulation. Thus, this method was compared to Dextran sedimentation method which removes only erythrocytes and plasma, to see if there is a significant difference in the levels of mPr3 on unstimulated cells. As above, CD16 was also used as marker of neutrophil activation.

Blood from volunteers was separated by both methods in parallel and under identical laboratory conditions. The levels of mPr3 and CD16 on unstimulated and stimulated (with maximal stimulation using cytoB and fMLP) cells were compared. Detection of mPr3 was demonstrated by MCF above isotype control background levels. Pr3 was detected on unstimulated neutrophils from both techniques but while the level on unstimulated Percoll-separated cells appeared higher than on Dextran-separated cells, the difference was not statistically significant. However, significant levels of mPr3 for both separation methods were only detected for the $mPr3^{high}$ population after cell stimulation. The level of increase was distinctly different between the alternate cell separation methods: Cells purified by Dextran-separation showed an 8-fold increase in mPr3 expression relative to unstimulated cells, while Percoll-separated cells only showed a 3-fold increase (figure 3.8A).

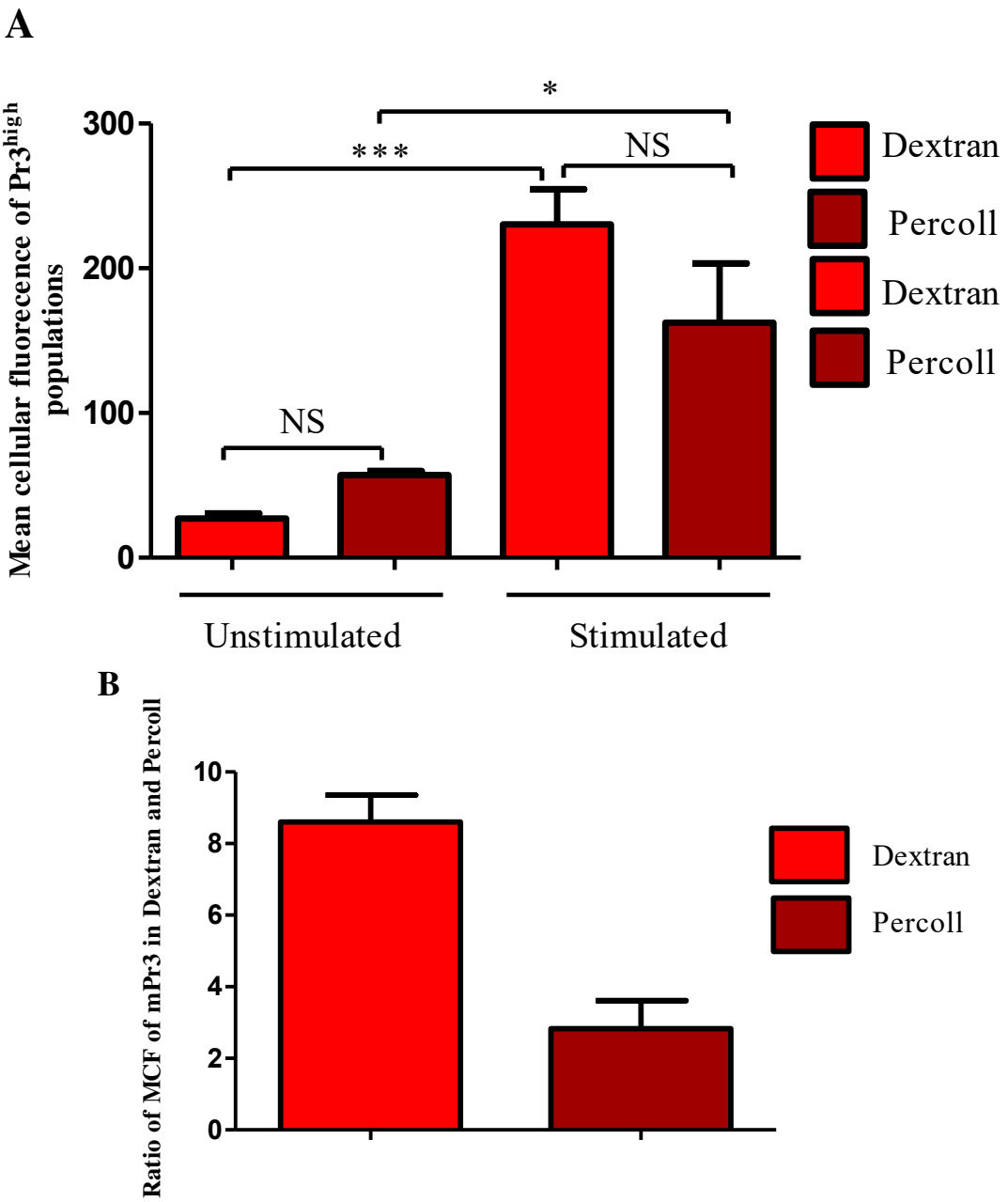


Figure 3.8 Comparison of Percoll and Dextran neutrophil isolation techniques. Neutrophils were isolated by either Dextran sedimentation or Percoll gradient. The cells were unstimulated or incubated for 15 minutes at 37C° with cytoB & fMLP and mPr3 detected by Pr3-G2 expressed as MCF. (A) Detection of mPr3 on Dextran and Percoll separated neutrophils. (B) Bar graph shows the comparison in the ratio of MCF of mPr3 from the two methods. Comparison is shown by dividing the level of detection of mPr3 on stimulated cells by the amount of mPr3 detected on stimulated cells in each method individually (n=3).

Figure 3.8B illustrates a significant difference (*P=0.01*) in the ratio of MCF mPr3 expression (on stimulated cells) between the two methods, suggesting that Percoll-separated cells are being activated more than the Dextran-separated cells in the isolation process and Percoll-separated cells are expressing less mPr3 due to unknown action of percoll itself on the neutrophils during the stimulation.

Figure 3.9 shows the MCF of CD16 expression on unstimulated cells and in response to stimulation with cytoB and fMLP for neutrophils isolated by both methods. CD16 expression on unstimulated Dextran cells was slightly higher with mean fluorescence of (2302±SEM434.9) compared to (1022±392.8) in Percoll unstimulated cells (P=0.05). There was a significant decrease (P=0.02) in CD16 expression on Dextran purified cells after stimulation while no significant change (P<0.08) was observed on the Percoll purified cells.

However, there was no significant difference in levels of CD16 expression comparing stimulated cells levels of CD16 across both methods. These results suggest that expression of CD16 on Percoll-separated cells was lower than Dextran cells and that they may activated or primed for activation which causes the shedding of CD16 from the neutrophil surface; however following maximal stimulation the end levels of CD16 were the same between the two methods.

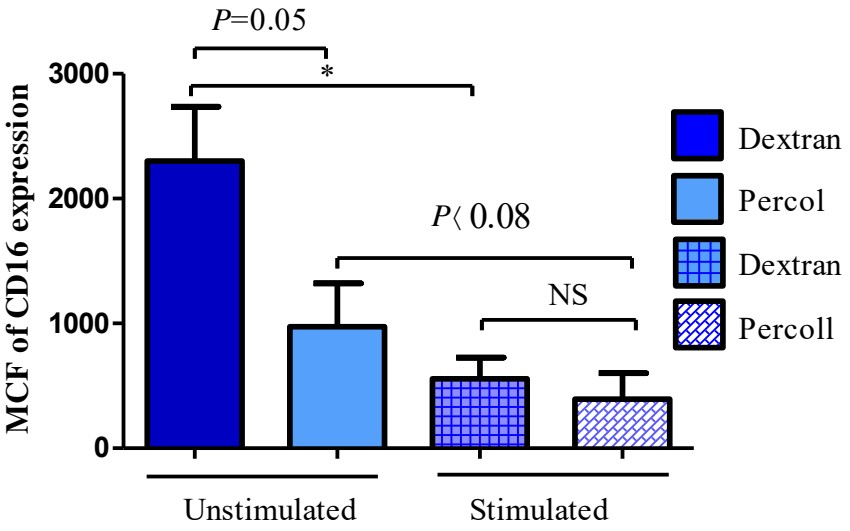


Figure 3.9 Effect of neutrophil purification method on CD16 expression as measured by flow cytometry. MCF for APC-conjugated anti-CD16 staining was measured on unstimulated and stimulated cells on Dextran purified cells and compared to Percoll gradient purified cells.

3.4 Discussion

In this chapter, I analysed the surface expression of CD177 and mPr3 in the presence of physiological inhibitors present in serum before and after neutrophil stimulation. It was confirmed that circulating human neutrophils have a bimodal distribution of Pr3 and CD177 on their plasma membrane which varies between individuals (Bauer *et al.,* 2007; Brachemi *et al.,* 2007; Von Vietinghoff *et al.,* 2007).

I did find that not all individuals have bimodal distribution of Pr3, but that these cells sometimes separated into a low and high mPr3 population after maximal stimulation. Hu and co-workers has demonstrated that isolated adult neutrophil samples change from monomodal to bimodal mPr3 expression following 15 minutes with TNF-α (Hu *et al.,* 2009). In contrast all CD177 expression was bimodal irrespective of stimulation. A study by Halbwachs-Mercarelli *et al.,* 1995 demonstrated that the proportion of freshly isolated neutrophils that expresses Pr3 varies considerably between donors (0–95%), but is extremely stable for each individual over prolonged periods of time.

Our data enhance the earlier funding that Pr3 is present on the surface of quiescent neutrophils (Halbwachs-Mecarelli *et al.,* 1995; Schreiber *et al.,* 2003). These results go against the findings of Yang *et al.,* (2000) who state that circulating neutrophils do not demonstrate mPr3. Many studies have found that the expression of Pr3 on CD177-positive neutrophil cell surfaces is upregulated by multiple proinflammatory mediators including TNF-α, PMA, LPS, fMLP and GMCSF (Csernok *et al.,* 1994; Halbwachs-Mecarelli *et al.,* 1995; Witko-Sarsat *et al.,* 1999; Hellmich *et al.,* 2000; Drewniak et al., 2008). Pr3 is a NSP that is stored in the granules of circulating neutrophils. Moreover, it is located within secretory vesicles that readily fuse with the plasma membrane.

CD177 is also found in the same intracellular compartments as Pr3 (secondary granule and secretory vesicles) and this supports the hypothesis that Pr3 and CD177 may share the same mode of trafficking (Von Vietinghoff S *et al.,* 2009; Goldschmeding R *et al.,* 1992; Bauer *et al.,* 2007). However, CD177 is also found in high abundance on the cell surface of some neutrophils, raising the possibility that intracellular CD177 and Pr3 translocate individually from different sources and associate at the membrane surface. The low levels of mPr3 and CD177 may translocate to the membrane as an already preformed complex from an intracellular pool; however, most of the surface CD177 is not associated with Pr3. So whether these complexes dissociate at the cell surface and following stimulation, Pr3 binds again to CD177 is unknown (Witko-Sarsat *et al.,* 2010; Choi M *et al.,* 2010). The hydrophobic patch predicted to be on the surface of the Pr3 molecule was shown to be important for its CD177 binding (Korkmaz *et al.,* 2008). The higher percentage of mPr3/CD177-double positive neutrophil is associated with an increased risk for and worse outcome of ANCA vasculitis. Therefore, dissociation of Pr3 and CD177, or disrupting Pr3/CD177 complexes, would have obvious therapeutic implication for Pr3/ANCA-mediated neutrophil activation.

Pr3 activity is inhibited by variety of natural inhibitors, such as AAT (a major physiological proteinase inhibitor in serum) and elafin. AAT binds covalently to all serine proteases and therefore exhibits effects that go beyond the Pr3-CD177 interaction. A previous study by Korkmaz has demonstrated that addition of purified AAT to Pr3-bound CD177 transfected CHO cells completely removed Pr3 from the surface of CD177 receptor-expressing CHO cells (Further explored in chapter 6). My results illustrate that serum has no effect on the levels of CD177 expression before or after stimulation for either $CD177^{low}$ or $CD177^{high}$ sub-populations. Levels of mPr3 on unstimulated neutrophils in HBSS and serum were similar (very low), while a significant reduction in mPr3 was noted following stimulation in the presence of serum when compared to cells stimulated in the absence of serum.

This suggests that Pr3 upregulated to the cell membrane following stimulation may be more easily removed than Pr3 that is already bound to the membrane before stimulation. Nonetheless, one should note that there is still a significant increase in the $mPr3^{high}$ population following stimulation in the presence of serum (which reflects the physiological conditions of neutrophil stimulation) indicating that regardless of the presence of physiological inhibitors, increased surface Pr3 would still occur with stimulation and may play an important role in the physiological processes of neutrophils. However, I have not ruled out the possibility that other molecules in serum may have protected the mPr3, or inhibited AAT function, or that given the bait-loop mechanism of AAT that the surface mPr3 may lack enzymatic activity. My result also indicated that the inhibitors in serum disassociated Pr3 only from the surface of neutrophils without removing the expression of the high affinity receptor (CD177). Korkmaz *et al.,* 2009 reported that constitutive mPr3 (mPr3 on resting neutrophils) is inactive and is not able to interacting with AAT so it can remain at the surface of quiescent circulating neutrophils even in the presence of huge amounts of inhibitor. In addition they suggested induced mPr3 (mPr3 after priming or stimulation of neutrophils) is inhibited and removed by AAT as was shown using cells that stably expressed the CD177 receptor. However, as I mention above stimulating neutrophil in presence of serum showed significant increase in Pr3 expression which suggested that AAT is not able to remove all induced mPr3.

CD16 (FcγRIIIb) is highly expressed on human neutrophils and is also found in a soluble form in serum and in other body fluids such as saliva, urine, and seminal fluid (Fleit *et al.,* 1992). Following neutrophil activation, CD16 is shed from the cell surface by proteolytic cleavage (Huizinga *et al.,* 1988; Homburg *et al.,* 1995). Shedding of cell-surface receptor may be essential to limit cell responsiveness to external ligands or to dissociate cells from ligands involved in the initial binding part of the transmigration process. Nevertheless

shedding may also represent a mechanism for the production of soluble receptors that convey signals to other cells in a manner analogous to cytokines (Tedder, 1991).

CD16 is a heavily glycosylated protein of 50–70 kDa that is linked to the plasma membrane via an easily cleaved glycosyl phosphatidylinositol (GPI) anchor. Surface expression of CD16 is controlled by the balance between the rates of shedding and mobilization of preformed intracellular stores to the cell surface (Fossati *et al.,* 2002). Activation of neutrophils with fMLP caused shedding of CD16 from the cell surface (Huizinga et al., 1988). It seems that serine proteases and metalloproteases are responsible for CD16 shedding (Middelhoven *et al.,* 1997; Middelhoven *et al.,* 2001). Tosi and Berger (1988) recognized that CD16 on neutrophils was sensitive to cleavage by elastase. The essential function of CD16 ligation seems to be ROS generation in response to ligation by soluble immune complexes in primed neutrophils (Fossati *et al.,* 2002).

In this work I found that stimulation of neutrophils with fMLP significantly decreased the expression of CD16 when cells were resuspended in HBSS, but the decrease was markedly blunted when stimulation of the cells occurred in presence of serum. The combination of fMLP + cytoB also greatly decreased CD16 expression, but CD16 shedding was significantly reduced if maximal stimulation occurred in the presence of serum. This suggests that exposure of neutrophil to serum could prevent CD16 shedding from the surface through endogenous serum inhibitors blocking the proteinases released or activated after neutrophil stimulation.

Neutrophils are highly activated and considered relatively fragile cells thus their isolation requires efficient methods to yield a good amount of cells in a short period of time. An additional consideration is the influence of the isolation and purification technique on neutrophil behaviour; especially neutrophils may become activated as a consequence of *ex*

vivo manipulation. In this chapter I also evaluated the influence of isolation methods in expression of mPr3 and CD16 on the surface of unstimulated and stimulated neutrophils. Methods of neutrophil isolation from whole blood can vary between laboratory groups, generating potential phenotypic differences in neutrophils even if retrieved from the same volunteer. This may result in alterations in neutrophil activation, which subsequently may lead to results in *ex vivo* studies being misinterpreted or even being incomparable between studies. There are currently a number of materials used for neutrophil isolation.

Percoll is presently the gradient material used by many laboratories, while other research groups use Histopaque. The Percoll-separation method involves many steps including repetition of centrifugation, wash steps and transfers between tubes which prolongs the time of isolation. Moreover, there was a possibility that Percoll itself stimulated neutrophils. Percoll consists of colloidal silica of 15–30 nm diameters which are coated with non-dialyzable polyvinylpyrrolidone (PVP) to avoid toxicity to cells and is used in combination with sodium chloride and water to create a gradient medium (http://www.sigmaaldrich.com). Therefore, this method was compared to Dextran sedimentation which removes only erythrocytes and plasma, to see if there is a significant difference in levels of mPr3 and CD16 on the surface of neutrophils.

The levels of mPr3 were evaluated on the surface of unstimulated neutrophils and levels following maximal stimulation, comparing neutrophils purified in parallel by dextran sedimentation and Percoll-gradient. The data suggested that Percoll-gradients increased the baseline levels of mPr3 on the surface of unstimulated neutrophils, but that the levels of mPr3 achieved much greater levels if the cells were purified by dextran sedimentation. However, neither of these differences achieved statistical significance (Figure 3.8A) it was only when the ratio of mPr3 between unstimulated and stimulated neutrophils was compared between

these two methods (Figure 3.8B) that this obvious difference was confirmed by statistical analysis.

Similarly, I found CD16 expression on unstimulated Dextran-sedimented cells was higher than that on unstimulated Percoll-separated cells ($p<0.05$, Figure 3.9) but that the levels of CD16 after maximal stimulation-induced shedding results in similar low CD16 levels. These results revealed that Percoll may cause activation of neutrophils in the absence of stimulation and Percoll-separated cells did not respond to stimulation to the same extent as Dextran-derived cells. Consequently, Dextran method is less time consuming and may be considered more representative of neutrophils *in vivo*. However, as neutrophils are not completely isolated in the dextran-sedimentation setting, require gating for investigation by including an APC-conjugated anti-CD16 antibody, and may be influenced by possible interactions with other cell types present, it may not always be the most appropriate method of preparing neutrophils for all types of experiments. For studies examining activation and degranulation, it should be preferred over Percoll-gradient purification, however.

3.5 Summary

In this chapter I have presented that mPr3 is still detectable on the surface of neutrophils in the presence of serum but it showed a marked decrease relative to levels bound in the absence of serum. Moreover the levels of mPr3 on stimulated cells (with fMLP or cytochalasin B + fMLP) in serum still demonstrated binding above background staining levels. This indicates that regardless of the presence of physiological inhibitors, Pr3 bound to the cell surface of neutrophils retains its presence, and because of that it may play an important role in the physiologic processes of neutrophils. In addition, the levels of CD177 showed no change in expression in the presence of serum, compared with the absence of serum, demonstrating decreased Pr3 surface staining is not an artefact of failure to detect surface neutrophil markers and that serum enhances the dissociation from the high affinity surface receptor. The results published by different research groups may be differentially influenced the method of neutrophil preparation which may alter mPr3 surface expression and CD16 shedding. The influence of alterations in separation protocols on subsequent *ex vivo* neutrophil behaviour and activation should be taken into consideration for experimental design.

CHAPTER 4

CELLULAR RELOCATION OF CD177, Pr3, MMP-8 AND MMP-9 DURING *in vitro* TRANSENDOTHELIAL MIGRATION

4.1 Introduction

The transmigration of neutrophils to the site of infection or tissue damage is crucial for innate immunity and inflammatory disorders. Transmigration of neutrophils involves firm adhesion followed by migration across the endothelium and the basement membrane. Degradation of extracellular proteins by neutrophil proteinases is a fundamental component of physiological processes, e.g. their egress from the vasculature and penetration of tissue barriers, tissue remodelling and repair, wound healing, and fibrinolysis. As a result of their proteolytic properties, it was originally thought that neutrophil proteases degrade constituents of the extracellular matrix, thus allowing cells to crawl through the gaps. Previous studies (Allport *et al.,* 1997; Del Maschio *et al.,* 1996; Moll *et al.,* 1998) demonstrated that neutrophil proteases are capable of rapidly degrading components of the vascular endothelial (VE)-cadherin complex during neutrophil-endothelial contact.

Among the proteases suspected to be involved in transmigration are serine proteases (including NE, Cat G, and Pr3) and matrix metalloproteases (such as MMP-8 and MMP-9). Both Pr3 and elastase can degrade extracellular matrix, ultimately leading to EC detachment (Ballieux *et al.,* 1994). Carden and Korthuis (1996) have demonstrated that inhibition of NE alone was sufficient to diminish neutrophil accumulation (without disturbing neutrophil adherence) in an animal model of ischemia/reperfusion in skeletal muscle. Furthermore, incubation of neutrophils with inhibitors of Cat G and NE together was found to reduce the adhesion of neutrophils to various surfaces (Delyani *et al.,* 1996; Murohara et al., 1995). Purified NE and Cat G can cleave the extracellular part of VE cadherin *in vitro* and specific inhibition of both NE and Cat G at the neutrophil surface significantly reduced neutrophil transmigration in an *in vitro* assay (Hermant *et al.,* 2003). Neutrophil adhesion and migration are accompanied by release of significant quantities of MMP-9 (Tschesche *et al.,* 1991). However, studies using serine protease inhibitors and MMP inhibitors indicated that neither

MMP nor serine protease digestion of sub-endothelial matrix was required for successful neutrophil transendothelial migration (Mackarel *et al.,* 1999). The generation of serine protease-deficient mice (NE−/− mice) additionally confirmed the hypothesis that neutrophil serine proteases were not required for *in vitro* chemotaxis as well as *in vivo* chemotaxis for some inflammatory models (Hirche 2004). However, a study by Wang S *et al.,* (2005) showed a defect in transmigration in NE-deficient mice was accompanied by a reduction in the levels of pro-inflammatory chemokines and cytokines. In addition, the results suggested that neutrophil migration through the laminin network within the perivascular basement membrane can be facilitated by increased expression of α6β1 (achieved via ligation of PECAM-1 at endothelial-cell junctions), and that NE activity was able to cooperate with this to enhance neutrophil transmigration. CD177 has been shown to be a heterophilic binding partner for the endothelial cell junctional protein, PECAM-1, as well as being the surface high affinity receptor for Pr3 (Sachs *et al.,* 2007). Disrupting the interaction between CD177 and PECAM-1 has been found to significantly inhibit neutrophil transendothelial cell migration on endothelial cell monolayers (Sachs *et al.,* 2007; Christopher *et al.,* 2012).

The heterophilic interaction between CD177 and PECAM-1 is roughly 15 times stronger than PECAM-1 homophilic interactions (Sachs *et al.,* 2007; Newton *et al.,* 1999; Christopher *et al.,* 2012). Recently, it has been demonstrated that Pr3 plays an important role in neutrophil transmigration under both static and flow conditions (Kuckleburg *et al.,* 2012). This requires Pr3 enzymatic activity and interactions with CD177. Furthermore, it was shown that CD177-positive neutrophils are selectively recruited to IL-1β activated endothelial cell monolayers, suggesting that under specific inflammatory conditions, tissues may accumulate high levels of neutrophil-expressed Pr3.

4.2 Aims

It is unknown if transmigration of neutrophils results in significant release of the proteinases Pr3, MMP-8 and MMP-9, or whether there is significant intracellular re-organisation of the proteinases or the Pr3/CD177 complex. Thus, the aims of the work described in this chapter were:

●To measure the surface expression of Pr3 and CD177 pre- and post-transendothelial migration *in vitro*.

●To measure the intracellular levels of Pr3, MMP-8 and MMP-9 pre- and post-transendothelial migration.

● Investigate the role of CD177 in neutrophil transendothelial migration by blocking CD177 with a monoclonal antibody.

4.3 Results

4.3.1 Endothelial monolayer integrity *in vitro* (FITC-Dextran impermeability)

In order to induce transendetheilal migration *in vitro*, endothelial cells were grown on a porous membrane which separted two chambers (transwell). It was important that the HUVECs monolayer formed an impermeant cell layer as a result of the formation of tight and gap junctions, so that neutrophils utilised physiological mechanisms to migrate across the cell layer. This was assessed by establishing the permeability of the cell monolayer to FITC-labelled dextran (average mol wt 4,000) added to the upper chamber and the appearance of the fluorescence in the lower chamber monitored. The passage of FITC-dextran through the transwell membrane with no cells was rapid, as expected for a porous membrane with mean pore size of 3μm (fig 4.1). However when endothelial cells were grown on the transwell membrane the passage of FITC-labelled dextran across the cell layer was significantly slowed demonstrating the diffusion barrier caused by the endothelial layer. It was concluded that the cell monolayer formed a contiguous layer with tight cell-to-cell contacts and was thus an *in vitro* mimic of the endothelial barrier across which neutrophils migrate during inflammatory responses.

4.3.2 Pr3 and CD177 surface expression during neutrophil transendothelial migration

Neutrophils contain Pr3 in the neutrophil azurophilic, secretory, specific granules, and at the plasma membrane of isolated resting neutrophils. CD177 is a neutrophil specific surface lipoprotein reported to be found on the plasma membrane and in secondary granules of neutrophils. It is largely thought to account for the dominant proportion of mPr3 on the neutrophil surface. To determine whether Pr3 and CD177 expression was increased on the surface of transmigrating neutrophils, neutrophils from healthy individual were allowed to

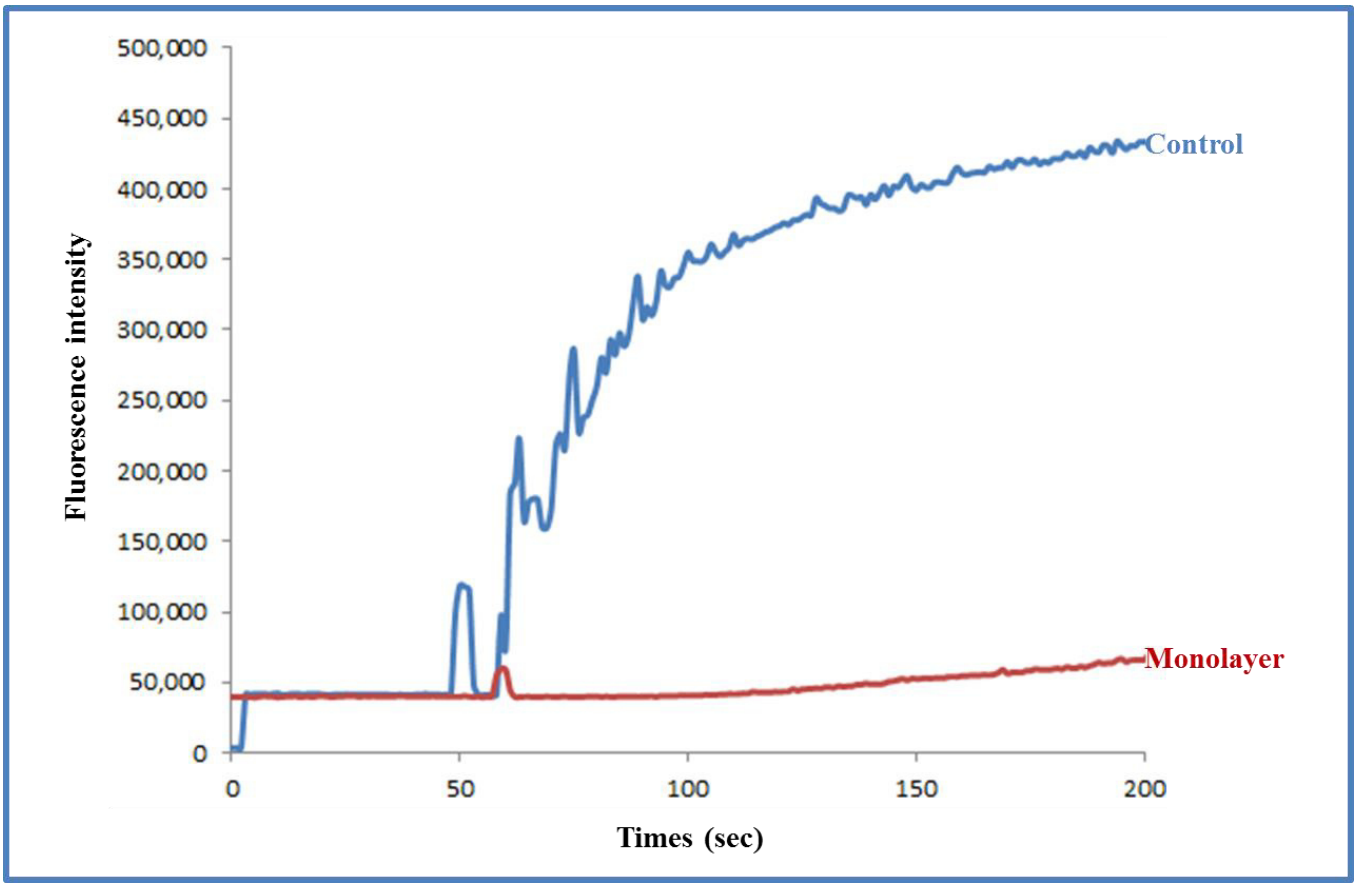


Figure 4.1 Flux of FITC-dextran across HUVEC monolayers. Endothelial monolayer integrity was determined by passage of FITC- dextran across intact monolayers and compared to inserts without cells. Cells were cultured for 48–72 h prior to analysis then a solution of FITC-dextran was used to replace culture medium in the upper well of the Transwell inserts. Influx was measured continuously by fluorometry of the lower chamber.

transmigrate through unstimulated and stimulated HUVECs (with TNF-α) cultured on matrigel-coated transwell inserts, toward the chemoattractant fMLP. Migrated neutrophils were then collected from the lower well of 24 Transwell plate and analyzed by flow cytometry for Pr3 and CD177 surface expression. To examine the direct influence by fMLP used as a chemoattractant I stimulated neutrophils ($1x10^6$) with fMLP, in the absence of transmigration as a control. Control neutrophils (unstimulated and stimulated with fMLP prior migration) and neutrophils that had crossed the endothelial layer into the lower chamber were stained with: 1) APC anti-CD177 and PE anti-CD16 and 2) anti-Pr3 mAb and PE-conjugated secondary followed by APC anti-CD16.

The influence of endothelial cell activation on neutrophil expression of mPr3 and CD177 was also examined. In some experiments HUVECs were pre-treated with IL-1β or TNF-α. Routinely, the histograms for unstimulated control neutrophils from all donors showed a clear bimodal distribution of Pr3 and CD177 compared with the unimodal low expression of the background fluorescence (isotype control). As shown in the representative set of histograms (Fig 4.2) populations of both Pr3 low and high neutrophils were found to transmigrate across HUVECs. No statistical significance was observed in the levels of mPr3 or the ratio of high and low Pr3 populations relative to the control cells, irrespective of stimulation of HUVECs with cytokines. The mean cellular fluorescence (MCF) of Pr3 for unstimulated control cells was 62.74 which increased after fMLP stimulation to 127.35, but the MCF was lower for cells exposed to fMLP that had migrated across the HUVECs to 72.57 and 71.58 (for untreated and TNF-α treated HUVECs, respectively).

Interestingly, in some individuals (n=3) while the migrated cells appeared to retain a bimodal Pr3 expression pattern, a third very low fluorescence neutrophil subset appeared to be absent in the transmigrated cells (Fig 4.3). As these cells were all gated for high CD16 expression, it is unclear what this small sub-population represents.

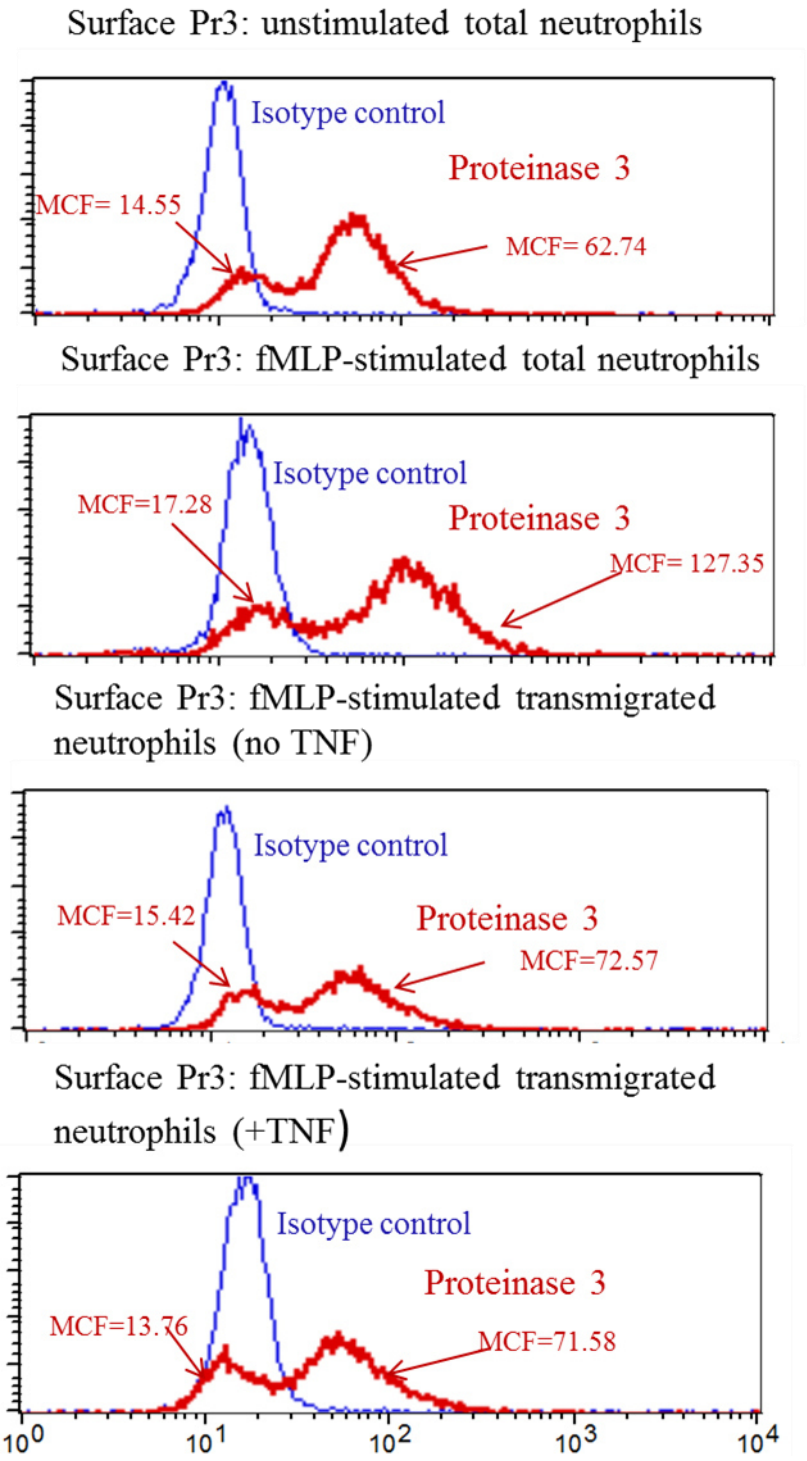


Figure 4.2. Detection of surface Pr3 expression on neutrophils before and after migration. Control cells (unstimulated or stimulated with fMLP) and the cells that migrated through HUVEC (not treated or treated with TNF-α) were labelled with anti-Pr3 mAb and PE-conjugated secondary. Only high expressing CD16 cells were gated for analysis. The isotype control is depicted in each panel as a blue line. Although Pr3 was up-regulated on the cell membrane (mPr3) following stimulation with fMLP (MCF=127.35) before transmigration (control cells) this was only slightly increased (not statistically significant) following transmigration (MCF=72.57 and 71.58).

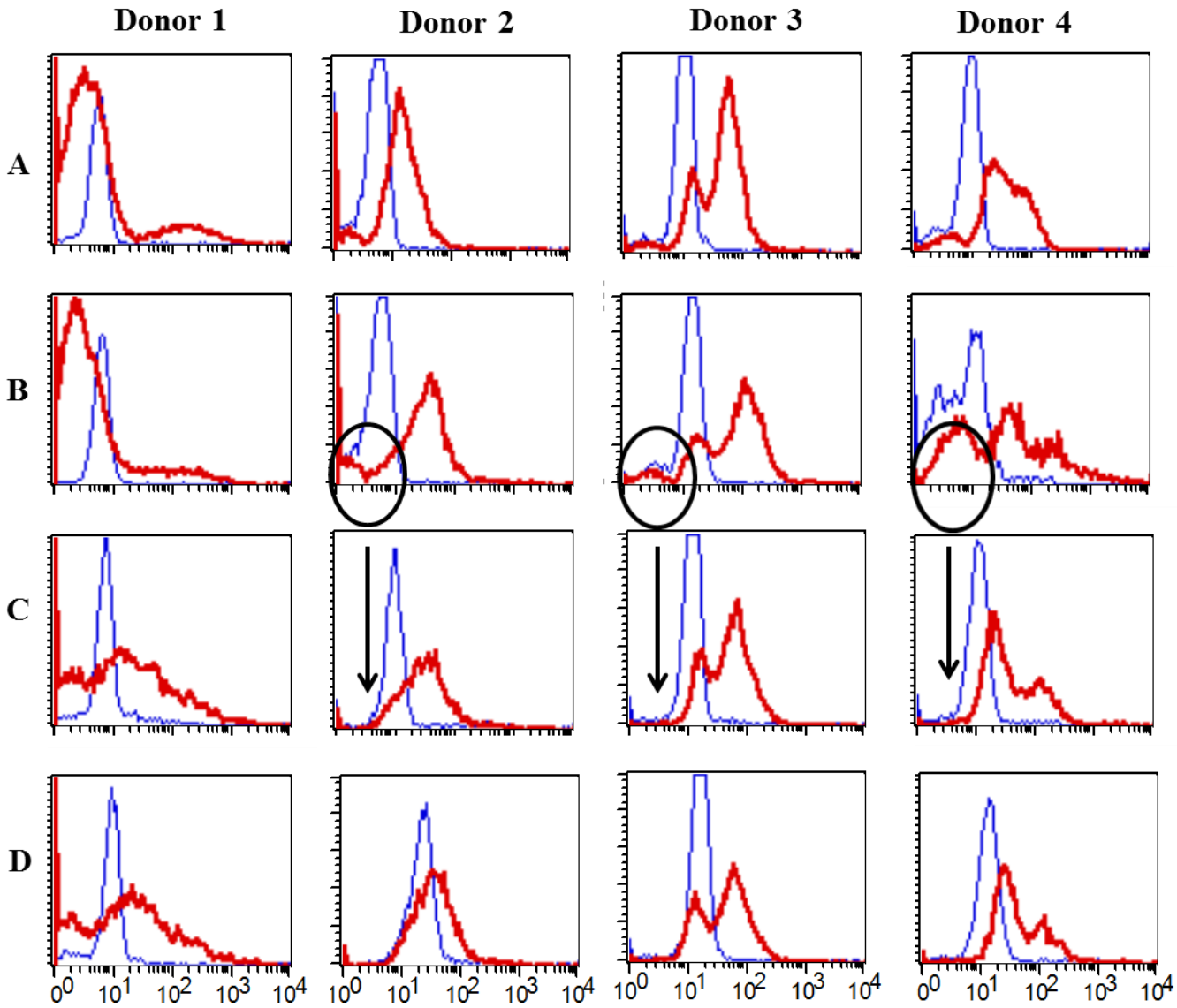


Figure 4.3 Overlay histogram of mean cellular fluorescence showing the expression of Pr3 (red) on unstimulated control cells (A), stimulated control cell with fMLP (B) migrated cells through un-activated HUVECs (C) and migrated cell through TNF-α activated HUVECs compared to the isotype control (blue). Histograms show representative results from four different donors. Circles identify the missing low fluorescent population in the transmigrated cells.

However, the same cells from these individuals expressed both low and high CD177 sub-populations after transmigration, suggesting that $CD177^{low}$ cells are unable to present Pr3 on the neutrophil surface following transmigration (Fig 4.4). Data from all experiments were analysed by converting the MCF to a ratio relative to the levels of Pr3 on the surface of unstimulated cells (Figure 4.5). While, incubation with fMLP alone (no transmigration) resulted in an overall increase of 50% in mPr3 levels, no significant change in transmigrated cells relative to the fMLP control was observed. For this analysis, total expression of mPr3 (i.e. not separated for low and high expression) was utilised as it is difficult to separate low and high populations on unstimulated cells. To determine if the type of cytokine used to pre-stimulate the HUVECs had an effect, pre-treatment with TNF-α and IL-1β were compared (Fig 4.6). As shown in figure 4.6A no alteration was seen in Pr3 expression after neutrophil transmigration through IL-1β treated endothelial cells. All donors showed expression of both high and low Pr3 populations after transmigration. Comparison in Pr3 expression between the neutrophils that migrated through TNF-α treated endothelial cells relative to those that migrated through IL-1β treated cells showed no significant deference (figure 4.6 B). These data confirm no alteration to mPr3 on the surface of neutrophils after migration.

As mentioned previously, CD177 has a variable distribution on the surface of unstimulated neutrophils, ranging from completely absent to uniformly high, but usually is observed as a bimodal mixture of expression. Neutrophils from all donors illustrated both subpopulations of CD177 expression before and after transmigration. To address the question whether CD177-positive (high) and CD177-negative (low) neutrophils behave equally in transmigration, I assessed the size of each neutrophil subpopulation after 2 hours of transmigration by analysing neutrophils after stimulation with fMLP or transmigration through unactivated and TNF-α activated endothelial cells.

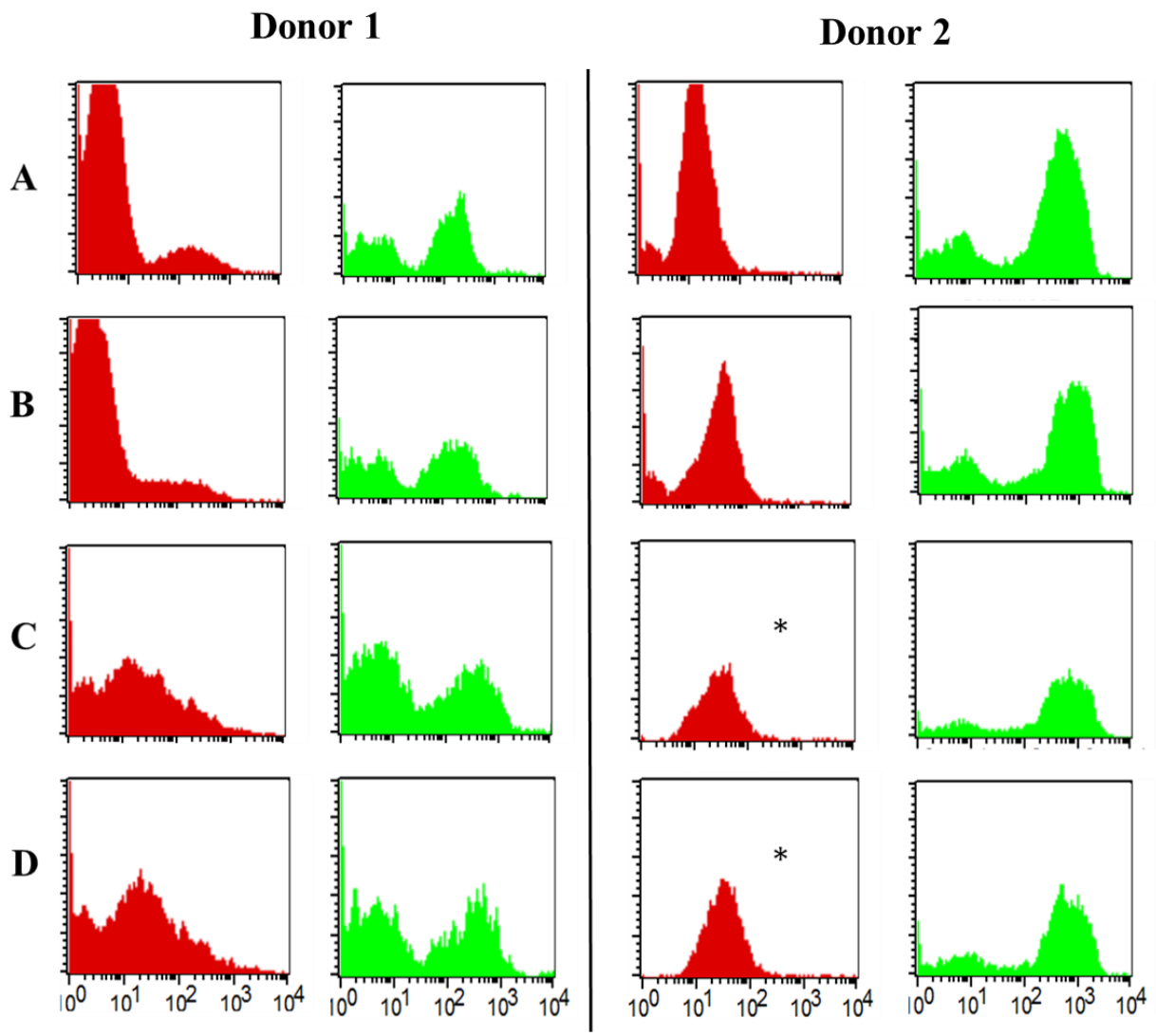


*Figure 4.4 Histograms show the comparison between mPR3 (red) and CD177 (green) expression on neutrophil surface of two different donors. Unstimulated control cells (A), control cells stimulated with fMLP (B), migrated cells in absence of HUVECs activation and migrated cells through activated HUVECs with TNF-α. * denotes the absence of $Pr3^{low}$ population expression on migrated cells of donor 2, but the ratio of high and low CD177 expressing cells remains the same (green).*

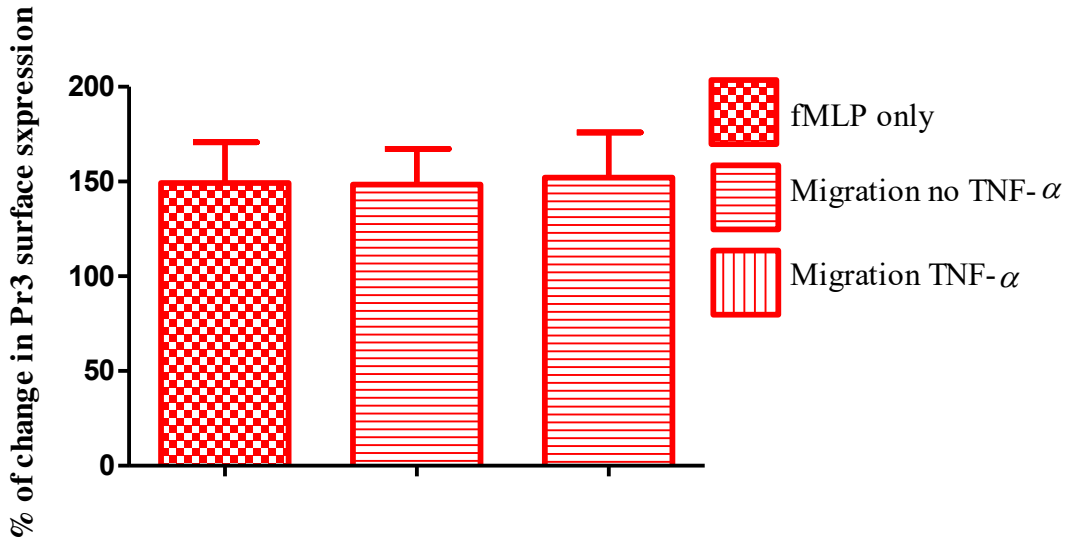


Figure 4.5 Percentage of change in mPr3 expression after stimulation with fMLP and transmigration. HUVEC cultured on transwells inserts (upper chamber) were stimulated with TNF-α (100 ng/ml) overnight and then neutrophils (1×10^5 cells) were added to the upper chamber of the transwell. After 2.5 hour, neutrophils were collected from the lower chambers and analyzed for Pr3 cell surface expression by flow cytometry. No statistically significant difference was detected in the expression of mPr3 after neutrophil stimulation with fMLP or transmigration. These data show alteration to surface Pr3 expression with migration relative to the effects of fMLP alone (n=6).

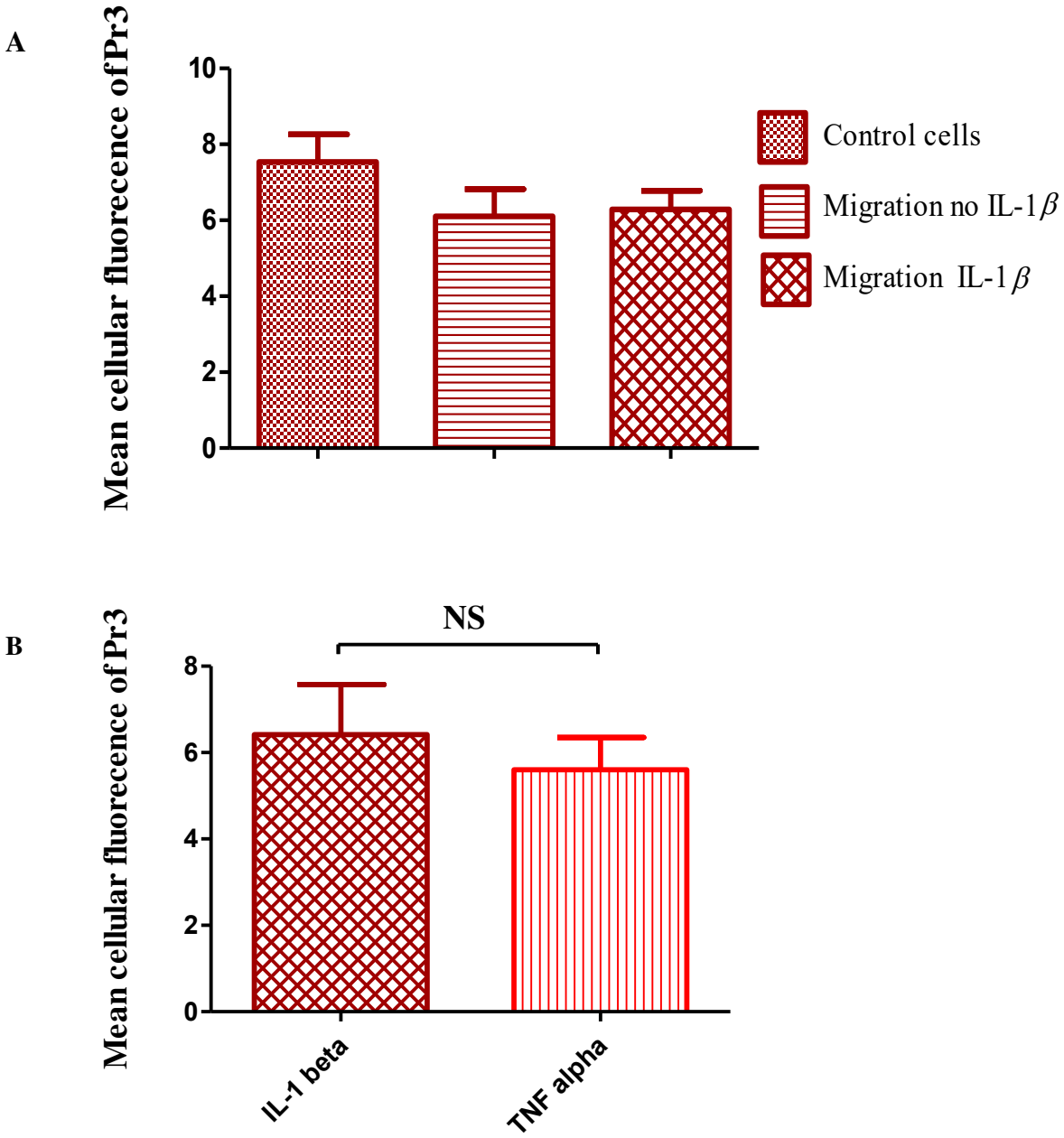


Figure 4.6 Detection of mPr3 on the surface neutrophil following transmigration cross untreated or treated endothelial cells with IL-1β (A). Neutrophils were incubated with HUVEC monolayers for 2.5 hours at 37°C. The data shows no significant change in the expression of mPr3 after neutrophil transmigration. No statistically significant deference detected in the expression of Pr3 between the neutrophil transmigration through TNF-α or IL-1β treated HUVECs (B).

Data from one donor showed nearly 60% of neutrophils were $CD177^{high}$ and 40% were $CD177^{low}$ prior to transmigration and after neutrophil stimulation and transmigration the proportion of both CD177 subpopulations showed no significant change. Moreover only the $CD177^{high}$ subpopulation showed an increase in CD177 levels after stimulation and transmigration (fig 4.7).

The $CD177^{high}$ expression population showed significant increase after stimulation with fMLP in the absence of transmigration (P=0.001) and after transmigration through HUVECs that were untreated or pre-treated with TNF-α (0.003 and P=0.0005, respectively; fig 4.8A). In addition, while the $CD177^{low}$ population appeared to show an increase in CD177 expression, it failed to reach statistical significance. Figure 4.8B shows cells that transmigrated through IL-1β pre-treated HUVECs also illustrated a similar result.

These data indicate that the presence of fMLP only increases CD177 expression, and additional significant increase in CD177 following neutrophil transmigration was observed. Thus, the ability of CD177-mediated neutrophil transmigration via heterophilic interaction with PECAM-1 or other molecules, to enhance the up regulation of CD177 remains unknown. $CD177^{low}$ cells were able to migrate as well as the $CD177^{high}$ cells in these *in vitro* assays. This indicates that $CD177^{high}$ cells have no more advantage for transmigration. Furthermore, this data showed an increase in CD177 expression, but not Pr3, which suggests that transmigration does not induce surface expression of Pr3 and CD177 as complex or that transmigration removes the Pr3 bound to CD177. Finally, the expression of these proteins is not dependent on the HUVECs activation mechanism.

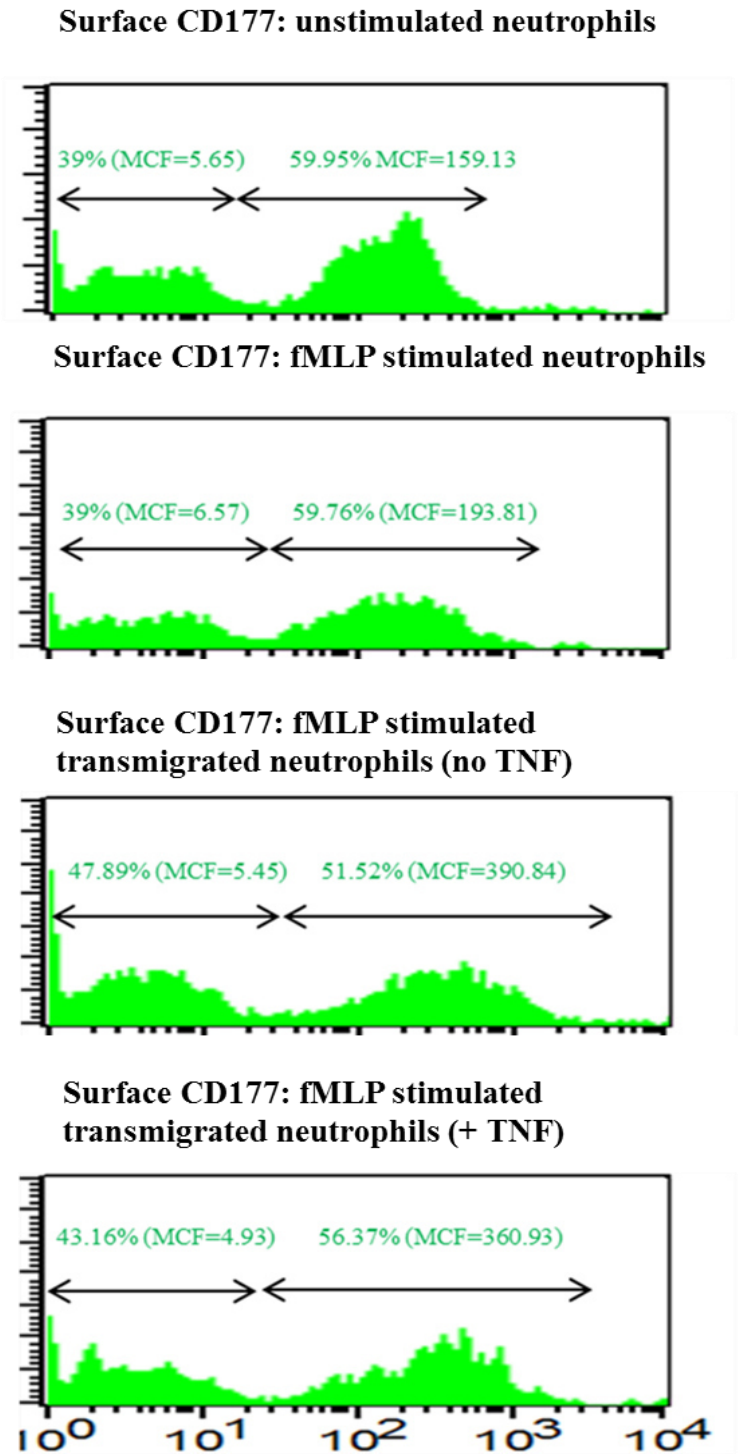


Figure 4.7 Histograms show the size of CD177-positive and negative subpopulations, and the MCF prior and post transmigration from one experiment. Neutrophils were incubated with APC anti-human CD177 antibody and neutrophils were identified in mixed leukocyte populations by gating for high CD16 expressing cells following co-staining with PE-conjugated mouse monoclonal anti-CD16.

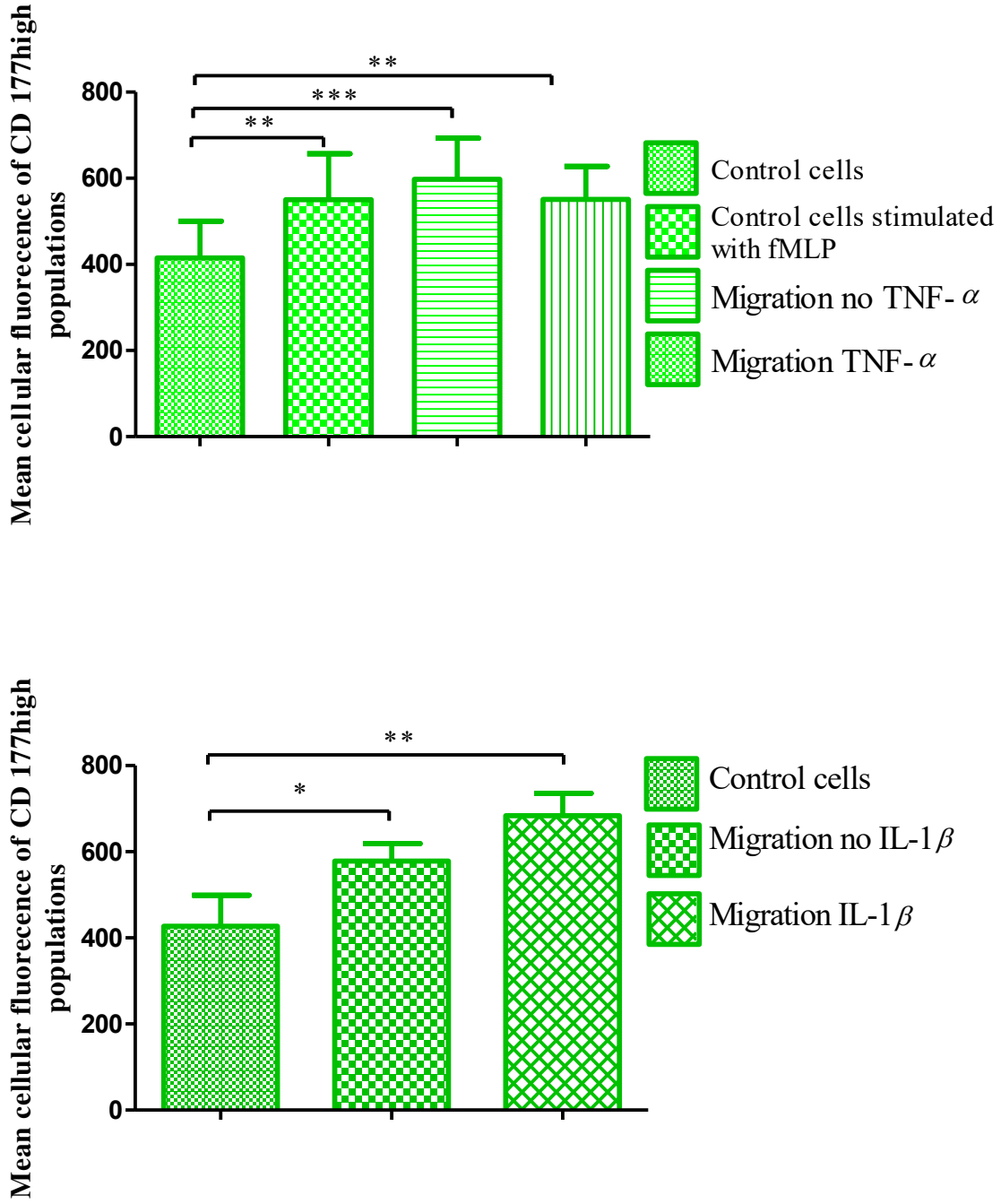


*Figure 4.8 Surface expression of CD177^{high} population prior to and post transmigration. Neutrophil transmigration resulted in a significant increase in the cell surface expression of CD177; however, this could be accounted for by the effect of stimulation with fMLP alone. Data expressed as mean, error bars demonstrate SEM, **=p<0.01; ***=p<0.001 (n=5).*

4.3.3 The surface expression of CD16 prior and after transmigration

As mentioned in chapter 3, neutrophil activation leads to an increase in the expression of many receptors (such as CR1 and CR3) while the CD16 receptor is shed from the cell surface. Therefore, I investigated the level of CD16 before and after transmigration. The obtained result illustrated that stimulating the neutrophils with fMLP caused significant reduction in the expression of CD16, but that no additional loss of CD16 occurred for cells that had undergone transmigration in absence of HUVECs treatment. Figure 4.10 showed that there was more shedding of CD16 expression after neutrophil migration through TNF-α treated HUVECs.

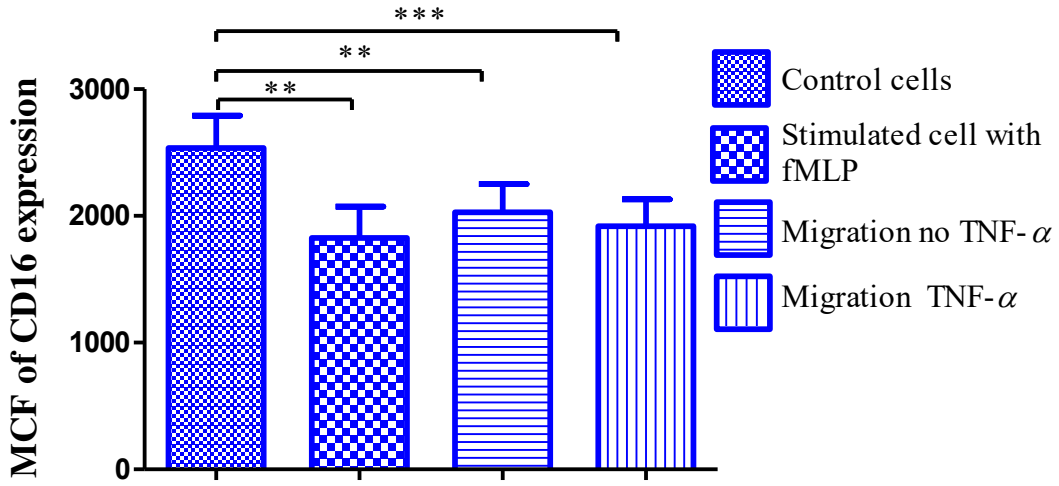


*Figure 4.10 Effect of neutrophil transmigration in CD16 expression as measured by flow cytometry. CD16 expression was measured as mean cellular fluorescence (MCF) on control cells, control cells stimulated with fMLP, as well as cells post migration across untreated HUVECs and HUVECs pre-treated with TNF-α. Expression shown as mean ± SEM **= p <0.01, ***= p<0.001 relative to compared to unstimulated control cells (n=7).*

4.3.4 Change in Pr3, MMP-8 and MMP-9 intracellular levels prior to and after transmigration

Parallel to measurement of extracellular surface Pr3 and CD177 expression, intracellular levels of Pr3, MMP-8 and MMP-9 were also evaluated in control and migrated neutrophils. All neutrophils were fixed and permeabilised as described previously (chapter 2) prior to intracellular antibody staining and assessment by intracellular flow cytometry. Here I investigated if neutrophil transmigration can promote neutrophils to mobilize more intracellular storage and release these proteins. Pr3 intracellular levels from all cells were very high compared to the isotype matched control background staining (fig 4.11A).

No significant change in intracellular levels of Pr3 was observed after stimulation with fMLP compared to unstimulated control cells. In addition, I found that no statistical difference in intracellular Pr3 levels after neutrophil transmigration through TNF-α or IL-1β pre-treated HUVECs despite the slight surface increase in Pr3 after transmigration (fig 4.11 B&C). This observation suggests that transmigration does not influence the release of Pr3. MMP-8 and MMP-9 play several roles in inflammation, including degradation of ECM components and regulation of cytokine activity. To determine the roles of MMP-8 and MMP-9 in neutrophil transmigration, I assessed the intracellular MMP-8 and MMP-9 contents in neutrophils prior to and after transmigration. Figure (4.12 A-D) show the pattern of MMP-9 levels in unstimulated neutrophils, in response to stimulation with fMLP, and after transmigration (both in the absence of HUVEC activation and after HUVEC cell pre-treatment with TNF-α). The data show a reduction in MMP-9 levels after stimulation and transmigration (representative data from one donor shown). When data from all experiments (from 9 experiments) were pooled and analysed, the mean intracellular fluorescence of MMP-9 for unstimulated cells was significantly higher than that of stimulated cells with fMLP (P=0.02 figure 4.E).

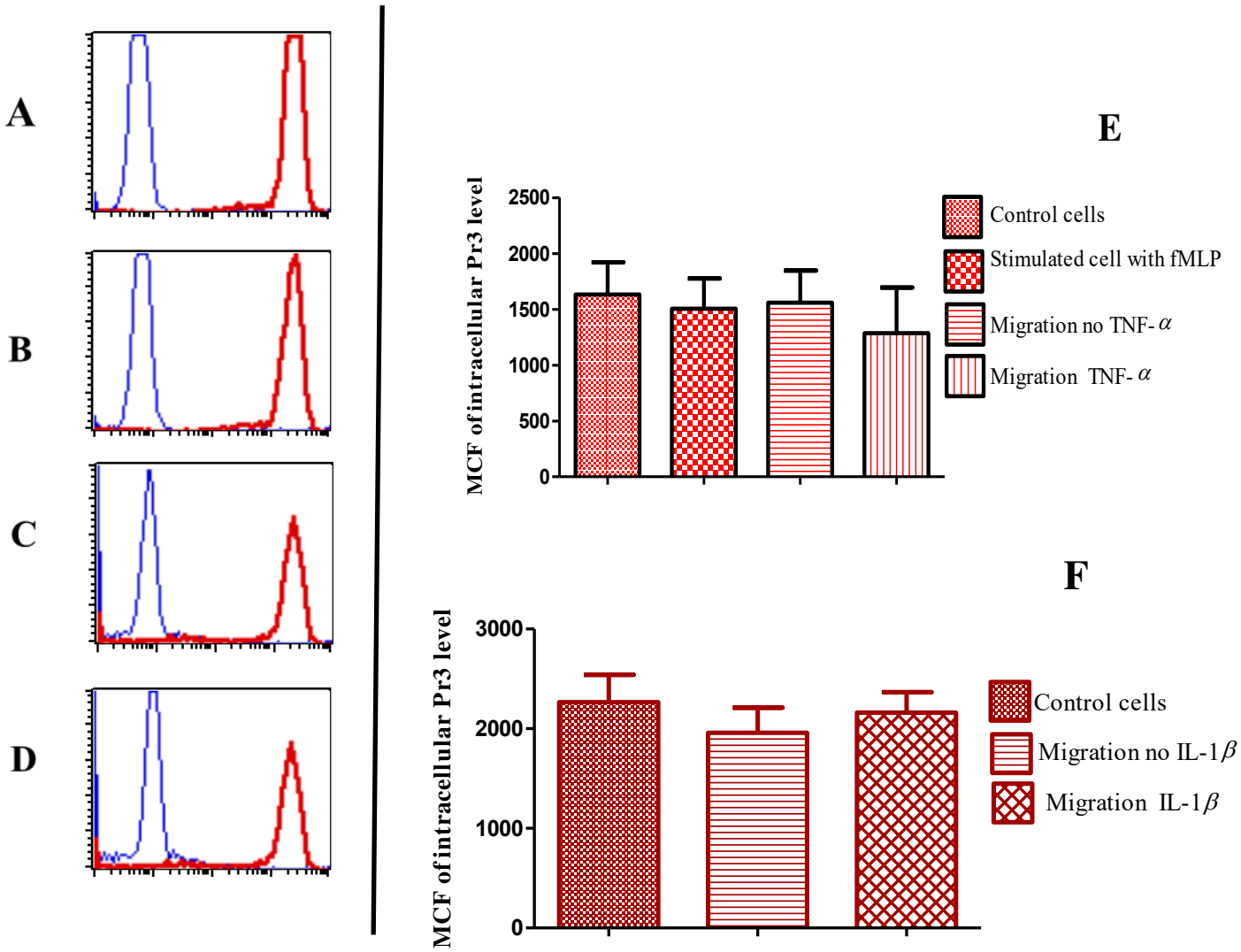


Figure 4.11 Intracellular levels of Pr3. Representative flow cytometry histograms show intracellular expression of unstimulated total neutrophils (A), fMLP-stimulated total neutrophils (B), neutrophils migrating towards fMLP in the absence of TNF-α endothelial cell pre-treatment (C), and fMLP transmigrated neutrophils following TNF-α pre-treatment of endothelial cells (D). Mean cellular fluorescence of Pr3 in control and cells that migrated across untreated or TNF-α (E; n=7) or IL-1β (F; n=4) pre-treated endothelial cells as measured by flow cytometry.

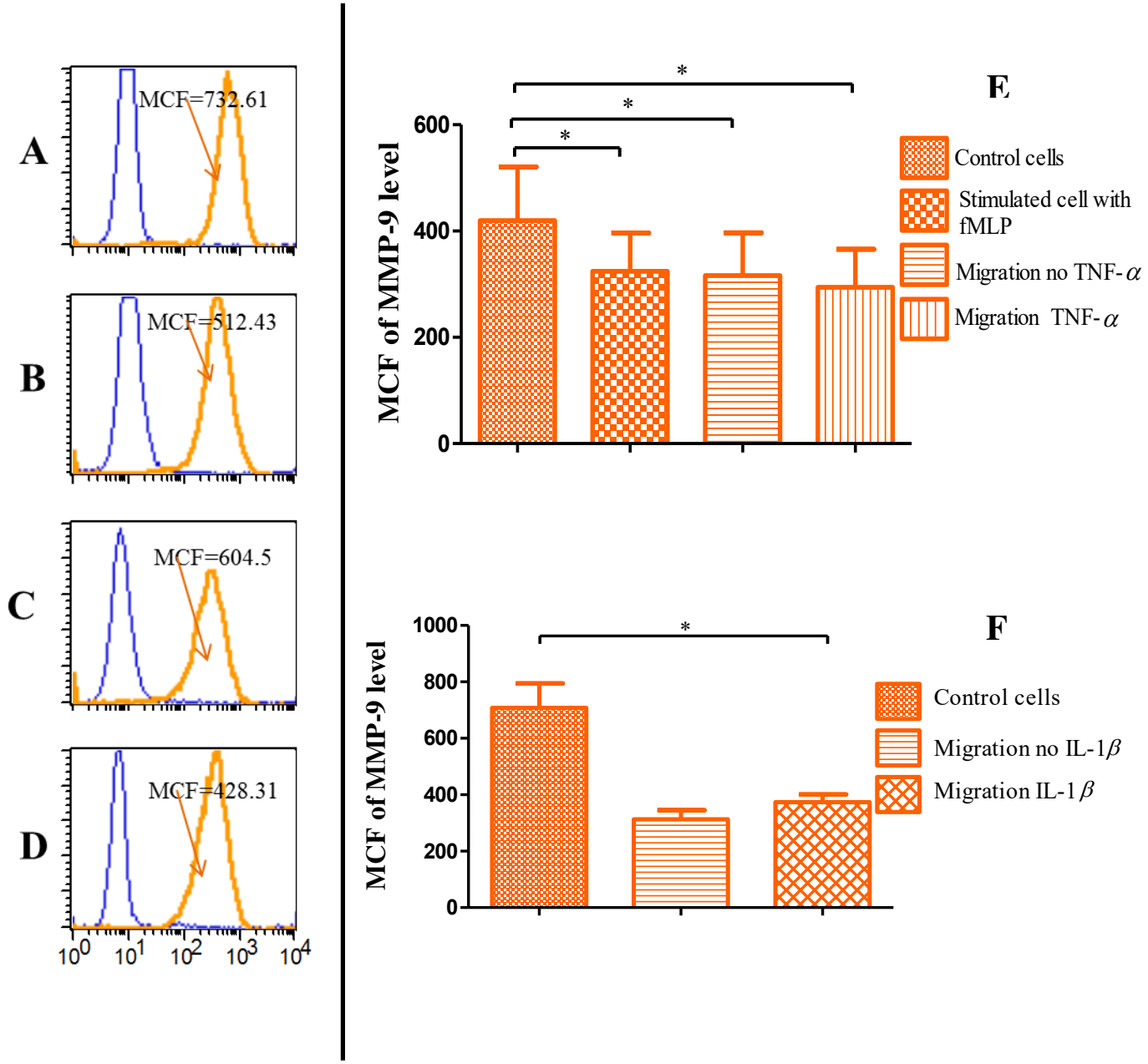


Figure 4.12 Intracellular expression of MMP-9. Representative flow cytometry histograms show intracellular expression of unstimulated total neutrophils (A), fMLP-stimulated total neutrophils (B), neutrophils migrating towards fMLP in the absence of TNF-α endothelial cell pre-treatment (C), and fMLP transmigrated neutrophils following TNF-α pre-treatment of endothelial cells (D). Bar graphs show the MCF of MMP-9 after stimulation and transmigration through TNF-α pre-treated (E; n=8) and IL-1β pre-treated (F; n=3) HUVECs. The data demonstrate a significant decrease in MMP-9 levels after neutrophil transmigration that is accounted for by the effects of fMLP alone ($P < 0.01$).*

Neutrophils that had migrated through untreated or cytokine pre-treated endothelial cells also showed a significant decrease in MMP-9 levels (P=0.03 and 0.01 respectively) relative to the unstimulated cells (figure 4.12 E/F). However, these effects were no greater than those observed for the effects of fMLP alone, indicating no additional release of MMP-9 in response to transmigration.

In contrast, pre- and post-transmigration MMP-8 levels showed no significant difference (fig 4.13 A-F). These results indicate that neutrophils are more prone to release MMP-9, but not MMP-8, in response to fMLP-driven migration across endothelial cells.

To assess the effect of maximum stimulation on MMP-9 and MMP-8 after transmigration, migrated neutrophils were collected and further stimulated with cytochalasin B (cytoB) and fMLP for 15 minutes at 37^{o}C. The results demonstrated a further significant decrease in the level of MMP-9. However, while a release of MMP-8 following maximal stimulation was apparent, it failed to reach statistical significance (p=0.10). However, increasing the number of experiment replicates (n=3 only here) would likely result in the release of MMP-8 in post-migrated neutrophils reaching statistical significance (Fig 4.14)

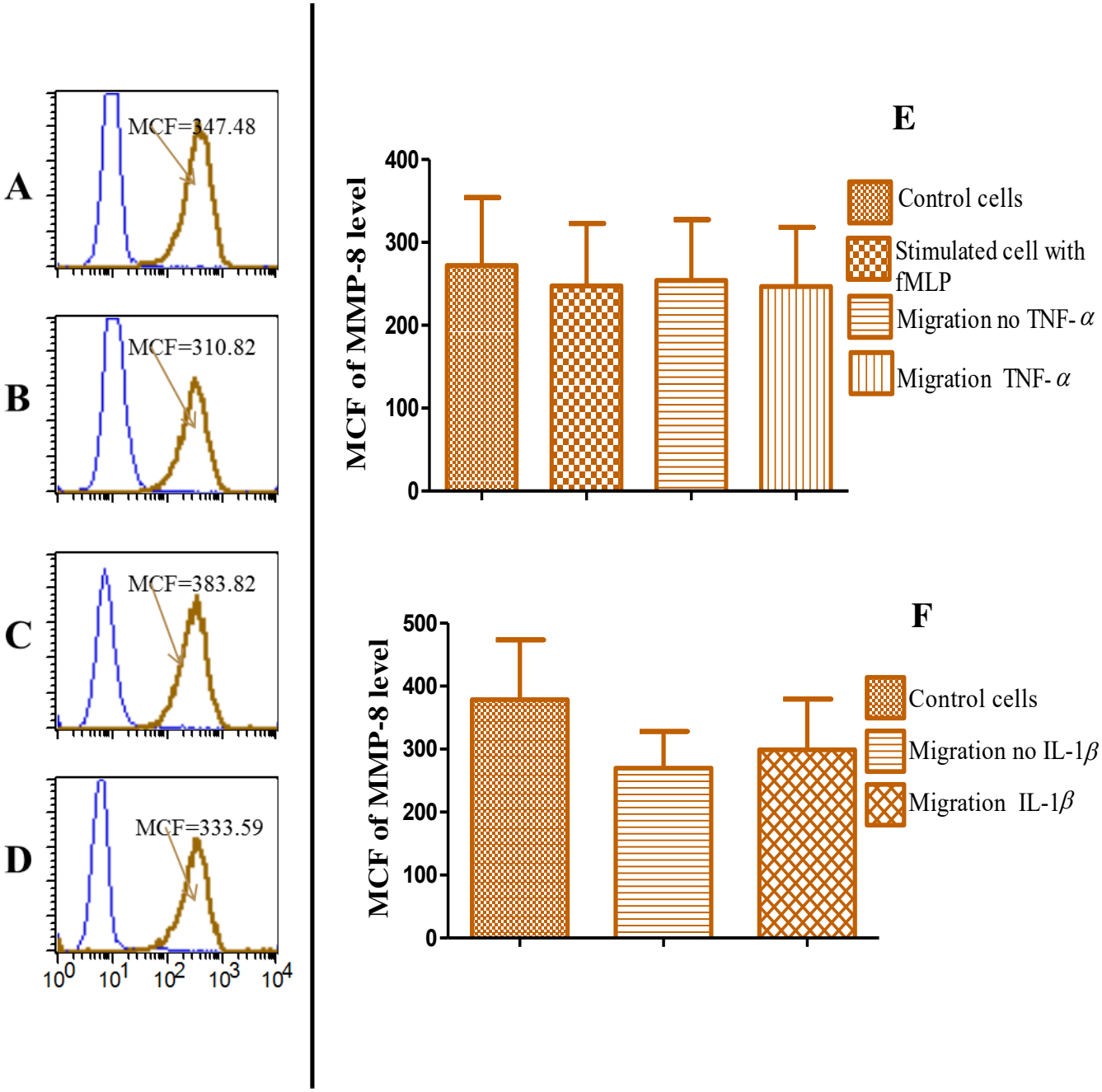


Figure 4.13 Intracellular expression of MMP-8. Representative flow cytometry histograms show intracellular expression of unstimulated total neutrophils (A), fMLP-stimulated total neutrophils (B), fMLP-stimulated transmigrated neutrophils in the absence of TNF-α pre-treatment of endothelial cells (C), and fMLP-stimulated transmigrated neutrophils following TNF-α pre-treatment of endothelial cells (D). Bar graphs show the MCF of MMP-8 after stimulation and transmigration through TNF-α (E; n=8) and IL-1β (F; n=3) activated HUVECs. No statistically significant difference was detected in the expression of MMP-8 after stimulation or transmigration.

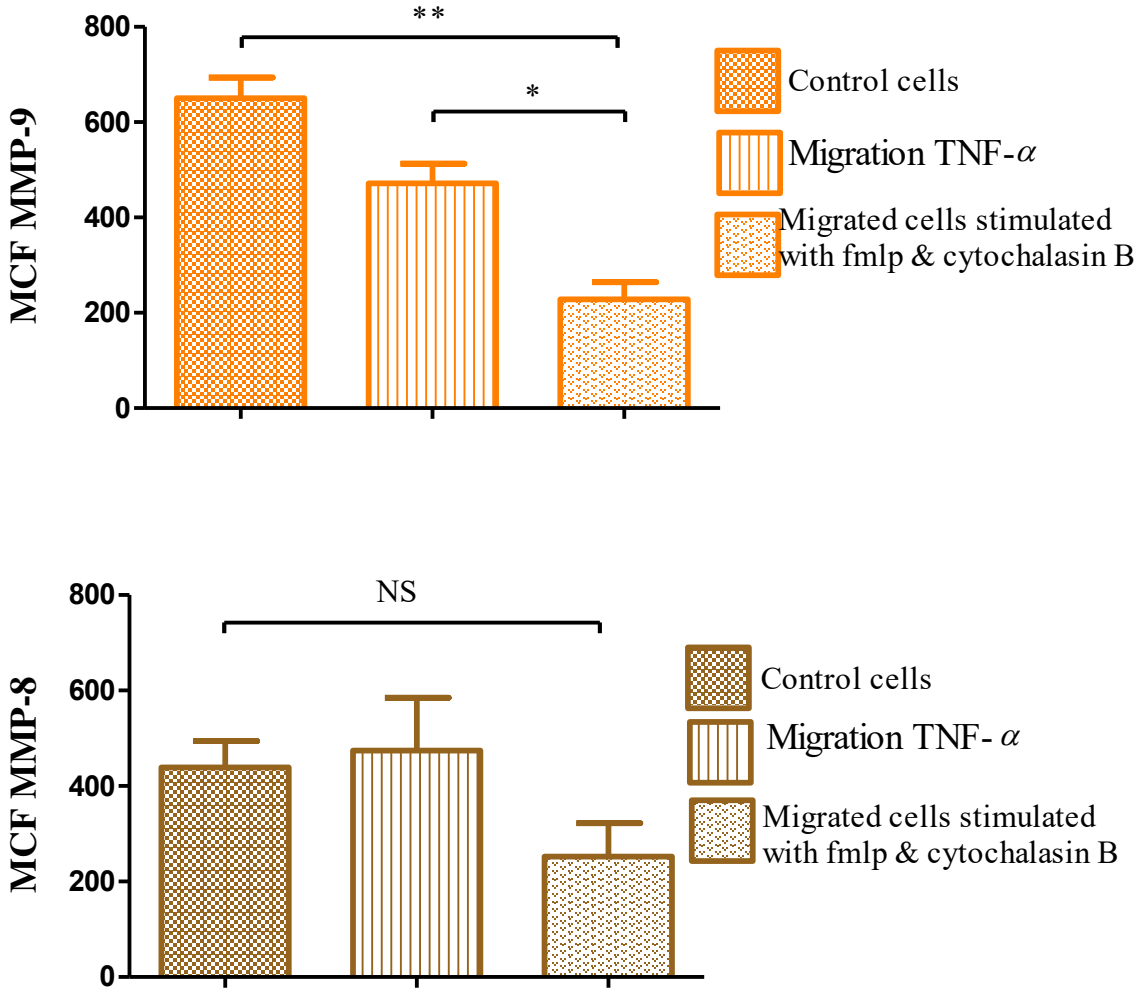


Figure 4.14 Effect of maximum stimulation on the levels of MMP-9 and MMP-8 following migration through endothelial cells. The levels were measured as MCF in unstimulated control cells, neutrophils following migration across unactivated and TNF-α pre-activated HUVECs as well as post-migrated cells further stimulated with cytochalasin B in combination with fMLP (n=3).

4.3.5 Effect of CD177 blocking on neutrophil transendothelial migration

In the present part of this chapter I addressed whether CD177 participates in neutrophil transmigration by using mAb MEM166 specific for CD177. Before being loaded into the upper well of the transwell inserts, neutrophils (1×10^6) were incubated with MEM166 (1µg for 20 minutes) then allowed to migrate through untreated and treated HUVECs in presence or absence of fMLP as a chemoattractant. The number of migrated cells in the lower chamber was then counted using light microscope. As shown in Figure 4.15, a significant inhibition of neutrophil migration was observed with mAb MEM166 (anti-CD177) in presence of the chemoattractant, but in absence of fMLP there was no significant inhibition. However, the numbers of cells migrating in the absence of an fMLP gradient were very low and this may obscure the ability to identify an effect. Thus, where a strong chemoattractant is driving neutrophil transmigration, this data demonstrated that CD177 is important.

To examine if MEM166 was lost on the migrated neutrophils, I examined the amount of cell bound MEM166 with PE-conjugated secondary antibody before and after transmigration. I also examined if I could increase MEM166 binding following transmigration by incubating with more MEM166 prior to incubation with PE-conjugated secondary antibody and analysis by flow cytometery. As shown in histograms in Figure 4.16, addition of MEM to neutrophils before migration through untreated endothelial cells reduced the MCF of MEM166 by 1.6-fold on the surface. This was not different if the neutrophils migrated through TNF-treated endothelial cells. As it is anticipated that exposure to fMLP would upregulate internal pools to the surface of neutrophils, I also examined if coating the neutrophils with MEM166 blocked fMLP-mobilisation of internal CD177 to the surface (Fig. 4.17). In the absence of transmigration, pre-incubation of neutrophils with MEM166 did not block the increased CD177 induced by incubation with fMLP (as detected by a second staining step after fMLP incubation). Therefore, the reduced MEM166 staining following transmigration of MEM166

coated neutrophils must be due to blockade of the highest expressing cells from passage through the endothelia or shedding of the GPI-anchored CD177+MEM166 complexes during migration.

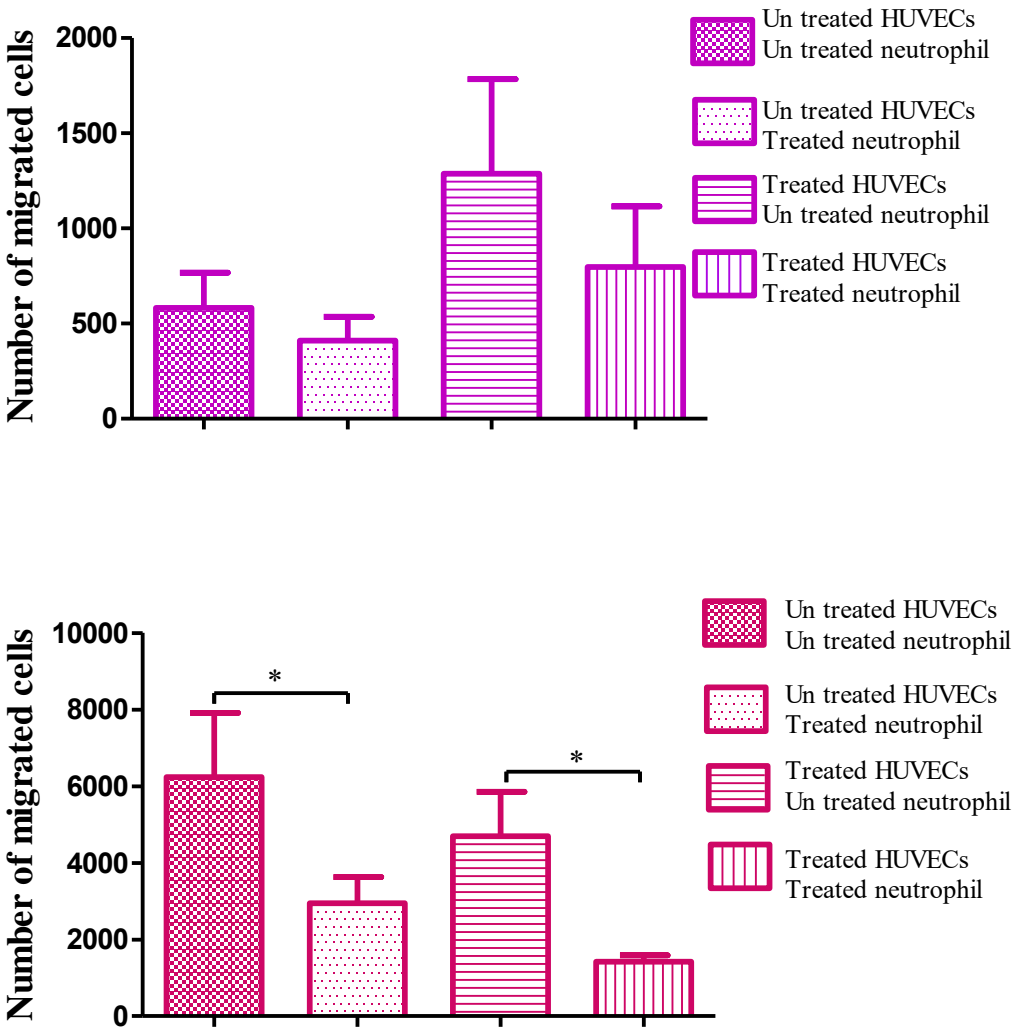


Figure 4.15 Role of CD177 on neutrophil transmigration through HUVECs. HUVECs cultured on transwell inserts were stimulated TNFα (100 ng/ml). One million of neutrophils were pre-incubated (or not for control) with mAb MEM166 specific for CD177 (1µg/ml). Untreated and treated neutrophils were loaded in transwell inserts and left to migrate in presence (bottom panel) and absence (top panel) of fMLP (n=5).

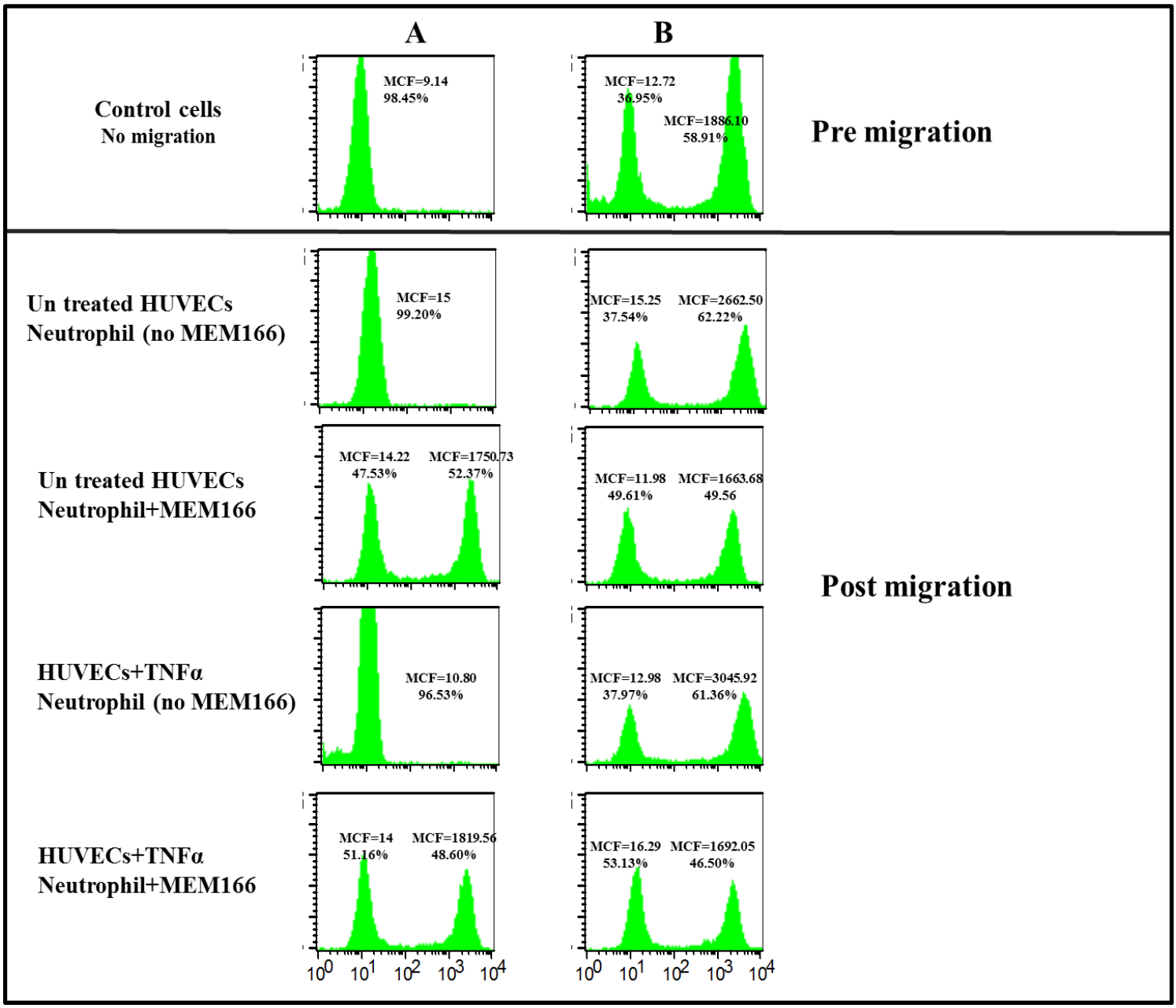


Figure 4.16 Histogram showing anti-CD177 antibody (MEM166) binding during migration inhibition experiments. Neutrophils were pre-treated (or not for control) with MEM166 monoclonal antibody specific for CD177 and allowed to migrate through untreated or TNF-α pre-treated HUVECs. All cells were stained with PE-conjugated secondary antibody for 20 minutes (A) or stained again with MEM166 and detected by PE-conjugated goat anti-mouse immunoglobulins (B).

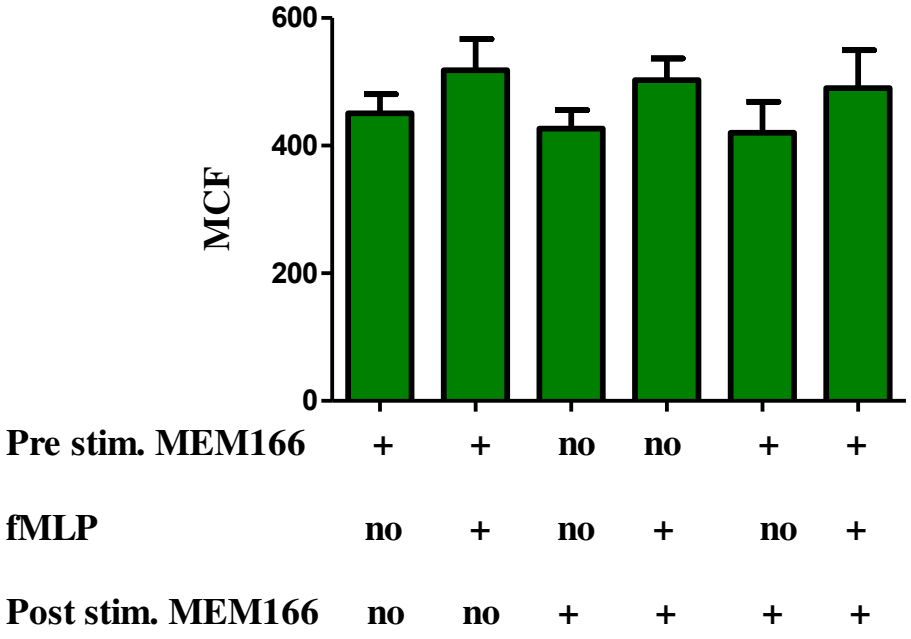


Figure 4.17 The effect of CD177 blocking pre- and post- neutrophil stimulation with fMLP. Unstimulated or stimulated neutrophils were pre-treated (or not) with MEM166 monoclonal antibody specific for CD177. All cells were stained with PE-conjugated secondary antibody for 20 minutes or stained again with MEM166 and detected by PE-conjugated goat anti-mouse immunoglobulins (n=3).

4.4 Discussion

Neutrophil transendothelial migration is a cardinal event in the immune and inflammatory responses. The event involves a series of molecular interactions between neutrophils and endothelial cells. Neutrophils express different sets of proteases such as NSPs and MMPs, which are believed to play an important role during neutrophil transmigration. In this study, I analysed the surface expression of mPr3 and CD177 after neutrophil transmigration and compared the surface expression to pre-migration levels. I also assessed the level of intracellular proteinases (Pr3, MMP-8 and MMP-9) before and following neutrophil transmigration.

NSPs aid the migration of neutrophils by activating endothelium and additionally lead to decreased intracellular connection through degrading several components of the extracellular matrix. NE a molecule closely related to Pr3 can cleave matrix proteins and is found at the leading edge of migrating neutrophils (Cepinskas *et al.,* 1997; Wang *et al.,* 2006). NE may also play indirect roles in facilitating neutrophil transmigration; for instance, via activation of other neutrophil proteases such as MMP-9 (Delclaux et al., 1996).

NE inhibitors have been shown to impede neutrophil migration (Delclaux *et al.,* 1996; Delacourt *et al.,* 2002). However, a number of studies have also indicated that inhibition or an inherent lack of NE have no effect on neutrophil transmigration (Huber and Weiss, 1989; Furie et al., 1987; Rosengren and Arfors, 1990; Mackarel et al., 1999; Allport *et al.,* 2002; Hirche *et al.,* 2004).

Pr3 also can digest several substrates, such as elastin, proteoglycans, IgG, fibronectin, laminin, vitronectin, and collagen type IV (Kao *et al.,* 1988; Dolman *et al.,* 1995; Rao *et al.,* 1991). Zen et al., 2011 indicated that Pr3 can cleave CD11b, thus it may be important in promoting neutrophil release from endothelial cell adhesion proteins during transmigration.

PR3 may also participate in enhancing IL-8 production by ECs, which additionally acts as a strong chemotactic factor and activates neutrophils (Berger et al., 1996). Pr3 and NE have also been shown to inactivate progranulin (PGRN), a molecule with anti-inflammatory properties (Kessenbrock *et al.,* 2008). Furthermore, EC express protease-activated receptors (PAR; a family associated with regulating vascular permeability), which are thought to be activated by Pr3. A study by Kuckleburg and Newman, (2013) showed that Pr3 is able to significantly enhance endothelial cell barrier function through a PAR-2-dependent pathway. In addition, they showed that Pr3 induced a sustained endothelial cell calcium signalling, while at the same time inhibiting the permeability changes and disruption of endothelial cell junctional proteins induced by PAR-1 agonists. Pr3 has also been reported to enhance the production of monocyte chemoattractant protein-1 (MCP-1) by HUVECs in a dose- and time-dependent manner, thus providing chemotactic and activating stimuli for both neutrophils and monocytes (Taekema-Roelvink *et al.,* 2001).

Based on the expression of membrane-bound proteinase 3 (mPr3), two subsets of neutrophils can be identified before neutrophil transmigration: neutrophils that hardly express surface-bound Pr3 ($mPr3^{low}$ neutrophils) and neutrophils that express high level of Pr3 ($mPr3^{high}$ neutrophils).

I have found that both mPr3 subsets from all donors are able to migrate across endothelial cells, with the exception of three donors where a very low mPr3 subset was excluded from the lower chamber following transmigration (Figure4.3). In addition, I found no significant change in Pr3 expression after neutrophil transmigration through cytokine (TNF-α or IL-1β) pre-treated or untreated endothelial cells. Pr3 is unique from other neutrophil serine proteases in that it is highly expressed on the surface of neutrophils via its interaction with a high affinity receptor such as CD177. CD177 has additionally been shown to be a high-affinity

heterophilic binding partner for endothelial cell PECAM-1, involved in mediating leukocyte transmigration (Sachs UJ *et al.,* 2007).

Kuckleburg *et al* (2012) reported that Pr3 expression and activity were significantly increased on CD177-positive neutrophils following transmigration, while neutrophils lacking CD177 demonstrated no increase in Pr3. CD177-positive neutrophils were also reported to transmigrate more readily than CD177-negative neutrophils from the same individual (Sachs UJ *et al.,* 2007). This is in contrast to my findings where all CD177 subsets were able to migrate, which suggests that a compensatory mechanism may exist in CD177-negative cells that adjusts for the absence of CD177. Moreover, the use of fMLP as a chemoattractant increased the levels of CD177 exclusive of transmigration, and neutrophils were found to cross HUVEC cells poorly in the absence of a chemotactic gradient. Therefore, it is not possible to determine if the process of transmigration actively increases the expression of CD177 on $CD177^{high}$. However, it would be expected that increased CD177 expression could enhance transmigration through interaction with other molecules such as PECAM, but that this process may be inhibited by CD177 binding by Pr3.

CD177-null individuals have been reported not to have impaired transmigration compared to normal individuals (Kuckleburg *et al.,* 2012). Furthermore, a study by Pliyev and Menshikov (2012) to investigate the binding of neutrophils to PECAM-1 expressing platelets showed no difference in neutrophil-platelet conjugation formation when examining CD177-negative and CD177-expressing neutrophils (Pliyev and Menshikov 2012).

Consistent with the results reported by others, I found blocking antibodies against CD177 significantly inhibited neutrophil transmigration across endothelial monolayers (Sachs *et al.,* 2007; Kuckleburg *et al* 2012). Surprisingly, I found the $CD177^{high}$ neutrophils were able to migrate across the HUVEC cell layer despite being coated with MEM166 antibody and that

no significant loss of MEM166 occurred during the transmigration. There was some effect of the MEM166 in that the ratio of $CD177^{low}$ cells increased when cells were pre-stained with MEM166, compared to parallel transmigrations where neutrophils were not labelled with MEM166. Obviously, I have not been able to determine if the epitope on CD177 that is bound by MEM166 is crucial to either Pr3 binding or to PECAM-1 binding, but one would expect that the steric hindrance associated with the high number of MEM166 on the surface would have a greater impact than that observed. Furthermore, it is interesting that while low levels of neutrophils migrated in the absence of fMLP gradient, it is unexpected that ANY neutrophils so highly coated with MEM166 would engage in transmigration across HUVECs.

Activation of neutrophils is known to induce CD16 shedding from neutrophils membrane via proteolytic shedding (Middelhoven *et al.,* 2001). Expression of CD16 has also been reported reduced at the surface of PMNs upon transmigration (Hofman et al., 2000). Similarly, I found that stimulating the neutrophil with fMLP and neutrophil transmigration prompted CD16 shedding (Tosi and Berger 1988; Hofman *et al.,* 2000).

In addition to NSP, neutrophils contain MMPs, such as MMP-9 and MMP-8; where MMP-8 is stored in specific granules and MMP-9 is stored in tertiary granules. MMPs are a family of Zn-dependent endopeptidases that regulate the release of proinflammatory cytokines TNF-α , interleukin (IL)-1β, the expression of adhesion molecules (CD31, β4-integrin, E-cadherin) and facilitate leukocyte extravasation by destroying components of the extracellular matrix (ECM) during an inflammatory response (Gearing *et al.,* 1994; Schönbeck *et al.,* 1998; Noe *et al.,* 2001; von Bredow et al., 1997). The majority of MMPs are synthesized and secreted as proenzymes, and their proteolytic activation occurs in the pericellular and extracellular space. Therefore one of the goals of this part in chapter 4 was to determine the effect of transmigration on the intracellular levels of MMP-8 and MMP-9 during the process of neutrophil transmigration.

Michelle *et al.* 2008 showed using a model of corneal inflammation that MMP-8 contributed to neutrophil migration through the dense collagenous ECM of the corneal stroma by generating chemotactic Pro-Gly-Pro (PGP) fragments during inflammation (Lin M *et al.,* 2008). The main substrates of MMP-8 are collagens I—III, VII, X, gelatin, proteoglycans, bradykinin, angiotensin-1, fibrinogen, substance P and aggrecan. In addition, MMP-8 can regulate inflammation by cleaving and activating chemokines (e.g. LIX) and it can inactivate alpha-2-macroglobulin (α2-MG) and the serpin proteinase, AAT which leads to increased serine proteinase activity (Owen et al., 2004; Sternlicht and Werb 2001).

As well as neutrophils, there are also other cellular sources of MMP-8 indicating that the regulation of tissue destruction by MMP-8 is more complicated than previously thought. Neutrophil-derived MMP-8 differs from interstitial collagenases expressed by other cells in that it is not synthesized *de novo* by mature PMN (Owen *et al.,* 2004).

In this work, I did not detect any significant difference in intracellular neutrophil MMP-8 levels after neutrophil transmigration through untreated or cytokine pre-treated HUVECs. This suggests that the neutrophil-derived MMP-8 does not play significant role during neutrophil transmigration.

MMP-9 is one of two major gelatinases in the MMP family, also known as gelatinase B and 92-kDa type IV collagenase. It has the ability to degrade ECM components such as collagens and elastins (Min *et al.,* 2002). MMP-9 is released from human neutrophils after stimulation with several mediators, including fMLP, TNF-α, C5a, and IL-8 (Chakrabarti and Patel 2005b; Chakrabarti *et al.,* 2006). Cell adhesion to the extracellular matrix is an additionally recognized stimulus for secretion of pro-MMP-9 and other MMPs. Ligation of selectin L and integrin CD11b/ CD18 provides stimulatory signals to neutrophils which induce secretion of MMP-9 that may facilitate their transmigration into sites of inflammation (Wize et al., 2008).

The binding of neutrophils to Thy-1 (expressed on activated EC) has been reported to stimulate the secretion and activation of MMP-9, resulting in increased transmigration through a basement membrane barrier. MMP-9 expression has been associated to various pathological conditions that incur disruption of the basement membrane, such as tumor invasion, arthritis, multiple sclerosis, systemic lupus erythematosus, and traumatic brain injury (Egeblad and Werb 2002; Tchetverikov *et al.,* 2003; Leppert *et al.,* 2008; Faber-Elmann *et al.,* 2002; Rylski *et al.,* 2008).

It has been hypothesised that MMP-9 plays a key role in neutrophil transmigration to infection and inflammatory sites through extracellular matrices (Delclaux *et al.,* 1996; Kolaczkowska *et al.,* 2006). MMP-9 is involved in the early recruitment steps of neutrophils and CD4+T cells, promotes the process of their transendothelial migration during hepatic ischemia-reperfusion (I/R) injury, and is required for motility of interstitially migrating leukocytes (Khandoga *et al.,* 2006). Leukocyte traffic and cytokine expression were markedly impaired in the liver of MMP-9 deficient animals and in the liver of mice treated with anti-MMP-9 antibody after (I/R) injury; however, initiation of the endothelial adhesion cascades was similar in both MMP-9 deficient and control animals (Hamada *et al.,* 2008).

My findings demonstrate that the intracellular level of human MMP-9 was reduced significantly after neutrophil transmigration through endothelial cells. However, this could be accounted for by the direct effects of fMLP used in the chemotactic gradient. I could not find any additional reduction comparing transmigrated and non-transmigrated cells exposed to fMLP for the same length of time. In addition, maximally stimulating the migrated cells with a combination of fMLP and cytochalasin B after transmigration caused a further significant reduction in the intracellular level of MMP-9 compared to control neutrophils. This indicates that not all mobilisable pools were utilised, nor were the signalling pathways required for degranulation disrupted by the pre-exposure to fMLP alone or migration across the HUVEC

cells. This confirms that MMP-9 release would be available to fight microbial infections *in vivo* following extravasation of neutrophils.

Nevertheless, MMP-9 is not essential to transmigration as previous studies have indicated that MMP-9-deficient (or NE-deficient) mice have no impairment in transendothelial migration *in vivo* or *in vitro* under flow conditions (Betsuyaku *et al.,* 1999; Allport *et al.,* 2002).

4.5 Summary

The findings indicate that transendothelial migration alone does not result in a significant increase in Pr3 expression. Moreover, all CD177-positive and negative neutrophils appear equally able to migrate through the endothelial monolayer. Neutrophils with surface Pr3-bound to CD177 do not have an advantage for transmigration, as no enrichment for $CD177^{high}$ or $mPr3^{high}$ cells was seen post-migration for either unstimulated and TNF-α or IL-1β pre-treated endothelia. However, neutrophils were more prone to release a proportion, but not all, of their MMP-9 stores, but none of their MMP-8 stores, during migration through the endothelial monolayer. This is consistent with the observation that tertiary granules have the lowest stimulation threshold for release.

CHAPTER 5

EFFECT OF EXTRAVASATION *IN VIVO*-ORAL NEUTROPHILS

5.1 Introduction

In the previous chapter, I examined the transmigration of neutrophils using transwell assay inserts. In this chapter, I compare the circulating neutrophils of volunteers to the neutrophils in the oral cavity of the same individuals. This provides two populations of neutrophils one circulatory and the other having transmigrated across the endothelial barrier. It is thus a model of inflammatory transmigration *in vivo*.

Peripheral blood neutrophils (bPMNs) and salivary neutrophils (sPMNs) are important cells that play an essential role in immunity and inflammation. Oral fluid is composed of saliva which contains not only constituents derived from the salivary glands but also other fluids for instance crevicular fluid (originating from crevicular sulci, i.e. the area between tooth and marginal gingiva) and the cells originating from oropharyngeal mucosa and/or the gingival crevice (epithelial cells, erythrocytes and leukocytes) (Vidović *et al.,* 2011).

The human oral fluid has a constant bacterial presence that is kept under control, in part, by a continual influx of neutrophils from the surrounding periodontal tissues (Bender *et al.,* 2006). The majority of salivary leucocytes were found to be neutrophils that enter the oral cavity through the gingival crevice (Lantzman and Michman 1970; Bender *et al.,* 2006). The human oral junctional epithelium is never sterile meaning that, even with optimal plaque control, neutrophils will still be stimulated to exit the gingival microvasculature, enter the periodontal tissues and, subsequently, migrate firstly toward endogenous, epithelial- (such as IL-8 and IL-1β) and serum-derived (plaque activated C5a) chemoattractants then preferentially toward exogenous chemotactic signals (such as LPS and fMLP) produced by plaque bacteria in the gingival crevice (Scott and Krauss 2012).

In the oral cavity, sPMNs have a major role against invading oral microorganisms such as *C. albicans* (Gasparoto *et al.,* 2009). In addition, sPMNs may play a crucial defence function

against periodontal diseases and oral ulceration such as oral lichen planus (Scully and Wilkinson 1985; Mizukawa *et al.,* 1999). High numbers of sPMNs constantly migrate from the bloodstream through the gingival crevice into the oral cavity (Schiött and Löe 1970; Ashkenazi and Dennison *et al.,* 1989). The main reason is to protect the oral environment from pathogens. The significant portion of the inflammatory mediated destruction of the tooth supporting tissues (periodontium) arises as a consequence of collateral damage initiated by the enzymes secreted by hyperactive neutrophils as they attempt to control the bacterial infection (Gangbar *et al.,* 1990; Lee *et al.,* 1995).

5.2 Aims

The aim of this chapter was to determine the neutrophil surface expression of CD177, mPr3, CD63, and CD16 and intracellular levels of Pr3, MMP-8 and MMP-9 in oral and circulatory neutrophils. This will allow a comparison to be made between neutrophils before and after extravasation. In addition, these data will be compared with those found in the in vitro system in the previous chapter.

5.3 Results

5.3.1 Level of Pr3 and CD77 Expression on bPMNs and sPMNs

bPMNs and sPMNs were collected from healthy donors as described in chapter 2 (section 2.1.1.2.2 and 2.1.2). The cells were purified, counted and then separated into unstimulated and maximal stimulation (cytochalasin B in combination with fMLP) groups. Flow cytometry was then employed with monoclonal antibodies to examine surface CD177 and Pr3 expression.

I have previously elaborated on the normal variation in the percentage of Pr3 and CD177 expression on the bPMNs surface amongst individuals ranging from 0-100%. The results obtained from this study showed that unlike the bimodal distribution of surface Pr3 and CD177 expression on unstimulated bPMNs in a given individual, all sPMNs expressed CD177 and Pr3 (Figure 5.1).

In addition the surface expression of Pr3 on sPMNs increased after stimulation (approaching statistical significance; P=0.10). Similarly, Pr3 on bPMNs also increased after stimulation (p=0.02). The levels of surface Pr3 on unstimulated sPMNs was, however, significantly higher than that of unstimulated bPMNs (P<0.0001 by unpaired t test). However, comparison of the surface Pr3 levels between these two groups following stimulation showed no significant difference following the increase of surface Pr3 on bPMNs under these conditions (figure 5.2). The histogram for isolated bPMNs and sPMNs showed a clear bimodal distribution of CD177 in the absence and following stimulation, and bPMNs showed increase in the expression after stimulation (figure 5.1).

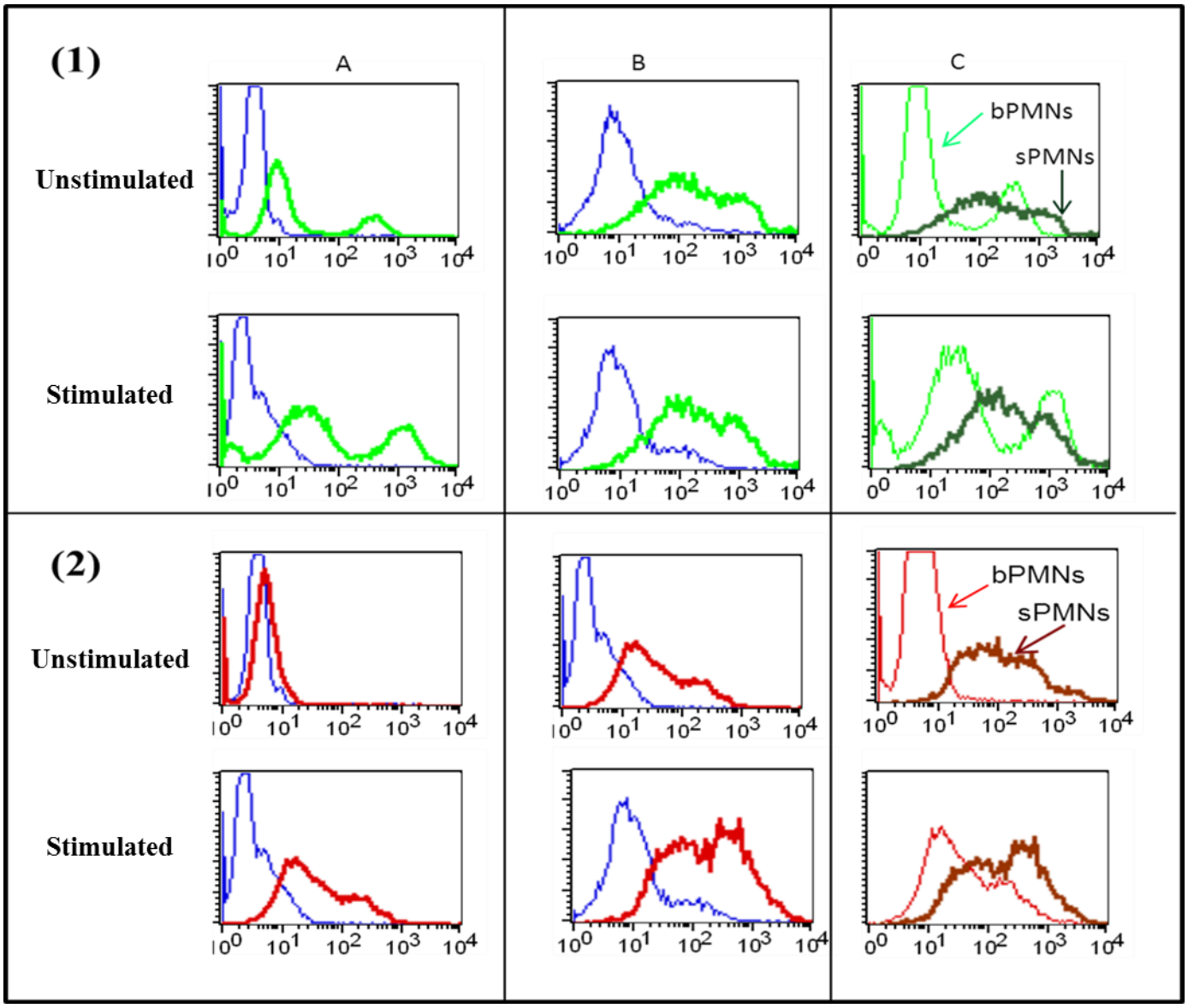


Figure 5.1 Representative histograms showing the expression of CD177 (1) and Pr3 (2) on the surface of unstimulated and stimulated bPMNs (A) and sPMNs (B) compared to the isotype control (blue line). Neutrophils were incubated with un-conjugated anti-CD177 (MEM166) or anti-Pr3 (G2) mAbs and after washing bound mAbs were detected with PE-conjugated goat anti-mouse antibody (rPE-GAM). Overlay histograms (C) illustrate sPMNs expressed only positive populations of CD177 and Pr3 whereas the expression on bPMNs ranged from 0 to 100% in a given individual.

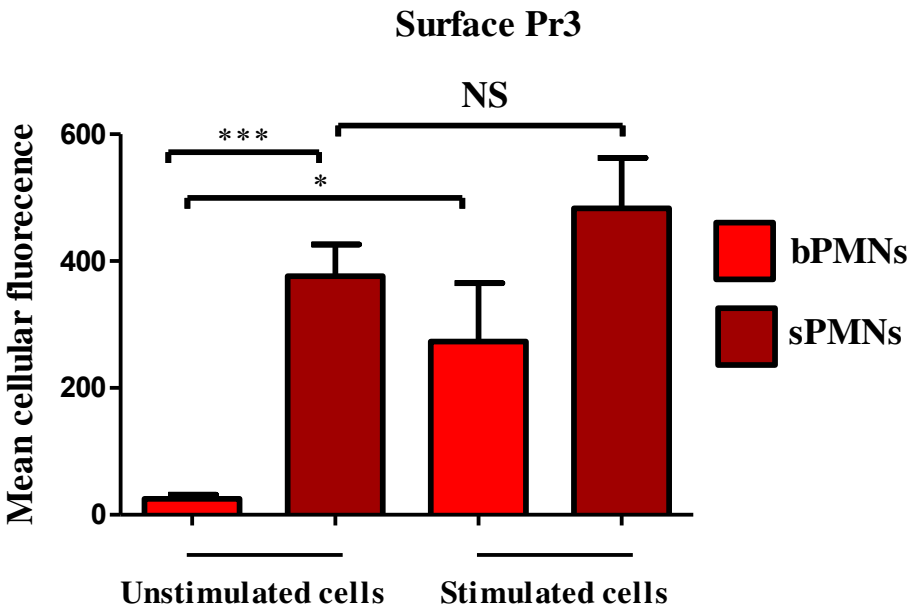


*Figure 5.2 Detection of surface Pr3 expression on matched neutrophils obtained from the saliva (sPMNs) and peripheral blood (bPMNs). Data expressed as mean, error bars demonstrate SEM, *= p< 0.05; ***=p<0.001(n=9).*

Stimulation of bPMNs caused a significant increase (P=0.0132) in the expression of CD177 protein on the $CD177^{high}$ sub-population of neutrophils (Figure 5.3); however, there was only a small increase in CD177on the $CD177^{low}$ sub-population which failed to achieve statistical significance. Stimulation of sPMNs illustrated no significant change in CD177 expression on both subsets. Similar to the Pr3 results, stimulation of the bPMNs resulted in high levels of surface CD177 that were not significantly different from the levels on unstimulated or stimulated sPMN levels for the $CD177^{high}$ subpopulation. However, the Pr3 levels on the surface of the $CD177^{low}$ subpopulation failed to achieve the levels seen on the unstimulated sPMNs and was significantly lower than the levels of stimulated sPMNs (P=0.0011), suggesting the $CD177^{low}$ subpopulation of sPMNs does not derive from the $CD177^{low}$ subpopulation of bPMNs. The scatter plots in figure 5.4 illustrate the surface expression of CD177 and Pr3.

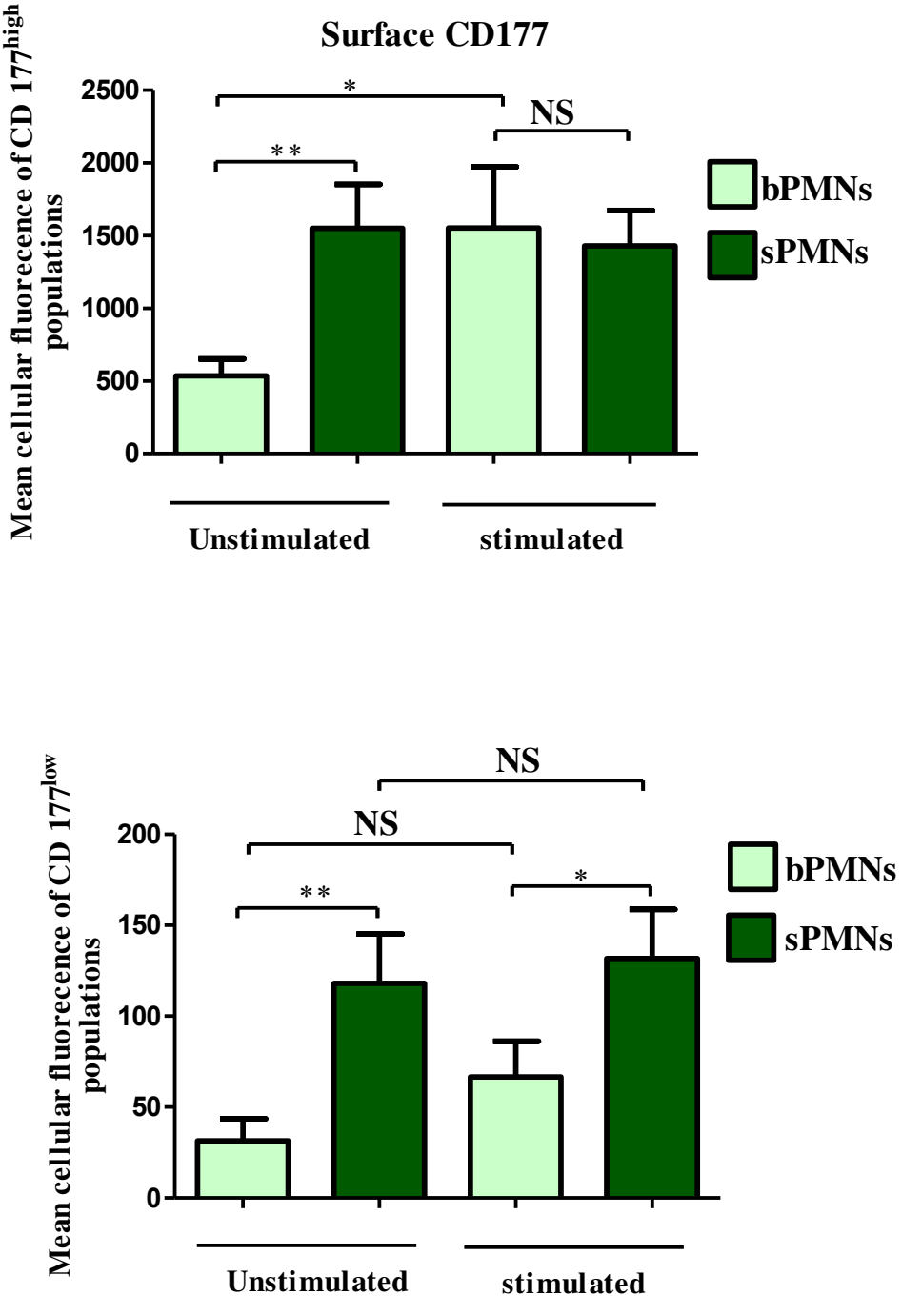


*Figure 5.3 Surface expression of CD177 on unstimulated and stimulated bPMNs and sPMNs. Data expressed as mean, error bars demonstrate SEM, *= p< 0.05; **=p<0.01; (n=10).*

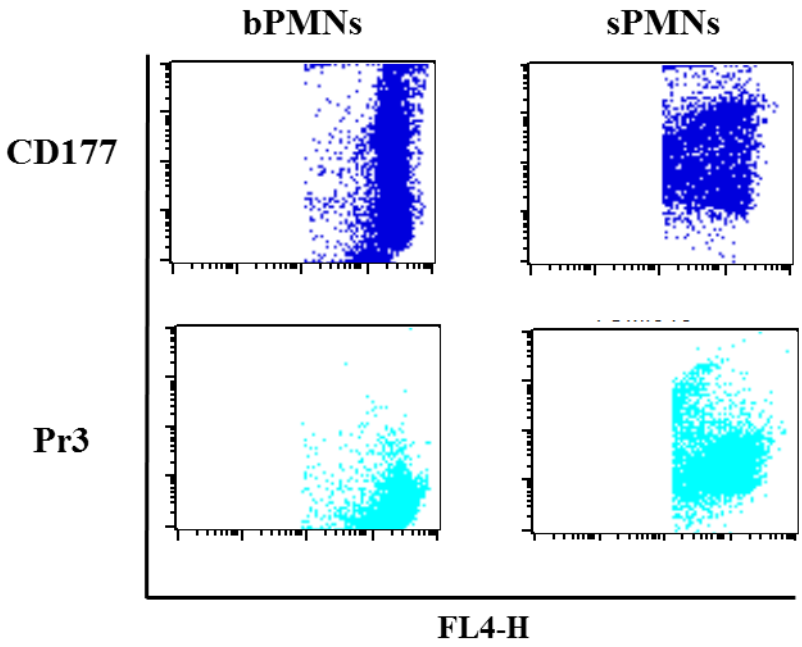


Figure 5.4 The surface expression of CD177 and Pr3 on unstimulated bPMNs and sPMNs. Both type of neutrophils were incubated with un-conjugated anti-CD177 (MEM166) or anti-Pr3 (G2) mAbs and after washing bound mAbs were detected with PE-conjugated goat anti-mouse antibody (rPE-GAM).

5.3.2 bPMNs and sPMNs expression of CD63 and CD16

As maximal stimulation resulted in the degranulation of the primary granules (containing most of the Pr3), which also results in the surface expression of the primary granule-specific marker CD63, I also examined the expression of CD63 along with CD16 shedding on these experimental groups. Neutrophil primary granule marker CD63 expression was assessed on the surface of neutrophils isolated from both blood and saliva. CD63 expression on unstimulated cells was significantly higher for sPMNs with mean fluorescence of 238.5 compared to 9.723 in bPMN unstimulated cells (p=0.0006). Stimulation of bPMNs caused a significant increase in surface CD63 expression.

The expression of CD63 on stimulated sPMNs was significantly higher than on unstimulated sPMNs (P=0.04) which suggests that further stimulation of sPMNs was capable of eliciting further degranulation of primary granules (figure 5.5A). However, *in vitro* maximal stimulation of bPMNs still failed to achieve the high level of surface CD63 found on unstimulated sPMNs suggesting that prolonged and multiple signalling pathways are engaged on these cells.

The mean fluorescence intensity of CD16 expression on unstimulated and stimulated bPMNs and sPMNs was investigated (figure 5.6). Stimulation of sPMNs caused a significant decrease in CD16 expression (Figure 5.6A) which showed that these neutrophils are capable of releasing the enzymes responsible for shedding CD16 from the surface of sPMNs. Despite the large decrease in CD16 on bPMNs following stimulation there was still a significant difference between bPMNs and sPMNs post-stimulation again suggesting prolonged stimulation and multiple signalling pathways involved for the sPMNs despite their ability to further reduce CD16 with further *in vitro* stimulation (P=0.009; figure 5.6 B).

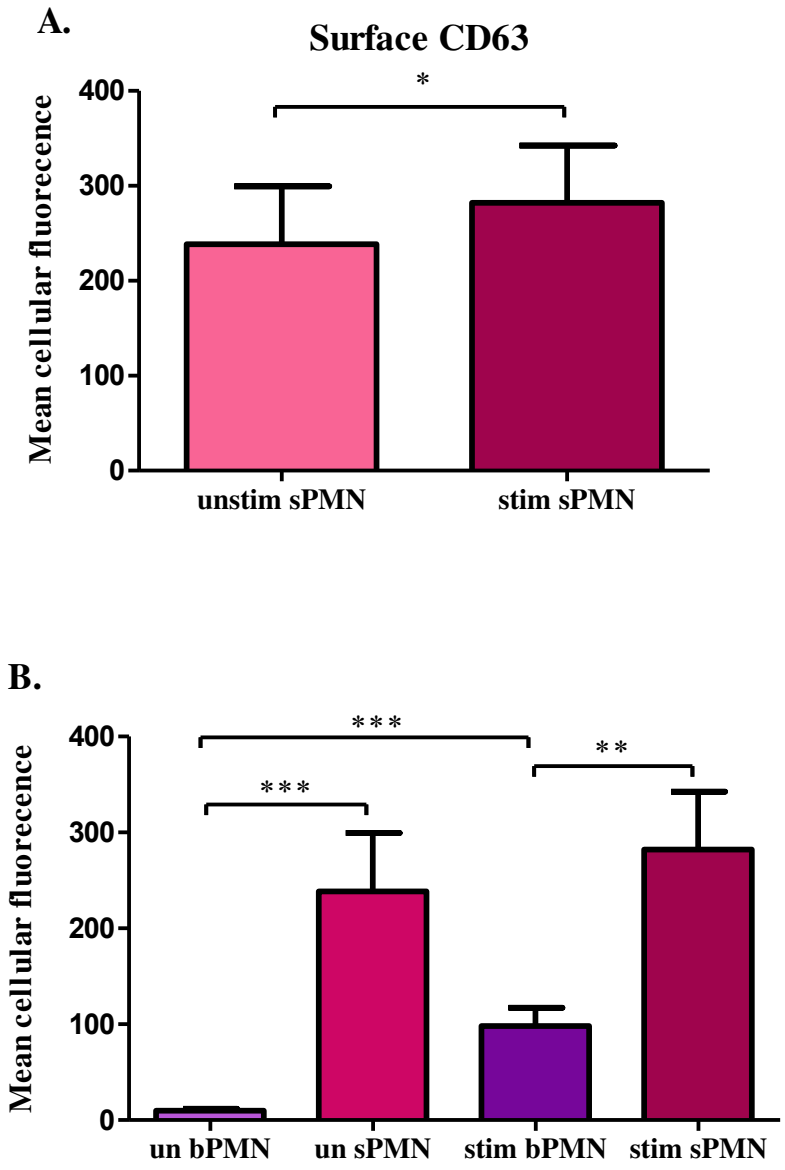


Figure 5.5 The cell surface expression of CD63 on bPMNs and sPMNs. Mean cellular fluorescence for FITC-conjugated anti-CD63 staining was measured on stimulated and unstimulated sPMNs (A) and Comparison of mean expression of CD63 on both stimulated and unstimulated bPMNs and sPMNs (B). Significant differences are shown (= P<0.05; **=P<0.01; ***=p<0.001 with bars showing the comparison groups, n=7).*

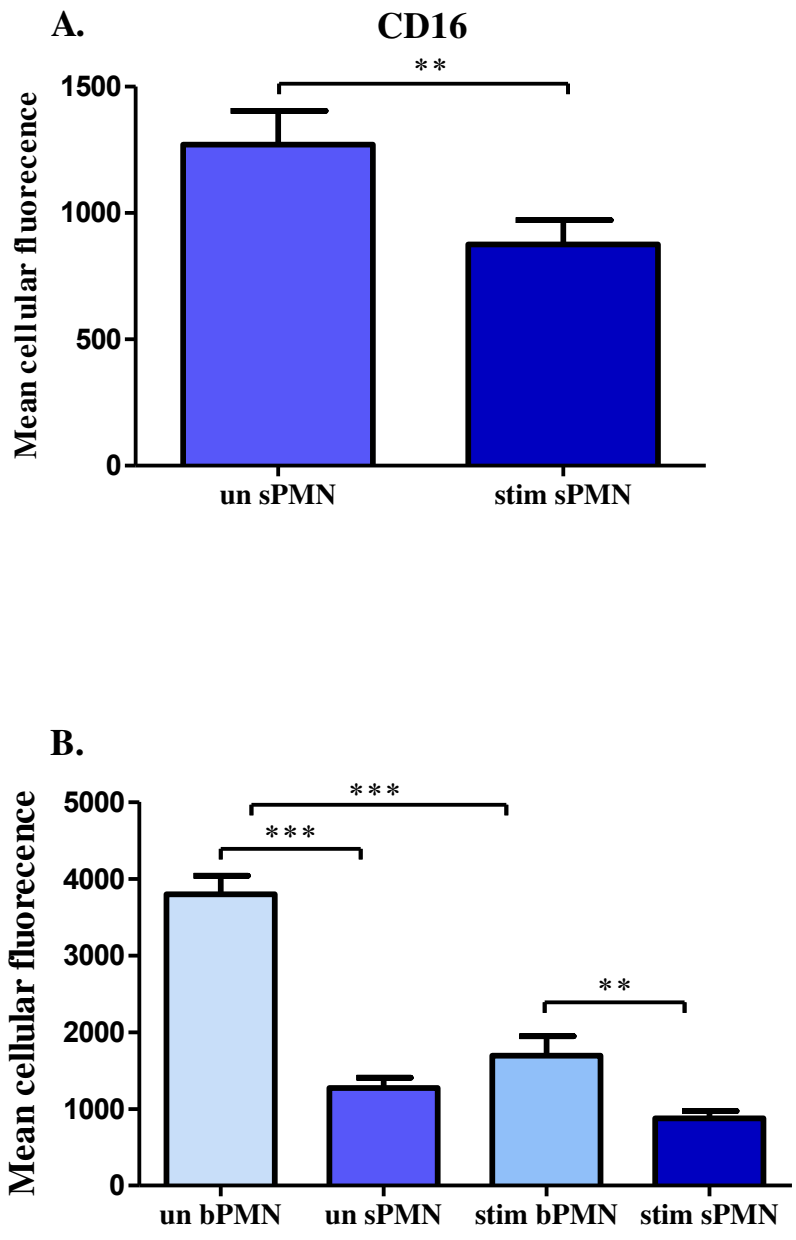


Figure 5.6 The expression of CD16 on pPMNs and sPMNs. Mean cellular fluorescence for APC-conjugated anti-CD16 staining was measured on stimulated and unstimulated sPMNs (A). Comparison of mean expression of CD16 in both stimulated and unstimulated pPMNs and sPMNs (B). Significant differences are shown (= P<0.05; **=P<0.01; ***=p<0.001 bars indicate the comparison groups, n=8).*

5.3.3 Detection of intracellular levels of Pr3, MMP-8 and MMP-9 in pPMNs and sPMNs

I next assayed intracellular levels of Pr3, MMP-8 and MMP-9 in sPMNs by flow cytometery and compared these levels to bPMNs levels. All neutrophils were fixed and permeabilised as described in section 2. The intracellular levels of Pr3, MMP-8 and MMP-9 were detected by incubation with anti-Pr3 (Pr3-G2), anti-MMP-8 (608) and anti-MMP9 (936). The background fluorescence was determined by staining with an isotype control.

The histogram for isolated sPMNs showed that the intracellular level of Pr3 was high relative to the isotype matched control background staining (figure 5.7.A). The levels of intracellular Pr3 in unstimulated sPMNs were significantly lower than levels in unstimulated bPMNs ($P=0.0002$). Following maximum stimulation of bPMNs, a significant decrease in intracellular levels of Pr3 was observed ($P=0.01$), whereas stimulation of sPMNs did not cause a further decrease in intracellular Pr3 levels (figure 5.7 B).

Levels of intracellular MMP-8 were higher in unstimulated bPMNs than in unstimulated sPMNs (Figure 5.8; $P<0.01$) and the intracellular levels in bPMN could be significantly reduced by maximal stimulation. However, these levels failed to achieve the low intracellular levels observed for unstimulated sPMNs. No additional decrease in intracellular sPMN MMP-8 levels could be mediated by further stimulation with cytochalasin B and fMLPecrease (Figure 5.8 A&B).

Similarly, MMP-9 levels were also significantly higher in unstimulated bPMNs compared to unstimulated sPMNs ($P= < 0.0001$). The levels of MMP-9 fell dramatically in bPMNs following stimulation ($P<0.05$), but still failed to reach the levels observed on unstimulated sPMNs and the levels of sPMN MMP-9 could not be further reduced following further stimulation (figure 5.9).

The intracellular levels of MMP-8 and MMP-9 in sPMNs were almost undetectable and could not be further altered following stimulation (unlike surface CD63 and CD16), indicating that most of these proteinases had been released either through transmigration or due to contact with oral microbes.

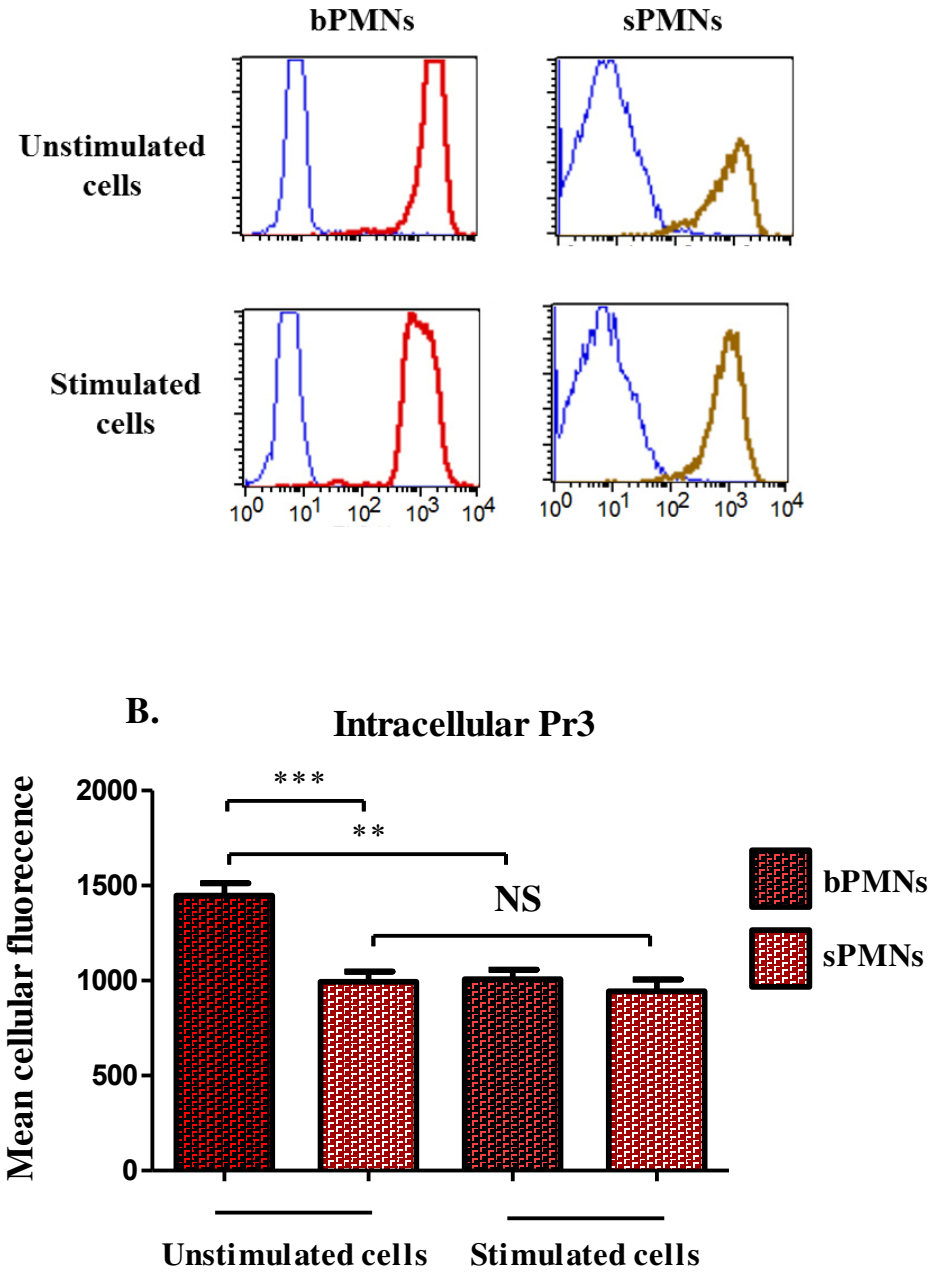


*Figure 5.7 Intracellular levels of Pr3. (A) Overlay histogram showing the expression patterns of intracellular Pr3 in unstimulated and stimulated bPMNs and sPMNs compared to the isotype control. (B) Comparison between blood and salivary neutrophil intracellular content of Pr3, before and after maximal stimulation with cytochalasin B combined with fMLP. Mean cellular fluorescence as measured by flow cytometry. Data expressed as mean, error bars demonstrate SEM, **=p<0.01; ***=p<0.001 (n=4).*

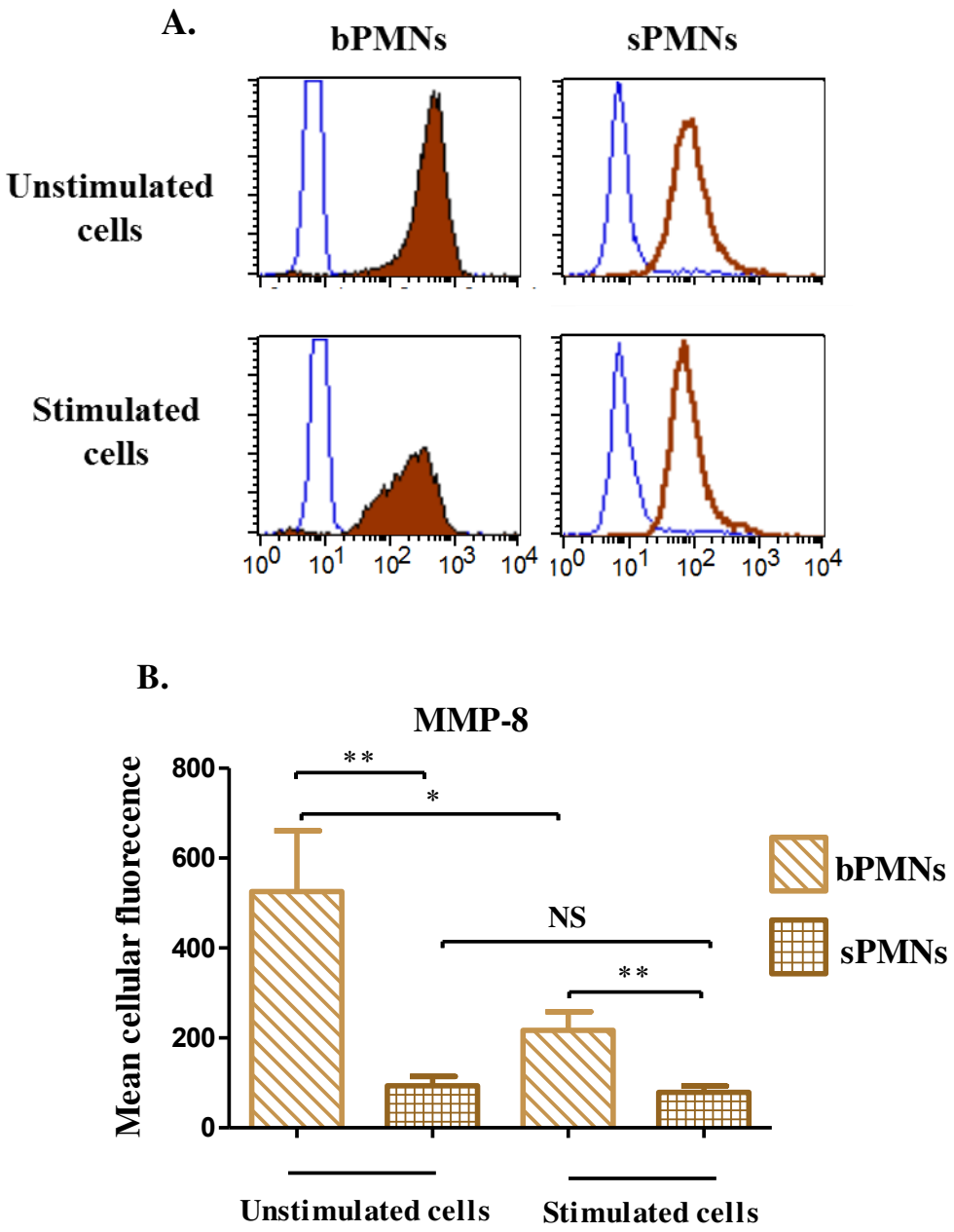


Figure 5.8 Intracellular levels of MMP-8 in bPMNs and sPMNs as measured by flow cytometry. Cells were either unstimulated or incubated for 15 min at 37°C with fMLP plus cytochalasin B. (A) Representative histogram showing the expression patterns of MMP-8 on unstimulated and stimulated bPMNs and sPMNs compared to the isotype control (blue). (B) Analysis of cumulative data for intracellular MMP-8. Note that stimulation of bPMNs induced significant decrease of MMP-8 ($p<0.05$) where as sPMNs show no significant decrease in the expression of MMP-8 after stimulation (B). n=5*

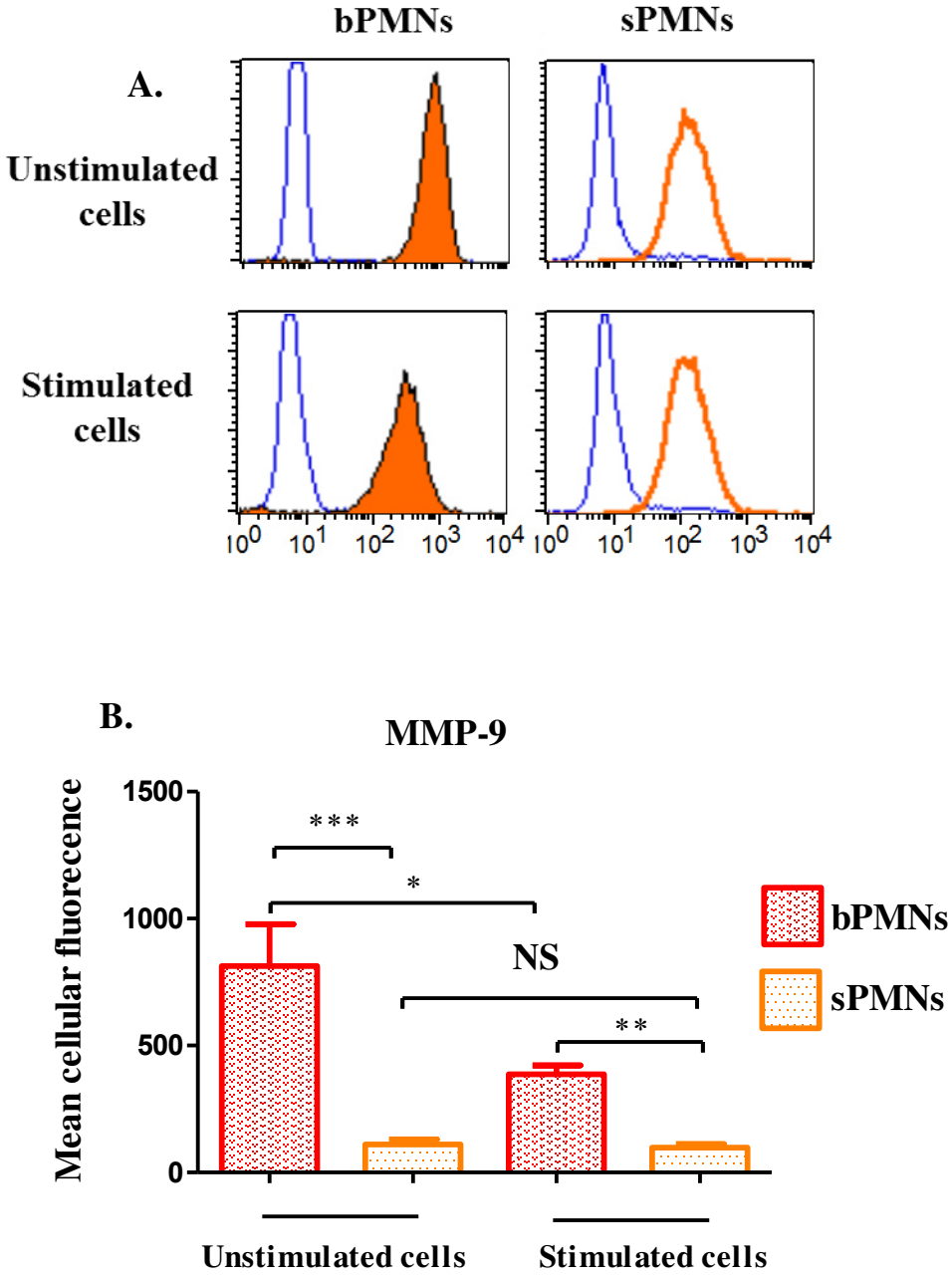


Figure 5.9 Intracellular levels of MMP-9 in bPMNs and sPMNs. (A) Histogram shows the expression patterns of MMP-9 on unstimulated and stimulated bPMNs and sPMNs compared to the isotype control (blue). (B) Bar graphs show the Mean intracellular fluorescence of MMP-9 in unstimulated and stimulated bPMNs and sPMNs. The data demonstrated that the levels of MMP-9 in unstimulated bPMN were remarkably higher than that in unstimulated sPMNs. n=5

5.3.4 Salivary neutrophil apoptosis

There appeared to be some discrepancy between the inability of sPMN to reduce intracellular Pr3, MMP-8 and MMP-9 following stimulation, relative to their ability to further decrease their CD16 surface expression and increase their CD63 expression following stimulation. Therefore in this section I have examined the level of salivary neutrophil apoptosis using annexin V-FITC in combination with propidium iodide measured by flow cytometery. Neutrophils were specifically analysed by gating on APC-CD16 expression. Figure 5.10A shows dot plots for unstained cells, cells double stained for annexin V-FITC and propidium iodide in presence of EDTA (which blocks annexin V binding to cells) and cells stained in presence of calcium. Figure 5.10B shows results for 2 additional experiments.

The externalisation of phosphatidylserine (PS) to the outer leaflet of the cell membrane is part of the early signs of apoptosis. Annexin-V is a member of annexin family which binds reversibly to PS-expressing membranes with high affinity in a calcium-dependent manner. Whereas increased permeability of cells for propidium iodide (PI) is consistent with necrosis, which can also occur as a consequence of apoptosis.

As shown in Figure 5.10, the percentage of viable cells (cell with no expression of PS or PI) ranged from 19.37-32.01%, apoptotic cells ranged from 28.05-57.61 with 22.38-39.80% necrotic cells. These results suggest that a high number of the sPMNs are apoptotic or necrotic, which may account for the failure to release intracellular Pr3 or increase surface expression of CD177 or Pr3 with further stimulation. However, it is interesting to note that these cells still show the capacity to actively shed CD16 and to externalise a small additional amount of CD63. There is also some variation in the levels of apoptosis/necrosis from person to person which may reflect the nature of inflammation in their oral cavity.

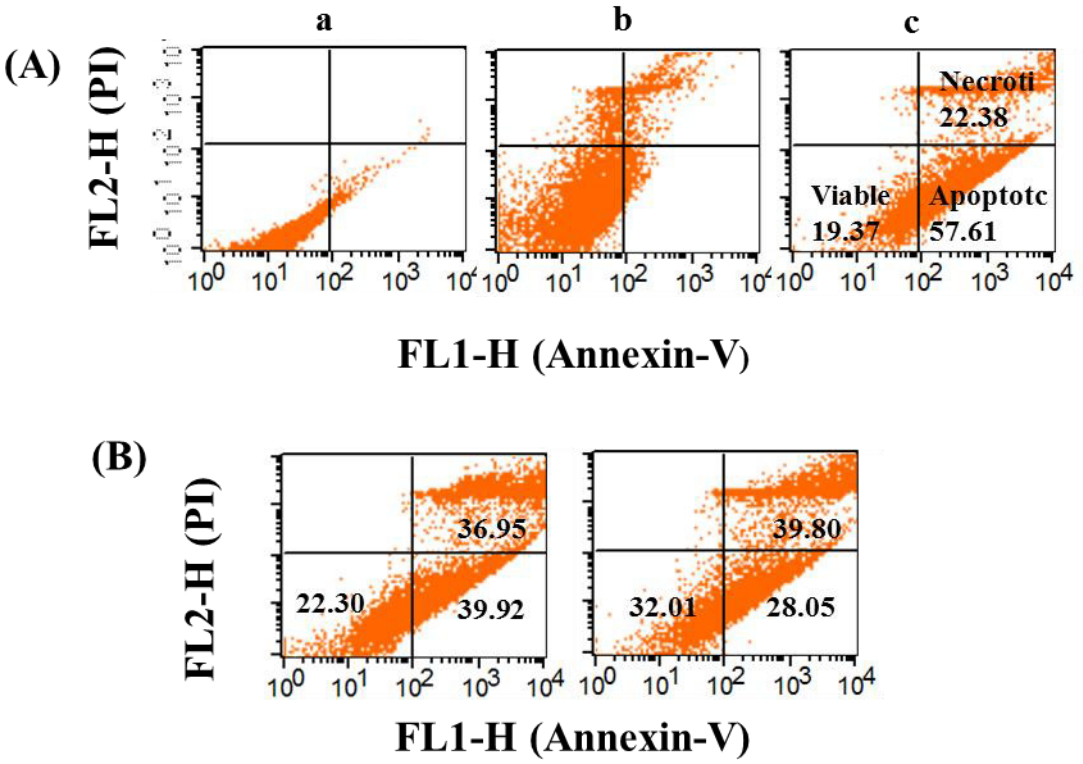


Figure 5.10 Assessment of neutrophil apoptosis in saliva sample. Dot plot images (A) show unstained cells (a), cells stained in presence of EDTA (no specific FITC-annexin V binding) (b) and in presence of Ca^{2+} (c) from one experiment. Dot plot images (B) show stained cells in presence of Ca from two additional experiments. Analysis of the APC-CD16 gated neutrophil population is shown. Sectors with percentages corresponding to apoptotic and non-apoptotic cells, as well as cell necrosis (PI positivity) are shown.

5.4 Discussion

sPMNs are essential contributors to keeping the balance between health and disease in the complex oral environment and are part of the host innate immune response in this environment. It has been supposed that more than 400 bacterial species exist in the oral cavity, and some cause inflammation (e.g. periodontitis; Sugawara *et al.,* 2002). Previous studies reported higher levels of salivary neutrophils in patients with teeth than in edentulous patients (i.e. lacking teeth). This suggests that dental plaque relating to oral bacteria is the chemotaxic source for neutrophils in the oral cavity (Wright *et al.,* 1986: Pink *et al.,* 2009). Neutrophils in the oral cavity produce reactive oxygen species, nitric oxide, and several antimicrobial peptides (including α-defensins) and release other enzymes (e.g. proteases).

In the previous chapter I found that all CD177-positive and negative neutrophils appear equally able to migrate through the endothelial monolayer neutrophils were more prone to release a proportion, but not all, of their MMP-9 stores, but none of their MMP-8 stores, during migration through the endothelial monolayer. In the present chapter, I compared the expression of selected proteases between bPMNs and sPMNs from healthy individuals by flow cytometry. My results showed for the first time that salivary neutrophils (*in vivo* transmigrated neutrophils) expressed only the positive subsets of Pr3 and CD177 which suggested that CD177 and Pr3 are important contributors for transmigration. A study conducted by Sachs *et al.* stated that CD177-positive neutrophils transmigrated more readily than did CD177-negative neutrophils from the same individual using *in vitro* assays (Sachs et al., 2007). Another study conducted by Kuckleburg *et al.* revealed that *in vitro* transmigrated neutrophils significantly increased surface expression of Pr3 and that CD177-positive neutrophils also transmigrated more efficiently than did CD177-negative neutrophils from the same individual (Kuckleburg *et al.,* 2012). However, neither study mentioned that the negative CD177 cells have impaired transmigration and Kuckleburg *et al* indicated that there

was no transmigration defect for CD177-null individuals. These results are consistent with my results from the previous chapter, where I found no bias between $CD177^{high}$ and $CD177^{low}$ populations using *in vitro* transmigration assays.

Pr3 is able to activate oral epithelial cells through G protein-coupled PAR-2 on the cell surface and actively contributes in the process of inflammation. PAR-2 activated oral epithelial cells produce bio-active IL-18, which is critical to Th1 and Th2 responses (Uehara *et al.,* 2002; Sugawara *et al.,* 2002). Herein, I found the levels of Pr3 expression on neutrophils were higher for sPMNs than in bPMNs, but further *in vitro* stimulation of sPMNs could not induce additional change in the mPr3 expression. Using a combination of cytochalasin B and fMLP to maximally stimulate bPMNs resulted in significant increase in bPMN surface Pr3; however, these levels did not achieve those observed on sPMN. Given the levels of apoptosis and necrosis in sPMN, it is likely that further mobilisation of intracellular pools of Pr3 and CD177 may have been restricted by changes in neutrophil signalling machinery under this physiological change. Equally, it may be that the entire mobilisable pool of these molecules had been externalised, which would be consistent with the baseline high levels of CD63 on unstimulated sPMN.

In neutrophils, Ca^{2+} signalling is essential for several cellular activities, including the generation of oxidants and the release of proteases. The changes in the cytosolic concentrations of free calcium are considered important for a number of neutrophil responses (Hallett 1997). Increases in free cytoplasmic calcium (up to close to 1 μM) are identified within seconds of stimulation by a variety of neutrophil agonists including chemotactic factors (Petroski *et al.,* 1979; Pozzan *et al.,* 1983). Receptors to fMLP, C5a, platelet-activating factor, and IL-8 are all G protein–coupled receptors (GPCRs) and convey through cytosolic free calcium signalling a number of pro-inflammatory activities such as chemoattraction, oxidase activation, and degranulation (Hallett 1997; Harfi *et al.,* 2005).

During apoptosis all neutrophil activities were greatly impaired including chemotaxis and spreading in response to fMLP stimulation, phagocytosis, and superoxide release in response to PMA and fMLP stimulation as well as opsonised zymosan binding (Whyte *et al.,* 1993). It has been shown that transient elevations of cytosolic Ca^{2+} delay apoptosis (Whyte *et al.,* 1993). Recently study by Francis et al. (2013), reported that neutrophils with externalized PS also have raised cytosolic-free Ca^{2+} and are subsequently prone to necrosis when Ca^{2+} is elevated further.

sPMNs spontaneously create superoxide radical and nitric oxide in the lack of any stimuli (Yamamoto *et al.,* 1991; Sato *et al.,* 1996; Nakahara *et al.,* 1998). Circulating neutrophils are primed by various ligands (such as LPS, IL-1β, and TNF-α) and during the migration into the oral cavity and spontaneously release reactive oxygen species. Salivary neutrophils are further activated by various ligands such as LPS which predominate in the subgingival environment (Sato *et al.,* 2008). Takubo *et al.* reported that there were no differences in the morphology and phagocytic activity between sPMN and bPMNs in healthy individuals, but that there was a significant difference in H_2O_2 production when stimulated with fMLP (H_2O_2 production by sPMN was significantly higher than that by bPMNs) but not PMA. In contrast, Lukac *et al.* found that the ability of sPMNs to ingest (yeast cell) was significantly lower compared to bPMNs in healthy individuals (Lukac *et al.,* 2003).

Yamamoto *et al.* stated that sPMNs have ability to show characteristic responses to various stimuli, and therefore sPMN display essential roles in the defence mechanisms in oral cavity (Yamamoto *et al.,* 1991). A study conducted by Sato *et al.* stated that cultured sPMN rapidly and spontaneously undergo apoptosis, whereas apoptosis of bPMN occurred more slowly (Sato *et al.,* 2003). In the oral environment, oxidative stress and/or redox regulation-dependent pathway(s) activated caspase-3 in sPMN, thus prompting their apoptosis (Sato *et al.,* 2008). My analysis of the surface expression of PS and permeability to propidium iodide

for sPMN showed that there was already a substantial baseline apoptosis/necrosis for sPMN. Therefore, there is a wide variation in the literature for the apoptotic status or ability to be induced to apoptosis for sPMN. The underlying conclusion from these studies indicates retention of some neutrophil functions, but alterations to other functions following transmigration into the oral cavity.

I have used surface expression of CD63, a member of the tetraspanin superfamily, to measure pre-existing and induced degranulation following stimulation for the different neutrophil populations. This protein is absent from the surface of freshly isolated unactivated neutrophils and is one of the membrane proteins of azurophilic granules. This granule subset represents one of the hardest to degranulate, therefore surface CD63 expression is a good indicator of maximal neutrophil stimulation (Cham *et al.,* 1994). It is interesting that I was able to induce a small but significant increase of surface CD63 on sPMN following stimulation. This suggests that it was possible to externalise some of the retained intracellular pools of proteases from sPMN. Similarly, I found that surface CD16 could be shed from sPMN following further stimulation which also suggested that sPMN proteases could be externalised. However, this was not confirmed by measurement of intracellular Pr3, MMP-8 and MMP-9 in sPMN. ,

MMPs are able to degrade extracellular matrix, and they are inducible enzymes depending on the inflammatory environment (e.g. periodontitis and bacterial infection in periodontal tissue). At the site of infection or inflammation, leukocytes release MMP-8 and MMP-9, which are activated locally. The major collagenase in periodontitis was human MMP-8, accompanied by MMP-9 (Sorsa *et al,* 1995; Sorsa *et al* 1988; Sorsa *et al.,* 2004). Throughout the beginning and course of inflammatory responses in periodontitis MMP-8 is up-regulated in affected tissues, and in the secreted, disease affected oral fluids: gingival crevicular fluid and saliva, as well as in serum and plasma (Sexton *et al.* 2011, Herr et al. 2007, Miller *et al.*

2006). Neutrophils have been recognized as an important source of MMP-9 and -8 in inflammatory periodontal diseases (Westerlund *et al.,* 1996; Sorsa *et al.,* 2004). However, production of MMPs in neutrophils cannot be upregulated and their entire contents are separated into different granule types. MMP-8 is located in the secretory or secondary granules and MMP-9 is located in the tertiary granules. Furthermore, there is a gradient of degranulation whereby tertiary granules are easier to externalise than secondary granules, which in turn are easier to externalise relative to azurophilic granules (Sengelov *et al.,* 1993; Bentwood *et al.,* 1980). This would account for my results with transmigration and fMLP only stimulation in the previous chapter where only intracellular MMP-9 levels were responsive to low levels of stimulation. However, the large amount of surface CD63 on freshly isolated sPMNs suggests that these neutrophils have been maximally stimulated and a majority of the granule contents have been released already. This is probably the reason for the inability to reduce the intracellular pools of MMP-9, MMP-8 and Pr3 from sPMNs, because those measureable intracellular amounts are not associated with granules. These data also suggest that although the transmigration of neutrophils is occurring continuously in all individuals in the absence of overt periodontal disease, the process should not be considered a "low inflammation transmigration" condition.

5.5 Summary

Migrated salivary neutrophils have released almost all of their MMP-8 and MMP-9 prior to collection and measurement. Only CD177-positive PMNs were found in the saliva, despite being bimodal in the periphery, and CD177 was found to have substantial amounts of Pr3 bound on the surface of these. While intracellular levels of MMP-8 and MMP-9 were completely depleted in sPMN, detectable levels of intracellular Pr3 were still present, although they could not be mobilised by further cell stimulation.

CHAPTER 6

GENERATION OF RECOMBINANT CD177-Fc FUSION PROTEIN AND CD177 RECOMBINANT PROTEIN EXPRESSION IN CHO CELLS

6.1 Introduction

Human CD177 gene is a member of the Ly-6 gene family (also known as the snake toxin family). Ly-6 genes were first described in mice as lymphocyte differentiation antigens (McKenzie *et al.,* 1977). The gene is located at chromosome 19q13.31 and is comprised of 9 exons and an open reading frame of 1311 bp which encodes 437 amino acids with an N-terminal signal peptide of 21 amino acids (Caruccio *et al.,* 2006; Kissel *et al.,* 2001; Bettinotti et al., 2002). It is highly homologous to another member of this gene family, which is polycythemia rubra vera-1 (PRV-1) and they only differ at 4 nucleotides (G42C, C390T, G1003A and T1171C) that result in four amino acid exchanges (Ala3Pro, Leu119Phe, Arg323Gln and Phe379Ser) (Kiessel *et al.,* 2002; Bettinoti *et al.,* 2002).

Stimulation of neutrophils with G-CSF or GM-CSF has been reported to up-regulate CD177 transcripts (Stroncek *et al.,* 1998) and also up-regulate CD177 gene expression in granulocyte progenitors upon inactivation by an estrogen-dependent form of E2a-Pbx1 oncoprotein (Sykes *et al.,* 2003). Other studies demonstrated that stimulation of neutrophils with G-CSF caused up-regulation of CD177 expression on CD177-positive cells but not on the fraction of CD177 negative neutrophils (Temerinac *et al.,* 2000; Wolff *et al.,* 2003). The overexpression of CD177 was also reported in neutrophils from patients with polycythaemia vera (Caruccio *et al.,* 2006)

Proteins encoded by the Ly-6 genes are classified in two subfamilies: one subfamily encodes GPI anchored proteins and the second subfamily encodes secretory proteins without a GPI anchor (Kong and Park 2012). CD177 of 58-64 kDa is only expressed on neutrophils, neutrophilic metamyelocytes, and myelocytes but no other blood cells (Stroncek et al., 1998 (1); Verheugt et al., 1977). Human CD177 plays an essential role in some immune mediated disorders (e.g. autoimmune neutropenia, and transfusion-associated lung injury, TRALI) (Bettinoti *et al.,* 2002). CD177 has an important role in the regulation of neutrophil migration

through interaction with PECAM-1 as a heterophilic binding partner of CD177 and prompts a cytokine signalling cascade including thrombopoietin- and IL-3-induced proliferation, and Pr3 expression (Sachs *et al.,* 2007; Korkmaz *et al.,* 2008). CD177 serves as the high-affinity receptor for Pr3 neutrophil membrane display. The binding of human Pr3 with CD177 involves six hydrophobic residues on Pr3 (Phe^{165}, Phe^{166}, Ile^{217}, Trp^{218}, Leu^{223}, Phe^{224}) that are not found on NE or cathepsin G, nor are these residues found in mouse or gibbon Pr3. The CD177-bound Pr3 on cellular membranes is accessible to physiological substrates and inhibitors, but only AAT is able to dissociate the Pr3 from membranes (Korkmaz *et al.,* 2008).

6.2 Aims

● Generate CD177 recombinant protein expression on transfected CHO cells to study isolated CD177 on a surface different from neutrophils, as well the interference of serum and purified AAT on Pr3 (both from stimulated neutrophil supernatant and purified commercial sources) binding to CD177 expressed on CHO cells.

● Generate soluble CD177-Fc fusion protein to be purified and used in assays of direct protein interactions, between CD177 and Pr3, by surface plasmon resonance technology (SPR).

6.3 Results

6.3.1 Generation of full-CD177 and CD177-Fc for expression in CHO cells

The cDNA encoding the entire CD177 open-reading frame (ORF) was provided in plasmid pCMV-SPORT6 as purchased from Thermo Scientific Ltd. Following propagation of the CD177 ORF in pCMV-SPORT6 in transformed *E.coli,* the plasmid was purified and used as a target for PCR amplification of the full-length CD177 cDNA or the extracellular portion of CD177 for ligation into the pTorsten vector which adds the human IgG1 Fc region in frame. PCR using specific primers and PCR products were purified as described in chapter 2. Amplified PCR products were cut using appropriate restriction enzymes that were engineered into the flanking regions of the primers: XbaI + EcoRV for full-length CD177 and XbaI + NotI for Fc CD177 cDNA then the products were purified and run on 1% agarose gel electrophoresis as seen in figure 6.1. PCR products for full-length CD177 cDNA and Fc CD177 cDNA were ligated into the pTorsten expression vector plasmid in the correct orientation to allow the EF-1alpha promoter to drive expression.

After ligation and transformation into *E. coli*, the plasmids from single colonies were grown up and purified. To test the appropriate insert size was successfully ligated into pTorsten, release of approximate correct size inserts were confirmed by cutting with the same REs used to create it and viewed on the gel as shown in figure 6.2. Correct addition of the Fc fusion was then confirmed by cutting Fc-CD177 plasmid with Xba1 and EcoRV: the right side of figure 6.2 shows the size of Fc-CD177 DNA was about 1900 bp whereas the size of full CD177 DNA was 1342. Plasmids from single colonies were then submitted for sequencing to confirm no mutations had been induced by the PCR amplification. The first sequence for Fc-CD177 identified a mutation, therefore the process was repeated until an error-free plasmid was identified for transfection and expression.

The plasmids from clones (full-length CD177 or Fc-CD177) were scaled up, purified and transfected into CHO cells. After transfection stable cells were selected in 400 mg/L hygromycin, and then screened for the production of soluble recombinant CD177-Fc fusion protein in supernatant by Western blot and the expression of CD177 on the surface of CHO cells by flow cytometry.

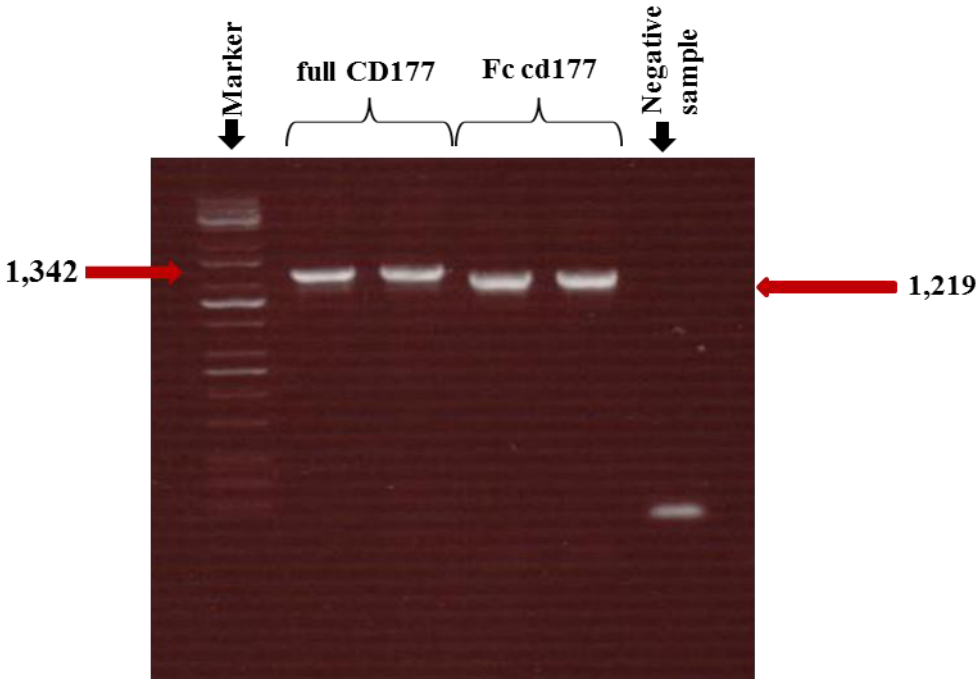


Figure 6.1 Analysis of CD177 DNA amplified PCR products by agarose gel electrophoresis. PCR amplicon products for extracellular region of CD177 (for Fc fusion protein creation) and full-length CD177 run along with DNA ladder marker on 1% Agarose gel. Lane 1: molecular marker, lane 2&3: amplified full CD177, lane 4&5: amplified CD177-Fc, negative sample was PCR run without CD177 cDNA containing template.

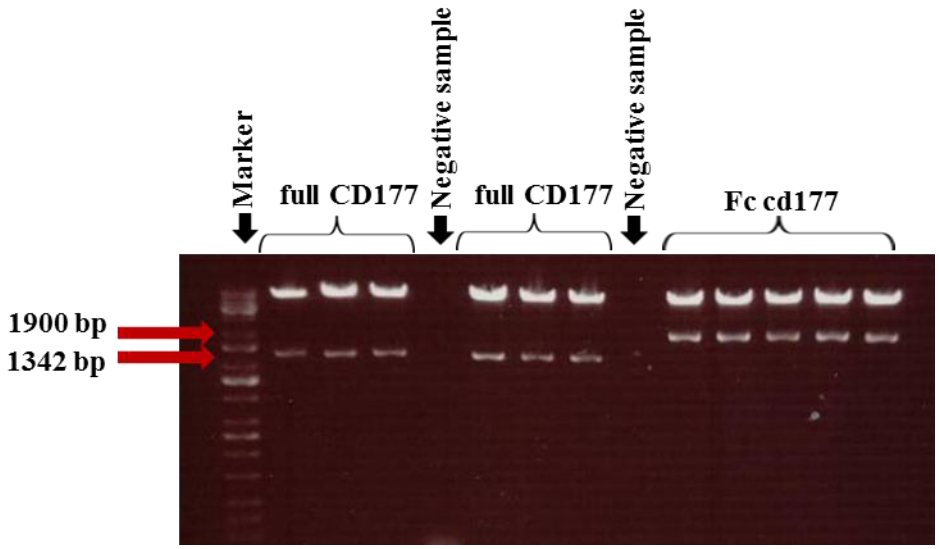


Figure 6.2 Restriction digest of plasmids to confirm insert size. Plasmids purified from different single colonies of transformed E.coli bacteria were run on 1% Agarose gel along with molecular marker (line 1). Positive plasmids for full-length CD177 are seen in lane 2, 3, 4, 6, 7, and 8 (cut with Xba1 and EcoRV). Positive plasmids for Fc-CD177 are seen in lane 10, 11, 12, 13 and 14 (cut with Xba1 and NotI).

6.3.1.1 Detection of recombinant CD177-Fc by Dot Blot

E.coli containing the plasmids with the Fc-CD177 fusion protein and a positive control (CD55-IgG) plasmid (Yanagawa *et al.,* 2003, Lab Invest83 (1):75-85) were scaled up, the plasmids purified using a Qiagen midi purification kit and transfected into CHO cells using Lipofectamine (as per manufacturer's instructions). After transfection, stable cells were selected in 400 mg/L of hygromycin B, and then surviving colonies of cells were screened for the production of soluble recombinant CD177-Fc fusion protein in medium supernatant. Supernatants were collected from all CHO cells (non-transfected controls and transfected cells) and initially screened by Dot Blot assay as described in chapter 2. Figure 6.3 shows results from representative dot-blot assays, using supernatant from positive control (CD55-Fc; +P) CHO transfected cells, supernatant from CD177-Fc transfected cells (P1,3,4,6) and supernatant from free CHO cells (negative control). Supernatants containing human Fc-IgG appeared as black spots as they react with anti-human IgG1 antibody whereas supernatant from free transfected cells showed no reactivity.

6.3.1.2 Detection of recombinant CD177-Fc by Western Blot

Western Blot was used to confirm correct size of the constructed CD177-Fc fusion protein. The CD177-Fc protein in transfected supernatant was also detected by Western Blot using MEM166 monoclonal antibody against CD177 and anti-human IgG. As shown in figure 6.4 the supernatant from positive transfected cells showed strong reactivity for a band at approximately 170 kDa. In contrast, anti-CD177 did not react with the CD55-Fc purified protein or the supernatant from untransfected cells confirming specificity.

After protein purification by the use of Protein G column, the purified CD177-Fc protein was analysed again using Western Blot to confirm enrichment and that no degradation had occurred during purification. Figure 6.5 shows the detection of the Fc-CD177 fusion protein

before and after purification compared to purified proteins, probed with anti-human IgG (left blot) and anti-CD177 antibodies (MEM166; right blot).

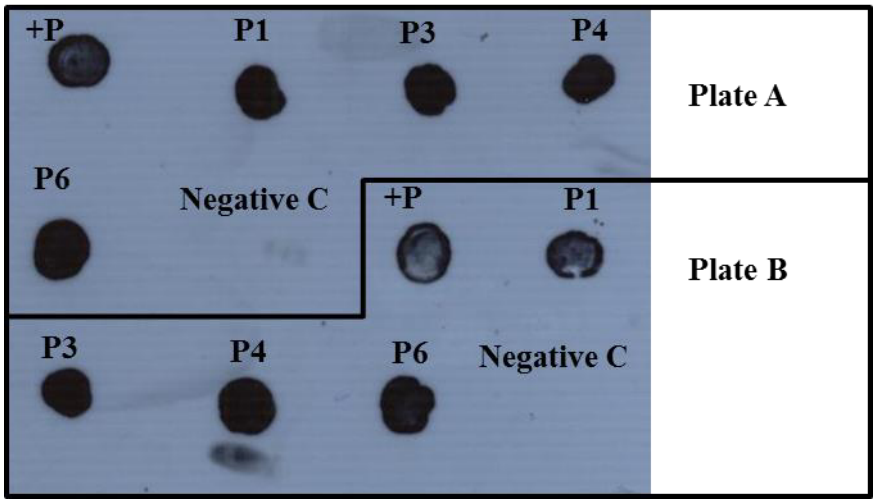


Figure 6.3 Dot blot analysis. Plasmids from four different positive transformed bacteria were purified and transfected into CHO cells. Cells were cultured in two 6-well plates with plasmids from positive control (CD55-Fc) included and untransfected negative control CHO cells included. The nitrocellulose membrane was probed with goat anti-human IgG1.

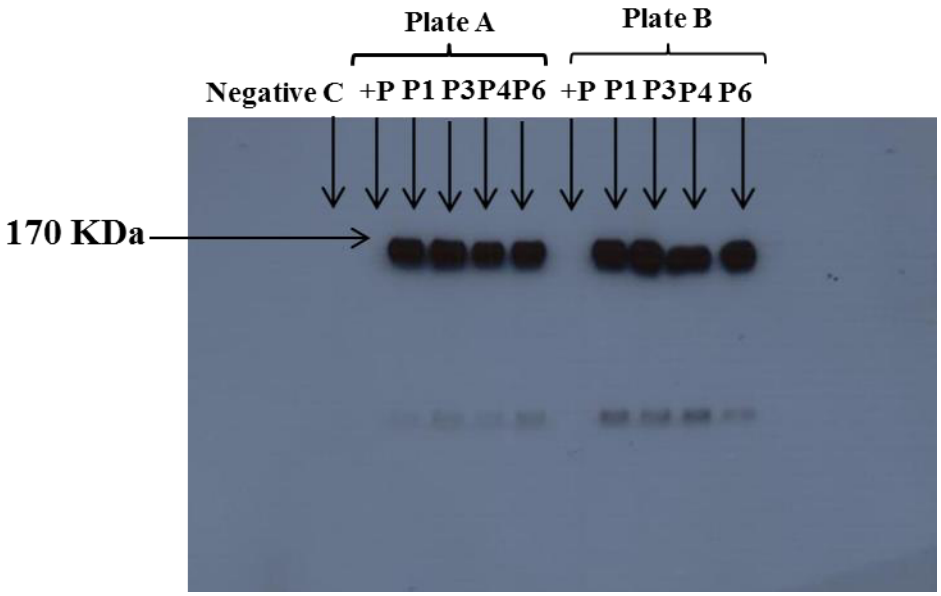


Figure 6.4 Western blot analysis of supernatants from selected stable expressing clones. Recombinant CD177-Fc protein was recognized by monoclonal MEM166 antibody raised against CD177 (which appears as a band of 170 kDa, a dimeric form under non-reducing conditions due to the properties of the Fc portion). No reactivity with untransfected CHO cell supernatant (negative) or CD55-Fc protein (expected size of 150 kDa) was observed.

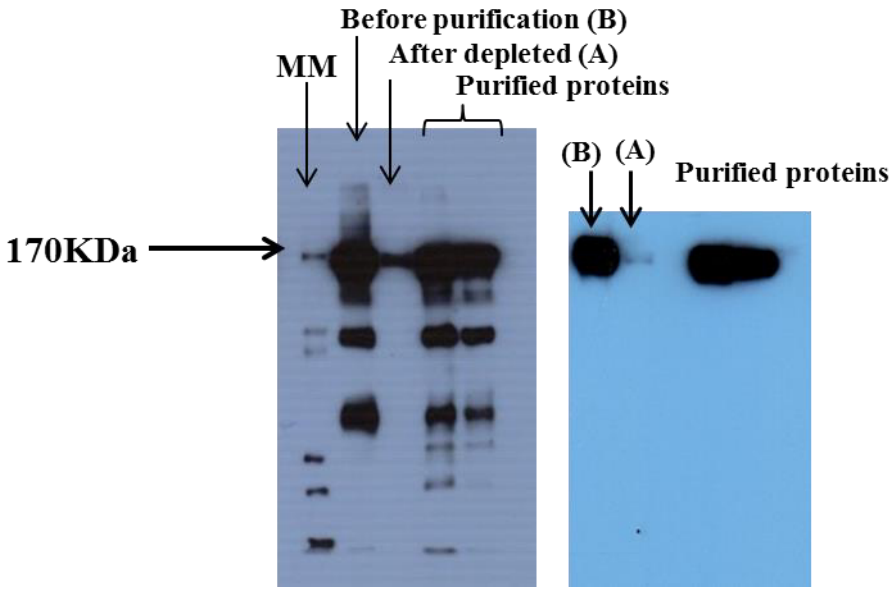


Figure 6.5 Western Blot analysis for soluble recombinant CD177-Fc protein separated by 7.5% SDS-PAGE before and after purification by Protein G-column. Soluble proteins were detected by anti-human IgG (left film) and anti-human CD177 (MEM-166; right film). Lane 1 represents the molecular marker (MM) Magic Mark XP, which is detected by the peroxidase secondary antibodies. The presence of released degradation fragments of IgG Fc is identified (and co-purified by Protein G) in the left blot.

6.3.1.3 Detection of recombinant CD177-Fc by ELISA

ELISA technique was used to determine CD177 fusion protein in the supernatant from CHO transfected cells. As mentioned in chapter 2, I used 3 different ELISAs in order to choose the most sensitive antibodies to detect Fc-CD177 protein. Firstly, the CD177-Fc protein was analysed in ELISA by capturing the Fc portion using monoclonal anti-IgG1 specific antibody immobilised to a 96-well plate. Bound CD177-Fc protein was then detected with Horseradish peroxidise (HRP) anti-human IgG antibody or Horseradish peroxidise (HRP) rabbit anti-human C177 antibody. Figure 6.6A shows that HRP anti-human CD177 antibody had a poor ability to detect CD177 protein.

The second ELISA was the same as the first, except that an additional secondary HRP-conjugated donkey anti-rabbit antibody was used to try to detect the HRP-conjugated rabbit anti-CD177 in case poor HRP activity or poor conjugation was responsible for the low signal. This did not improve the ELISA result (figure 6.6 B).

However, the use of capture and detection of IgG does not assess undegraded CD177-Fc, therefore a third assay was created where MEM166 was immobilised to 96-well plates to capture the CD177 portion and the Fc was detected by the HRPO-conjugated anti-IgG used in the first 2 assays (Figure 6.6C). The MEM166 antibody gave more sensitive results than the antibody used in previous two experiments.

After protein purification, ELISA was also used with the same steps in the third experiment (figure 6.7).

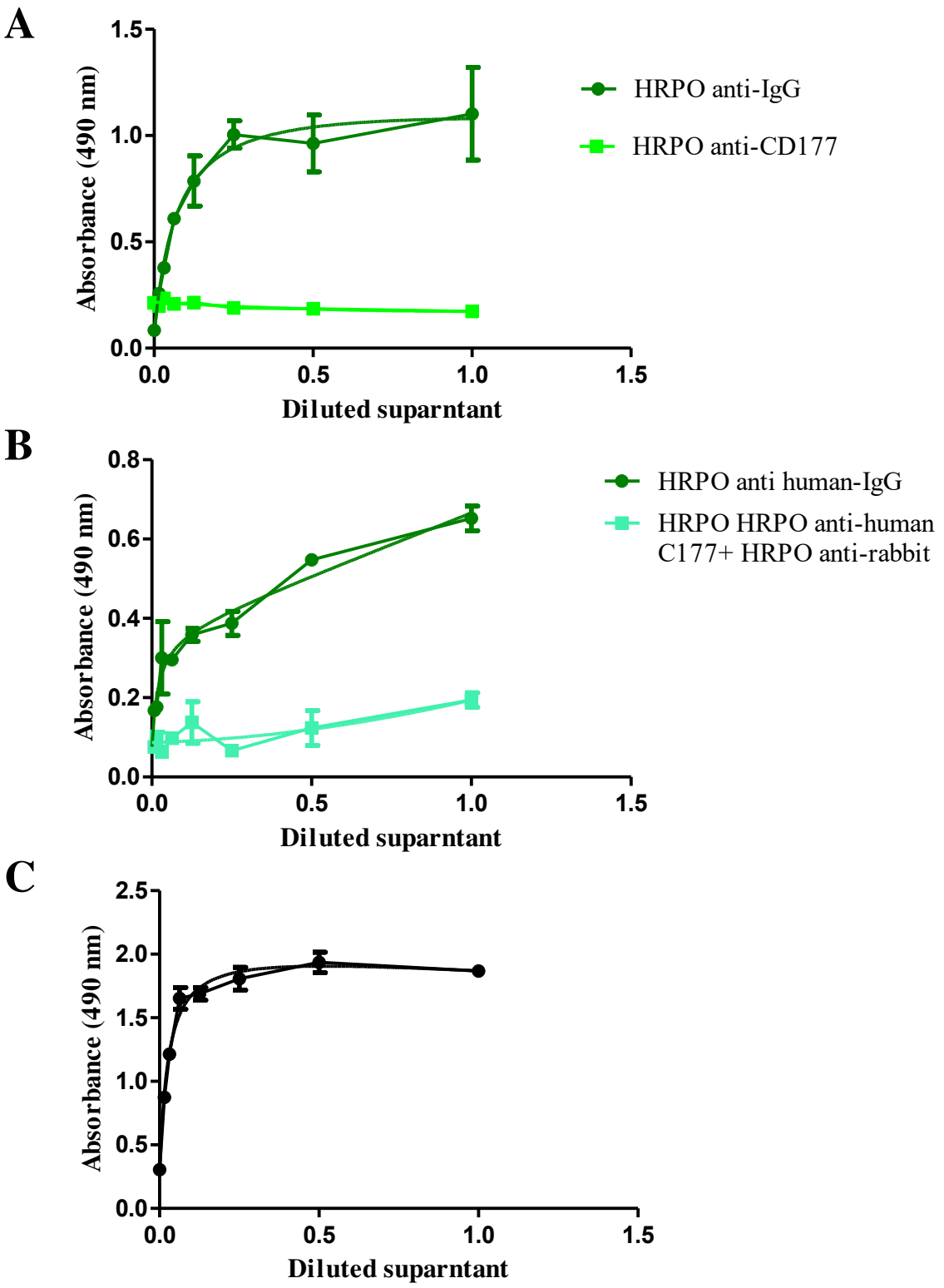


Figure 6.6 ELISA detection using different antibodies to capture Fc-CD177 fusion protein. A) Capture using monoclonal anti-human IgG1 antibody and detect with HRPO rabbit anti-human C177 or HRPO goat anti-human IgG antibodies. B) Capture using monoclonal anti-human IgG1 antibody and HRP goat anti-human IgG, but try to improve the signal of HRPO rabbit anti-human C177 antibody by addition of HRPO donkey anti-rabbit antibody. C) Capture using monoclonal MEM166 antibody and detect with HRPO goat anti-human IgG antibody.

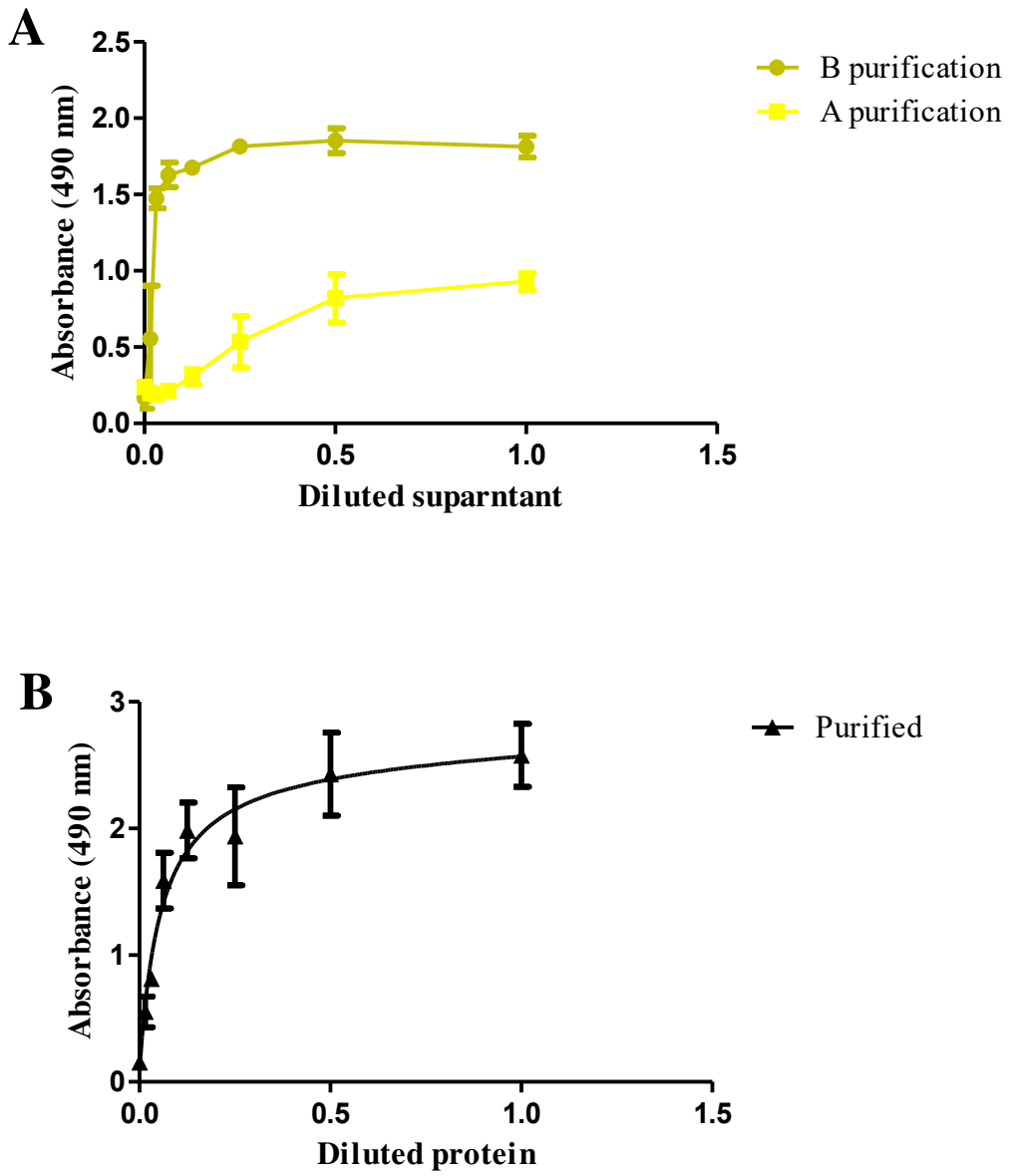


Figure 6.7 Using the optimised ELISA to measure the amount of CD177-Fc protein in pooled transfected CHO cell supernant (A) and confirm depletion (and determine remaining presence) of CD177-Fc post-Protein G column (B) (upper panel). ELISA was also used to confirm intact CD177-Fc after elution from Protein G-column, followed by buffer exchange and concentration. (lower panel)

6.3.1.4 Colloidal Coomassie blue staining

Coomassie blue staining allows the detection of proteins at nanogram concentrations. Protein purity, yield and quality are easily influenced by processing conditions such as temperature and time. Here I used this method to analyse the purity of CD177-Fc fusion protein using SDS-PAGE followed by Coomassie blue staining. As seen in figure 6.8 a majority of the protein is intact dimeric CD177-Fc fusion protein, however there were two smaller, lower intensity bands that are likely monomeric CD177-Fc and released Fc from minor degradation during purification, as all three bands are recognised by the HRPO-anti-human IgG antibody. One of the purified protein samples appears to have other minor lower molecular mass contaminants that may represent further degradation products.

6.3.1.5 Detection of recombinant CD177-full expression on CHO cells by Flow Cytometry

Stable transfected CHO cells and untransfected CHO cells (background control cells) were cultured until they reached the 80-90% confluency post-hygromycin B selection were then stained for CD177 using unconjugated monoclonal anti-CD177 (MEM-166) antibody. Bound MEM166 was then detected by PE-conjugated goat anti-mouse antibody as described in chapter 2. An isotype IgG control was also used as a primary antibody to detect background fluorescence. Different clonal cell populations were expanded and separately analysed. As shown in figure 6.9 the cells transfected with full-CD177 cDNA produced fluorescence above background, whereas untransfected CHO cells did not display significant fluorescence compared to the isotype control. In addition the different transfected clones showed different levels of CD177 expression ranging from high and moderate and negative expression. The high expression cells were re-cultured and used to investigate the binding of CD177 to purified Pr3 or Pr3 in stimulated neutrophil supernatant and also to explore the effect of AAT and serum on the CD177/Pr3 complex.

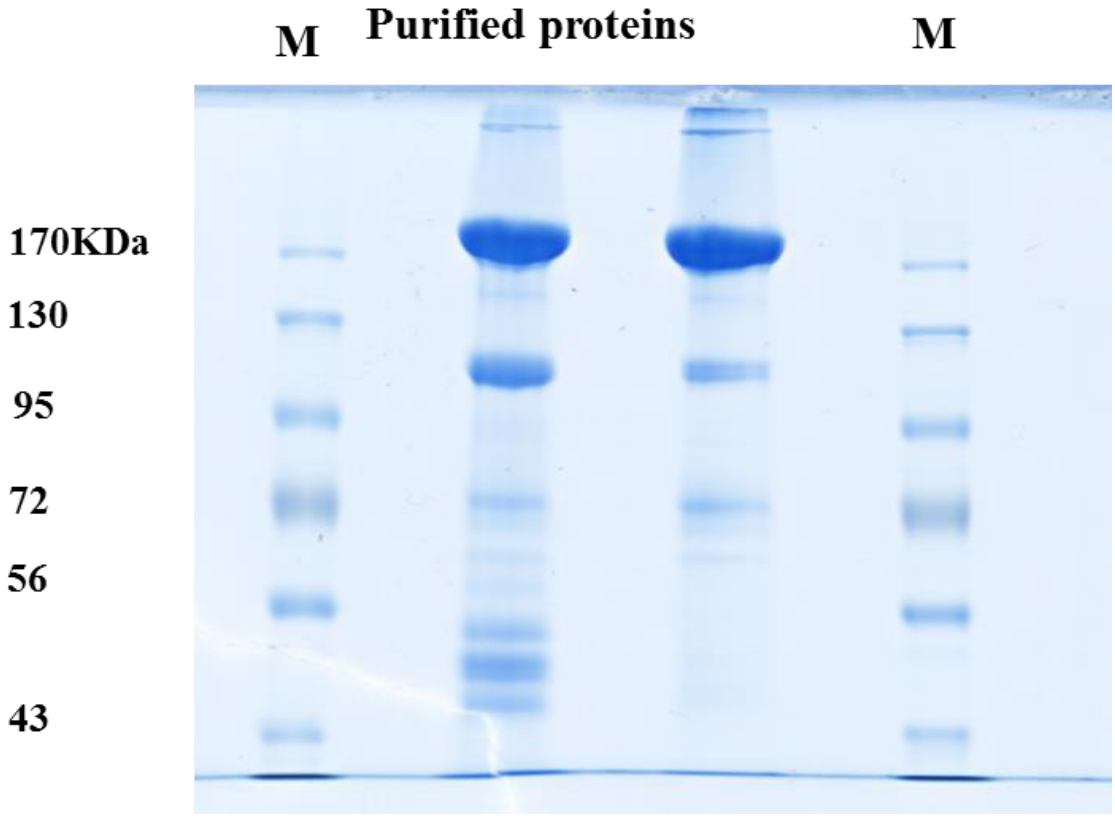


Figure 6.8 CD177-Fc proteins purified by Protein G–Sepharose chromatography were analysed by SDS–PAGE and stained with Coomassie blue. The gel scanned using an Image Scanner with MagicScan software to record electronic image. Lanes: M, protein molecular weight marker, 2 and 3, purified proteins.

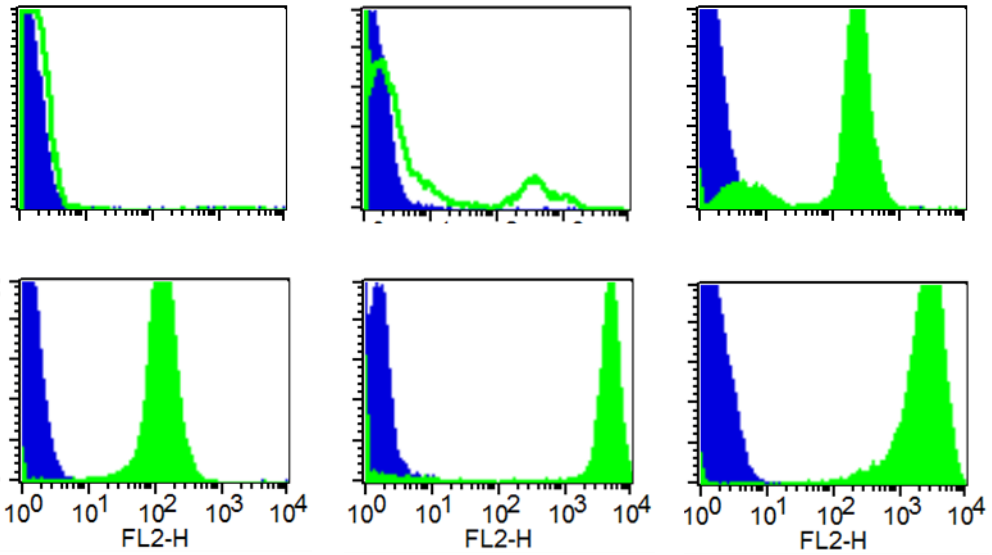


Figure 6.9 Flow cytometric detection of full-CD177 expression on CHO cells. CHO cells were transfected with full-CD177 DNA according to Lipofectamine® 2000 DNA transfection reagent protocol and cultured in 6-well plates. Top Row: Overlay histograms show the expression patterns of CD177 expression on different transfected CHO cell colonies (green) compared to isotype control (background; blue). The transfected cell clones showed different levels of CD177 expression. The first panel illustrates the lack of reactivity of MEM166 anti-CD177 with untransfected CHO cells. The second and third panels show clones with bimodal and incomplete CD177 expression. Bottom Row: Unimodal expressing clones ranging from moderate expression (bottom left) to high expression (last 2 panels) of CD177 on transfected CHO cells.

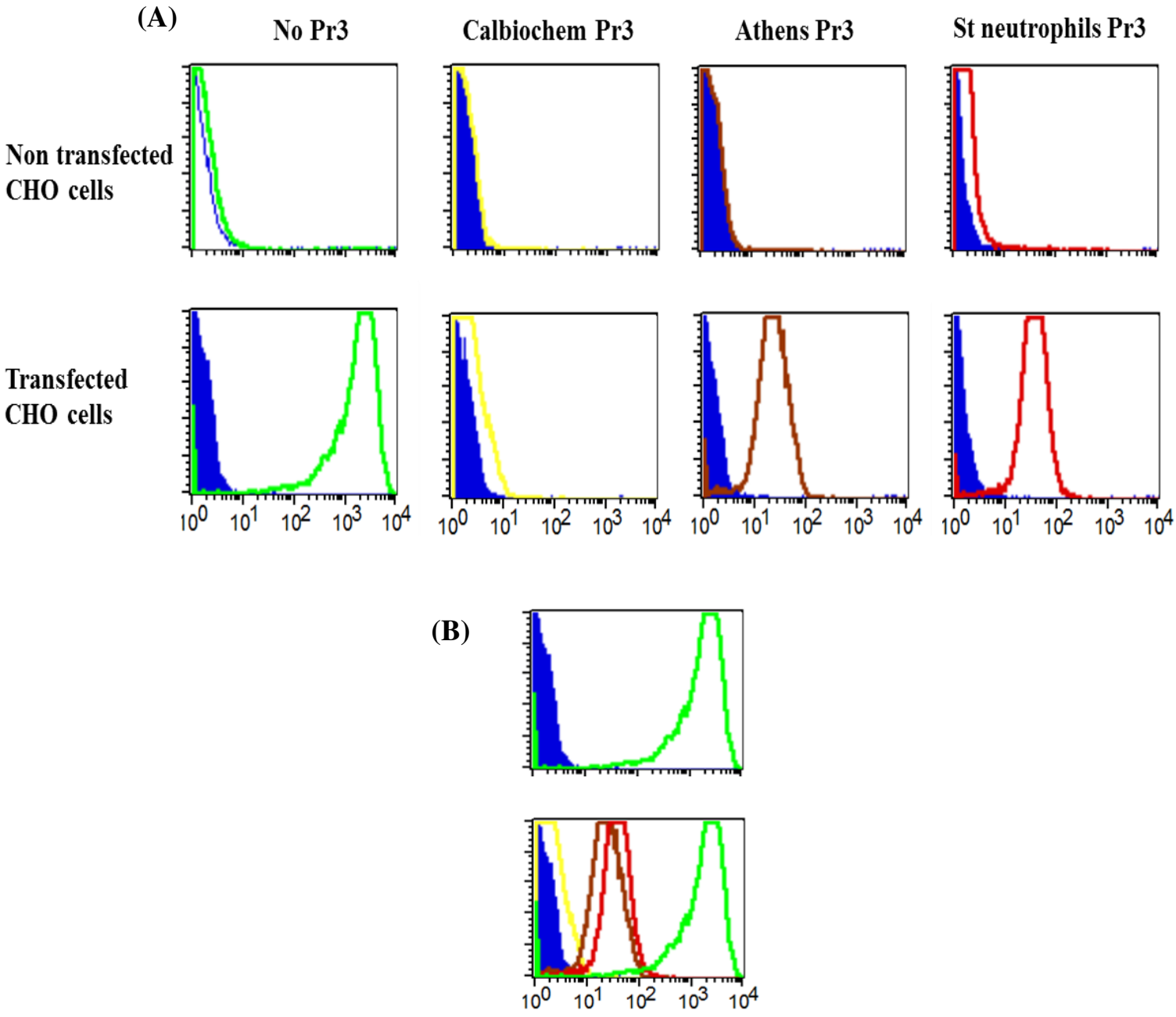
6.3.2 Binding of Pr3 (purified and PMNs stimulated supernatant) to CD177 expressed on transfected CHOs

Transfected CHO cells with high CD177 expression were re-cultured until they reached confluence. Once the cells reached a confluence of approximately 80-90%, the medium was aspirated. Human purified Pr3 from two different sources (Calbiochem and Athens Biotechnology) and supernatant from stimulated neutrophils were incubated with the transfected CHO cell monolayer for 20 min at 37˚C in a humidified 5% CO2 incubator. CHO cell layers were then disaggregated with flow cytometry medium (containing 15mM EDTA) and staining for Pr3 and CD177 using monoclonal antibodies, then the samples were analysed by flow cytometry as described in chapter 2.

Positive staining of transfected CHO was observed with monoclonal anti-Pr3, but not on non-transfected CHO cells indicating specificity of Pr3 binding. The histogram for isolated CHO cells showed that untransfected cells were not able to bind Pr3 from any of the sources. However, transfected cells illustrated a clear binding of Pr3 from Athens Biotechnology and Pr3 from stimulated human neutrophils supernatant, expected to occur via the surface expressed CD177 on CHO cells, but Pr3 from Calbiochem showed low binding compared to the other Pr3 sources (Figure 6.10A). Confirmation of the high level of expression of CD177 for the cells used in these experiments is shown in Figure 6.10B. Analysis of cumulative data presented in Figure 6.11 shows significant binding of Pr3 from Athens Biotechnology and stimulated fresh neutrophils to CD177-expressing CHO cells, but failure of the Calbiochem Pr3 to bind to these cells relative to the control cells (without Pr3).

6.3.3 Inhibition of CD177-bound Pr3 by AAT or human serum

In order to investigate the effect of AAT in inhibition of Pr3 binding to membrane-expressed CD177, CD177-transfected CHO cells with high expression were cultured until confluent and incubated with stimulated neutrophil supernatant in the presence of AAT (final concentration



Figur2 6.10 Flow cytometry investigation of Pr3 binding to CD177-expressing CHO cells. A) Non transfected and transfected CHO cells were incubated with Calbiochem Pr3 (yellow), Athens Biotechnology Pr3 (dark red), Pr3 from stimulated neutrophils supernatant (light red) compared to the isotype control (blue). B) Overlay histogram of MCF showing CHO cells expressing the membrane form of CD177 binding to different sources of Pr3 compared to the isotype control.

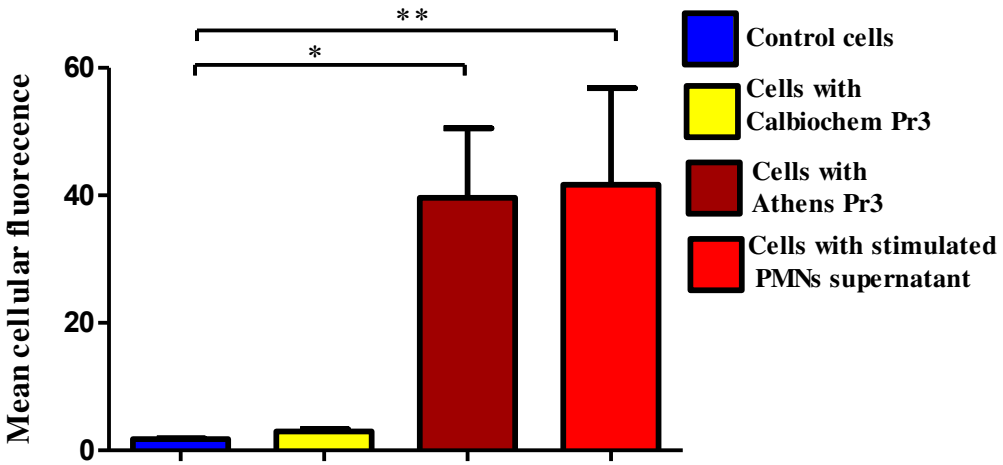


Figure 6.11 Bar graphs showing comparison of mean cellular fluorescence between different sources of Pr3 binding to CDI77 expressed on transfected CHO cells. The data are presented as average mean cellular fluorescence ± SEM from n=3 independent experiments.

2mg/ml added to the supernatant) for 20 min or incubated with stimulated neutrophil supernatant for 20 min then incubated with 2 mg/ml AAT for another 20 min. This was done to determine if bound Pr3 behaved differently than if Pr3 and AAT were both present during CD177 binding. Figure 6.12 illustrates that there was a significantly decreased binding of Pr3 to the surface of CD177-expressing CHO cells after treatment with stimulated neutrophil supernatant in presence of AAT. This indicated a loss of the Pr3 from the cellular surface. Moreover, adding AAT after Pr3 from neutrophil supernatant had bound in the absence of AAT also showed a decrease in the Pr3 binding. Although post-binding addition of AAT appeared not to reduce the Pr3 binding to the same degree as AAT present during the binding, these differences were not significant (Figure 6.12A).

Next I examined the effect of decreasing AAT concentration on the ability to inhibit Pr3 binding to CD177-expressing CHO cells (Figure 6.12B). A stepwise decrease in Pr3 binding inhibition was observed indicating 2 mg/ml may not have the maximum effect. All concentrations of AAT did cause shedding of Pr3 from the surface of CHO cells but 2 mg/ml and 2μg/ml of AAT resulted in a significant reduction (figure 6.12B). The physiological concentration of AAT is 3 mg/ml in serum and similar effects were seen when serum was used as the source of AAT (Figure 6.12C).

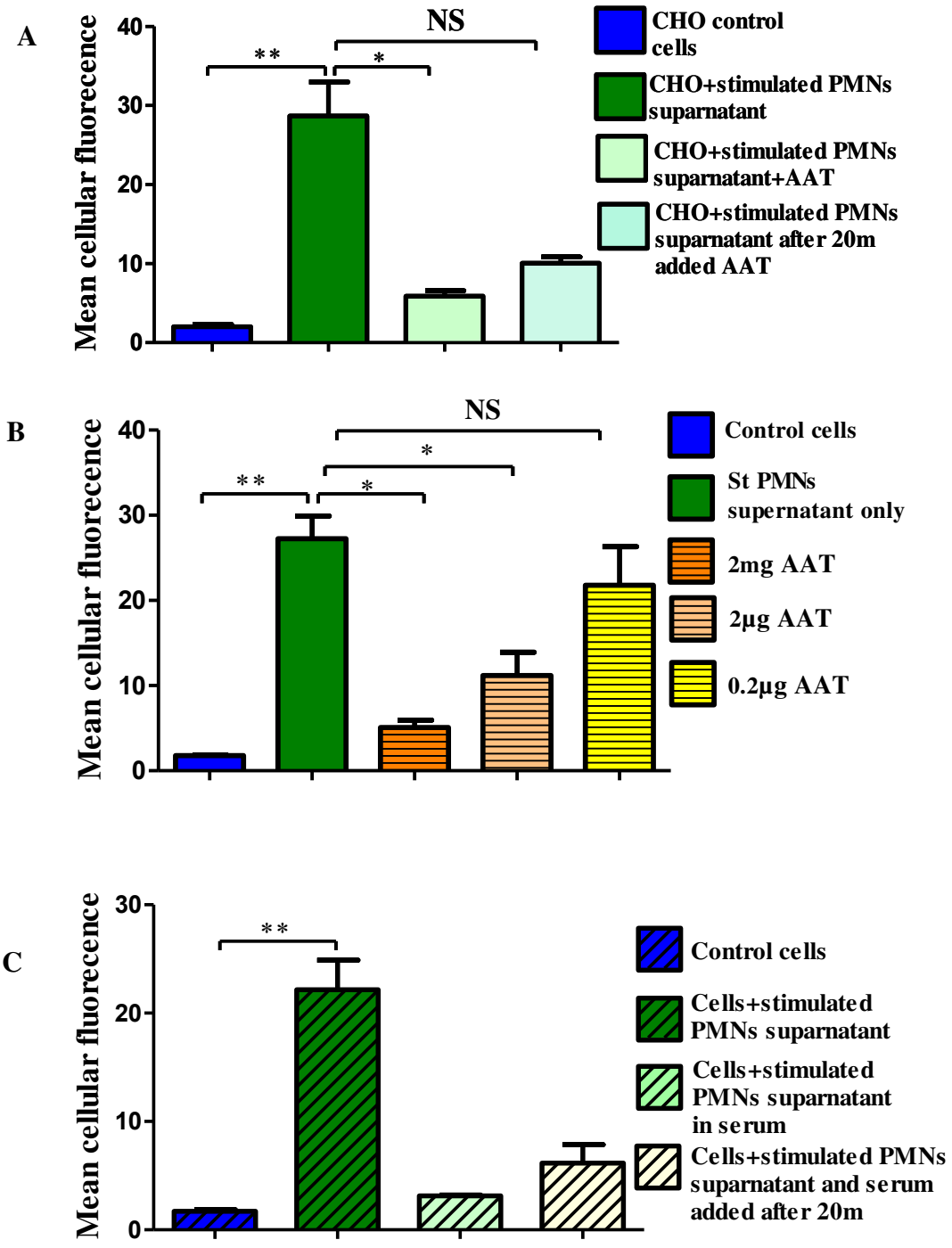


Figure 6.12 Influence of AAT or serum on Pr3 binding to CD177. Neutrophils were stimulated with maximum stimulation in absence or presence of human purified AAT or 100% autologous serum. A) CD177 transfected CHO cells were incubated with stimulated neutrophils supernatant only or with 2mg/ml purified AAT for 20 min or incubated with neutrophil supernatant for 20 min and washed then AAT added for another 20 min. B) Different concentrations of AAT were used to examine the most effective concentration of AAT. C) Serum was used as a physiological source of AAT.

6.3.4 Analysis of CD177-Fc and Pr3 Interaction by SPR technology

CD177 protein-protein interaction with Pr3 was examined in real time using surface plasmon resonance (SPR) technology on a Biacore 2000 machine. The preliminary work up was performed by Dr. David Cole (Infection and Immunity) for these studies. CD177-Fc could be immobilised very well either directly or indirectly (via immobilised anti-human IgG1 antibody) to CM5 chips. Unfortunately, incubation of Pr3 with the blank control cell on the CM5 chip resulted in significant increasing non-specific binding which made determining an accurate association and disassociation constants impossible. Unfortunately, due to the dimeric nature of the CD177-Fc fusion protein, affinity could not be determined only avidity. Therefore, despite 4 separate preparatory experiments with an experienced SPR investigator, further experiments were not pursued.

6.4 Discussion

Pr3 is one of the four serine protease homologues that are localized in granules and on plasma membrane of neutrophils. Following neutrophil priming, further amounts of Pr3 reach the extracellular environment as a freely secreted or as a membrane-bound protease. The surface-exposed fraction of secreted Pr3 is directly accessible to circulating c-ANCA, as seen in some individuals. Several membrane partners of Pr3 have been identified, such as CD16/FcγRIIIb, phospholipid scramblase-1, CD11b/CD18 and human neutrophil antigen NB1/CD177. CD177 also lacks a transmembrane domain. CD177 has been shown to present Pr3 on the neutrophil surface and permits Pr3-ANCA binding to the $CD177^{pos}$/$mPR3^{high}$ subset (von Vietinghoff *et al.,* 2007; Bauer *et al.,* 2007). Therefore, in this study we elucidated the binding and distribution of existing CD177–Pr3 membrane complexes. To achieve this goal we created the CD177-Fc fusion protein and CD177 expression on CHO cells by recombinant technology.

CD177 was first described in 1971 as a neutrophil-specific antigen in a case of neonatal alloimmune neutropenia (NIN) (Lalezari et al., 1971). It is exclusively expressed on human neutrophils and it has a mass of 58 to 64 kD on analysis by SDS-PAGE and 50.5 kDa as determined by MALDI-TOF mass spectrometry (Kissel *et al.,* 2001). Direct physical interaction of CD177 with PECAM-1 on endothelial cell membrane was demonstrated, thus CD177 plays a role in neutrophil transmigration (Sachs *et al.,* 2007). Göhring *et al* (2004) reported that the CD177 surface expression is greater in patients with bacterial infections, polycythemia rubra vera and neutrophils of G-CSF stimulated donors compared with healthy donors. The expression of CD177 and Pr3 ranges from 0 to 100%. A previous study by Korkmaz *et al.,* using gibbon/human Pr3 hybrids mapped a Pr3 region of closely clustered hydrophobic residues Phe166, Trp218 and Leu223, and showed its importance for the binding to human CD177 and adding AAT caused dissociation of Pr3 from the CD177

receptor, most probably by conformational distortion of the hydrophobic loop on Pr3. Preventing the association of Pr3, or disrupting established cell surface complexes, would have obvious therapeutic implications for Pr3-ANCA-mediated neutrophil activation. AAT binds covalently to all SNP and therefore has effects that go beyond the Pr3/CD177 interaction.

In this study I analyzed the binding of the different Pr3 sources to CD177 on CHO cells (Athens Biotechnology, Calbiochem and supernatant of stimulated neutrophils), in the absence of any other potential neutrophil proteins that could also act as Pr3 receptors. All sources of Pr3 were detected on the plasma membrane of CD177 receptor-expressing CHO cells, but Pr3 from Calbiochem showed low binding. No interaction was observed with non-transfected CHO cells using all three sources of Pr3 (Figure 6.10), therefore any residual Pr3 that could not be removed by serum or exogenous AAT must be associated with CD177 on the transfected CHO cells. Korkmaz *et al.,* 2008 reported that at high concentrations (15µg/ml), human Pr3 adhered to membranes of CD177 receptor negative CHO cells, which suggested that an additional mechanism of hydrophobic binding direct lipid insertion, was operating under these conditions when present in concentrations above physiological levels.

In this study we used stimulated neutrophil supernatant as a source of Pr3 to investigate the effect of AAT on CD177/Pr3 complex. Herein we stimulated neutrophils in the presence of AAT or we added AAT after 20 minutes to examine if there was a difference in the ability of AAT to remove Pr3 after binding CD177. Consistent with the results reported by others, Pr3 binding to CD177 expressed on the surface of CHO transfected cells was cleared from the surface by AAT at a concentration of 2 mg/ml (Korkmaz et al., 2008). In addition, I found that there was no statically significant difference in the ability of AAT to disrupt the binding of Pr3 to CD177 if added during neutrophil stimulation or after Pr3 binding to CD177.

However, there was a consistent trend to higher levels of Pr3 being bound to CD177 if added after binding, despite the fact that it did not reach significance.

Also I examined different concentrations of AAT on CD177/Pr3 complexes and the result showed a stepwise increase in Pr3 loss from the surface of cells and AAT concentration of 2 mg/ml and 2µg/ml resulted in a significant decrease in CD177-bound Pr3. These data were confirmed when human neutrophils were used as the target of Pr3 binding as shown in chapter 3. However, even at the concentration of 2 mg/ml or 2µg/ml, AAT did not remove Pr3 completely from the surface of CHO cells or neutrophils. Given that untransfected CHO cells could not bind significant amounts of Pr3, the residual binding must be in the context of CD177-Pr3 complex.

6.5 Summary

In this chapter I have presented that physiological and recombinant AAT are able to remove Pr3 from CD177 receptor-expressing CHO cells. Further experiments are needed to finish investigating CD177 protein-protein interaction with Pr3 by SPR technology

CHAPTER 7

THE CELLULAR LOCATION OF NEUTROPHIL PROTEINASES WITHIN NEUTROPHILS

7.1 Introduction

Neutrophils contain 3 separate classes of proteinase containing granules: primary (azurophilic) granules (containing granule marker CD63 and NE, Pr3, and cathepsin G), secondary granules (containing granule marker CD66b and MMP-8) and tertiary (MMP-rich) granules (contraining MMP-9) (Borregaard *et al.,* 1997). It has become dogma that these types of granules are fundamentally different. A great majority of the neutrophil functions are dependent on the mobilization and release of these granules and their content that convert a circulating neutrophil into an active participant in the immune and inflammatory responses (Sengeløv *et al.,* 1995).

Upon exposure to inflammatory mediators, each neutrophil granule is exocytosed in a hierarchic manner and sequential order. These granules either release their contents (by exocytosis or degranulation) into the phagosomes or they fuse with the plasma membrane and release to the extracellular space. Exocytosis in human neutrophils is a complex event. The granules fuse independently of each other with the plasma membrane (simple exocytosis which is the predominant mechanism in human neutophils) or fuse first with other granules in the cytosol prior to their subsequent fusion with the plasma membrane (compound exocytosis) or one granule fuses initially with the plasma membrane, followed by fusion of a second granule to the membrane of the granule already engaged in fusion, thus forming a degranulation sac (cumulative fusion) (Lollike *et al.,* 2002).

Using confocal microscopy, the intracellular distribution of neutrophil proteinases was compared with the location of the 2 granule subsets prior to and after stimulation. Primary conjugated monoclonal, and secondary conjugated monoclonal antibodies were used for single or double staining. Confocal microscopy was used to test whether two fluorescently labelled molecules are associated with one another.

Co-localisation analysis was performed to quantify the overlap of two fluorescent signals at the same sub-cellular location. Co-localisation analysis of two fluorescence (dye) channels (two proteins) broadly divides into two categories. The first category are methods that simply consider the presence of both fluorophores in individual pixels, which known as Qualitative Co-localisation analysis (such as Dye Overlay). This method is both simple and widely used but has a number of drawbacks (Li Q et al., 2004). In contrast, quantitative co-localisation uses the intensity vales of each pixel to calculate the interdependence of the two variables as a pixel by pixel correlation coefficient.

7.2 Aims

The aim of the work presented in this chapter was to establish whether specific proteinases were located within particular granule sub-types within human neutrophils. The distribution of neutrophil proteinases and markers of 2 granule subsets was imaged by confocal fluorescence microscopy. The co-localisation of a particular protease with particular granule markers was quantified to draw conclusion about proteinase location within granule sub-types.

7.3 Results

Neutrophils, isolated from blood of healthy volunteers analysed as unstimulated or following stimulated with maximum stimulation before fixation and permeabilization as described in chapter 3. Neutrophils were stained for Pr3, NE, CD177, MMP-8 and MMP-9, individually with CD63 or with CD66b. FITC and Cy3 were used as secondary antibodies in combination with rabbit and mouse monoclonal antibodies. Granule specific markers (CD63, and CD66b) were co-stained with proteinases of interest.

After confocal images were taken, the degree of co-localisation was statistically determined. This was done using ImageJ software with two different Plugins; intensity correlation analysis (ICA) and Just Another Co-localisation Plugin (JACoP). Both plugins provide statistical data for colocalization analysis (such as Pearson's coefficient and Mander's Coefficients)

The JACoP software automatically thresholds images removing user bias and providing a quantitative value of the extent of co-localisation (Richer 2011). The Pearson's correlation coefficient (r(obs); in JACoP), also known as Rr; in ICA), measures the covariance between the intensities of each channel in each pixel, and is not sensitive to background or co-localised pixel intensity. It has a linear regression range of -1 to 1, with -1 being total negative correlation, 0 being a random correlation, and 1 being total positive correlation (where all the pixel intensities correspond). Pearson's coefficients values higher than 0.5 are considered to be indicative of co-localisation, however, the most important parameters to note are the r(obs), r(rand), and their p-value. These three values were required to statistically determine the extent of colocalisation between two markers (Richer 2011). In this study I used CD63 as marker of primary granules and CD66b for secondary granules. I found that the size of CD63 granules was about 900nm whereas the size of CD66b granules was about 400-600nm.

7.3.1. Co-localisation of NE with CD63

Unstimulated and stimulated neutrophils were incubated with unconjugated monoclonal anti-NE which was detected by CY3-conjugated donkey anti-mouse antibody. CD63 was detected by a third staining step using FITC-conjugated monoclonal CD63. In the absence of stimulation, the co-localisation between these molecules was good with Pearson's correlation coefficient = 0.7 in both methods of analysis (fig 7.1). This agrees with the accepted view that CD63 and NE are both located in the primary granules. Stimulation of neutrophils did not alter the level of co-localisation (r=0.67 or 0.68).

7.3.2 Co-localisation of MMP-8 with CD63

The sub-cellular locations of neutrophil primary granule marker CD63 and MMP-8 was evaluated in unstimulated and stimulated neutrophils. There was no co-localisation between these two proteins in either the absence or presence of stimulation (fig 7.2). The correlation coefficient being 0.469 and 0.445 in unstimulated cells and 0.497 and 0.476 in stimulated cells.

7.3.3 Co-localisation of MMP-9 with CD63 in unstimulated but not stimulated cells

In the absence of stimulation, MMP-9 and CD63 were co-localised in neutrophils, (fig 7.3). with correlation coefficients of 0.7-0.5. This was surprising, as it is well established that the majority of neutrophil MMP-9 is stored in tertiary granules, but CD63 is a membrane marker of azurophilic granules. After stimulation, the co-localization between the two proteins was lost or significantly reduced, with correlation coefficients of 0.269 and 0.599.

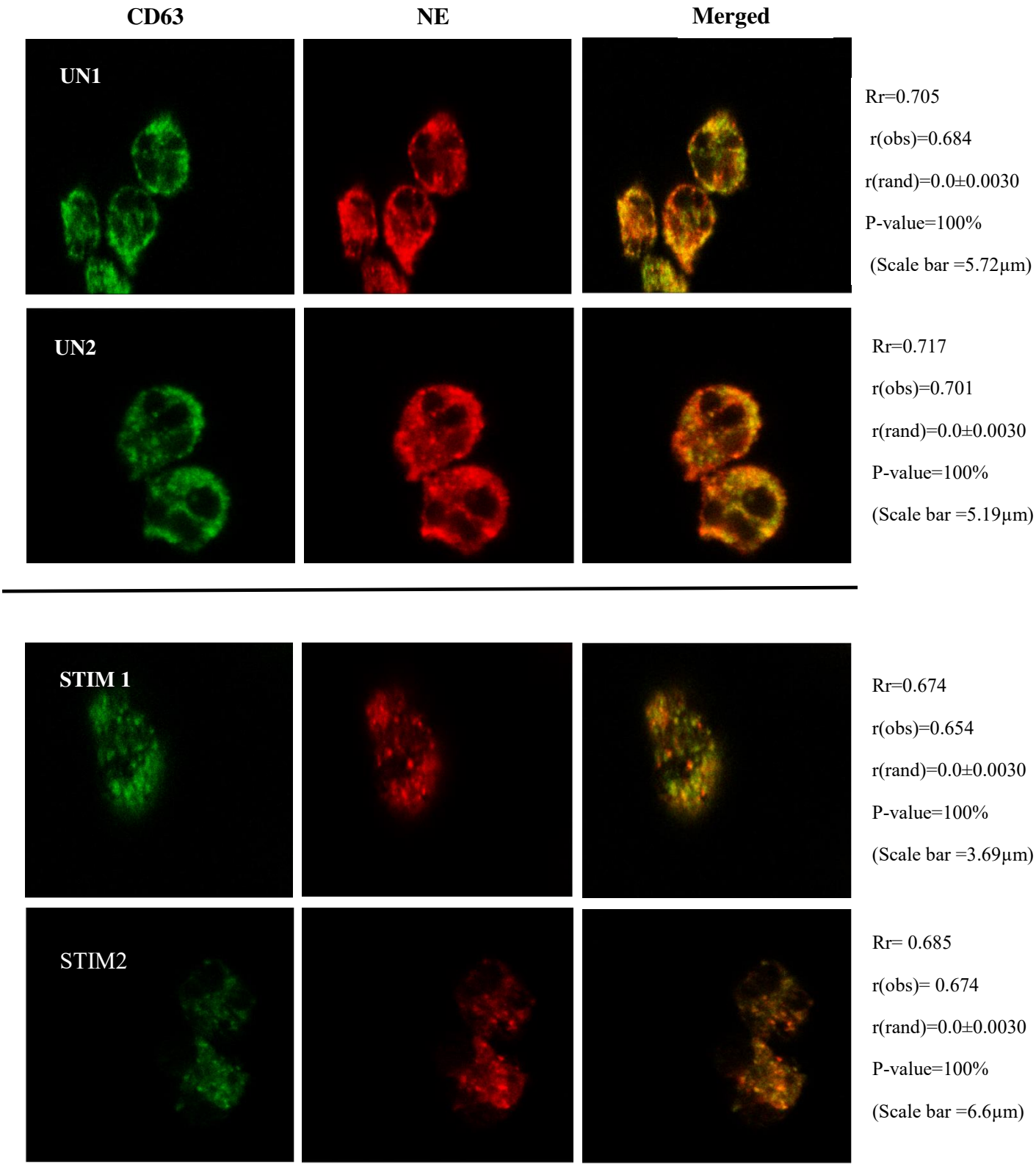


Figure 7.1 Co-localisation analysis of NE and CD63 in unstimulated (UN1 and UN2) and stimulated neutrophils (STIM1 and STIM2). Neutrophils were stained for CD63 (green) and NE (red). Overlay images demonstrate co-localization of green and red-stained molecules by a shift toward yellow colour in some areas inside the cells (n=2).

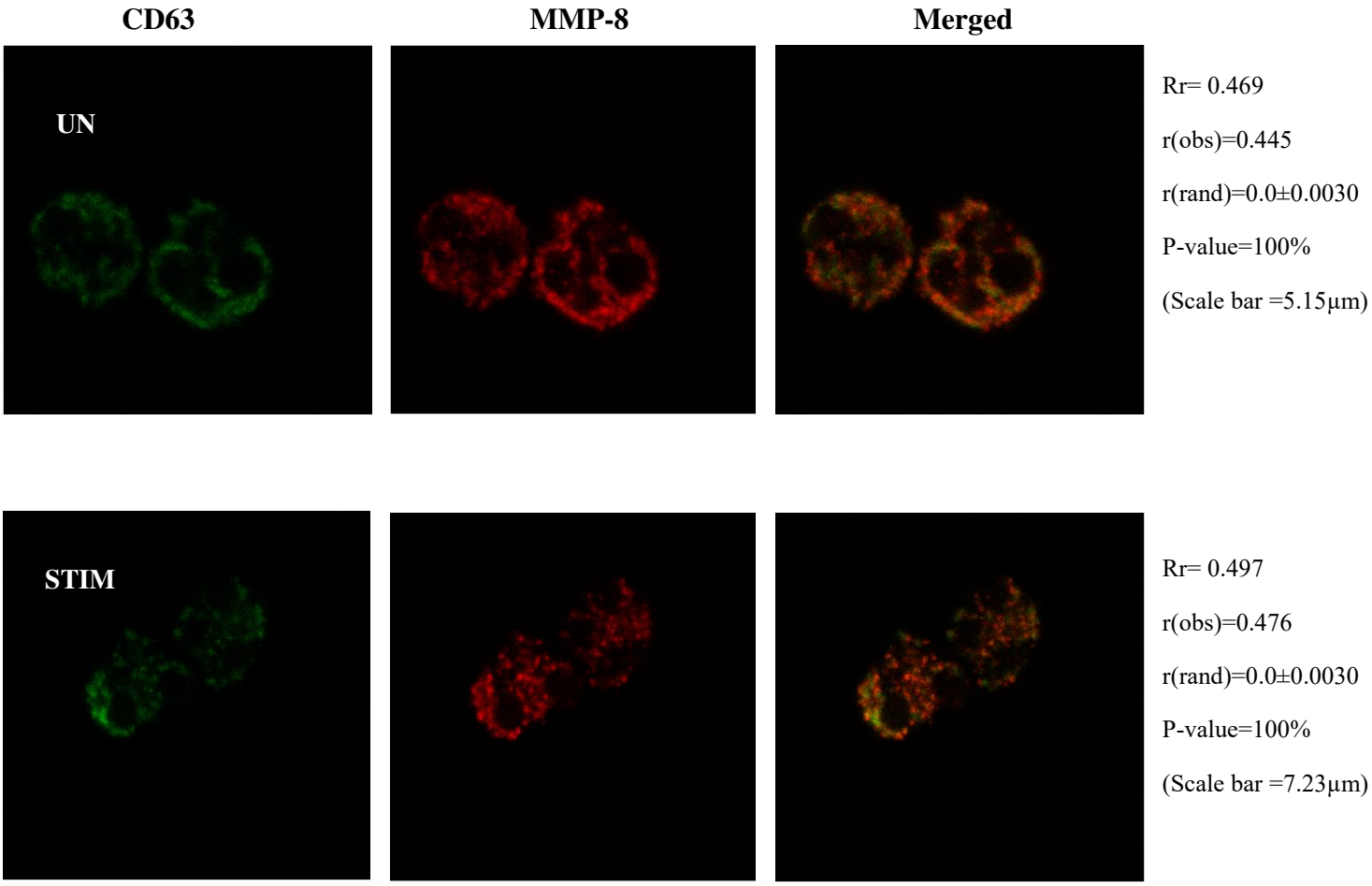
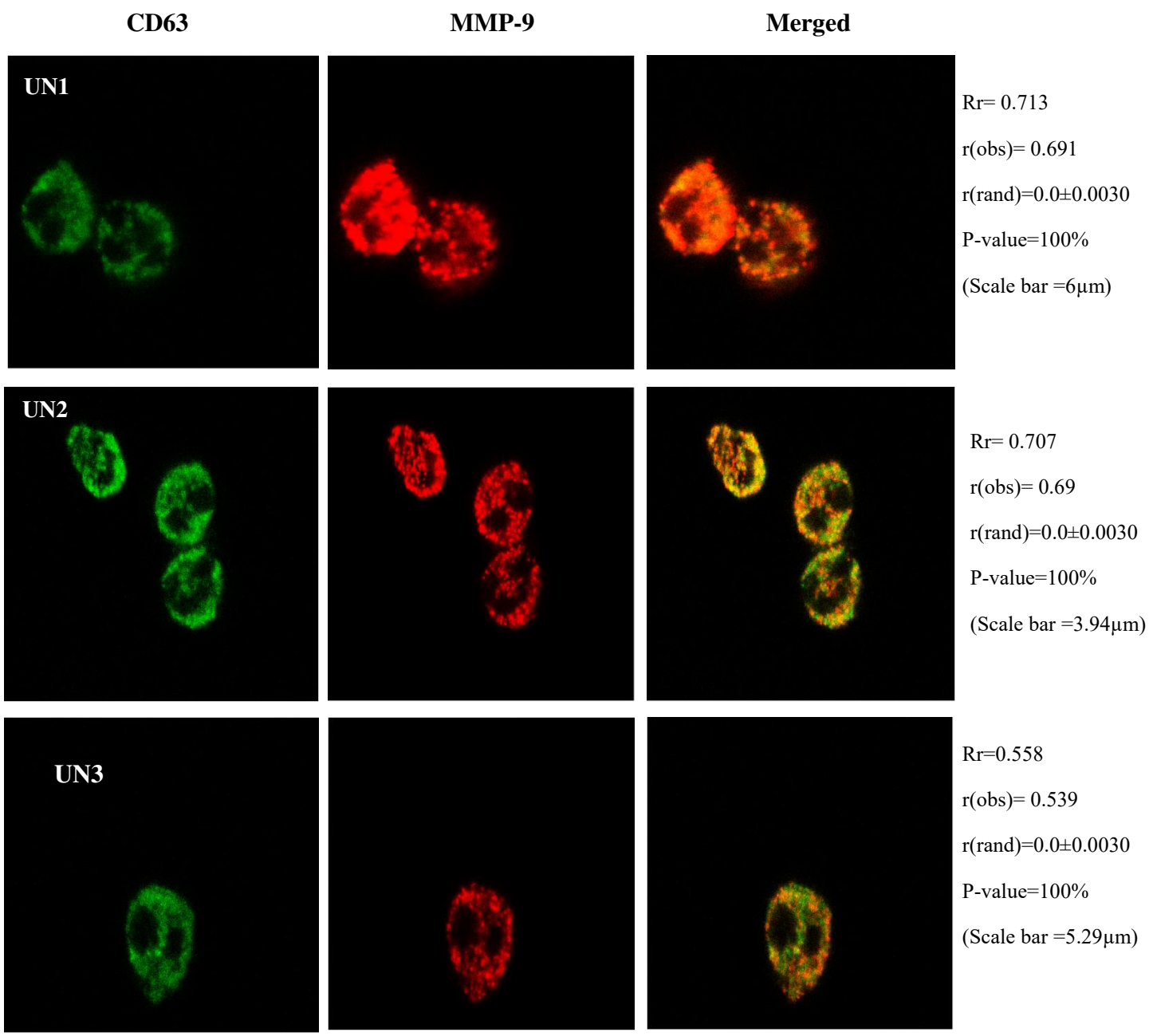


Figure 7.2 Co-localisation analysis of CD63 and MMP-8 in unstimulated (UN) and stimulated neutrophils (STIM). The merged images of CD63 and MMP-8 are shown on the right, with no yellow colour indicating no co-localisation between the two molecules (n=3).

(A)


CD63
MMP-9
Merged
UN1
Rr= 0.713
r(obs)= 0.691
r(rand)=0.0±0.0030
P-value=100%
(Scale bar =6µm)
UN2
Rr= 0.707
r(obs)= 0.69
r(rand)=0.0±0.0030
P-value=100%
(Scale bar =3.94µm)
UN3
Rr=0.558
r(obs)= 0.539
r(rand)=0.0±0.0030
P-value=100%
(Scale bar =5.29µm)

(B)

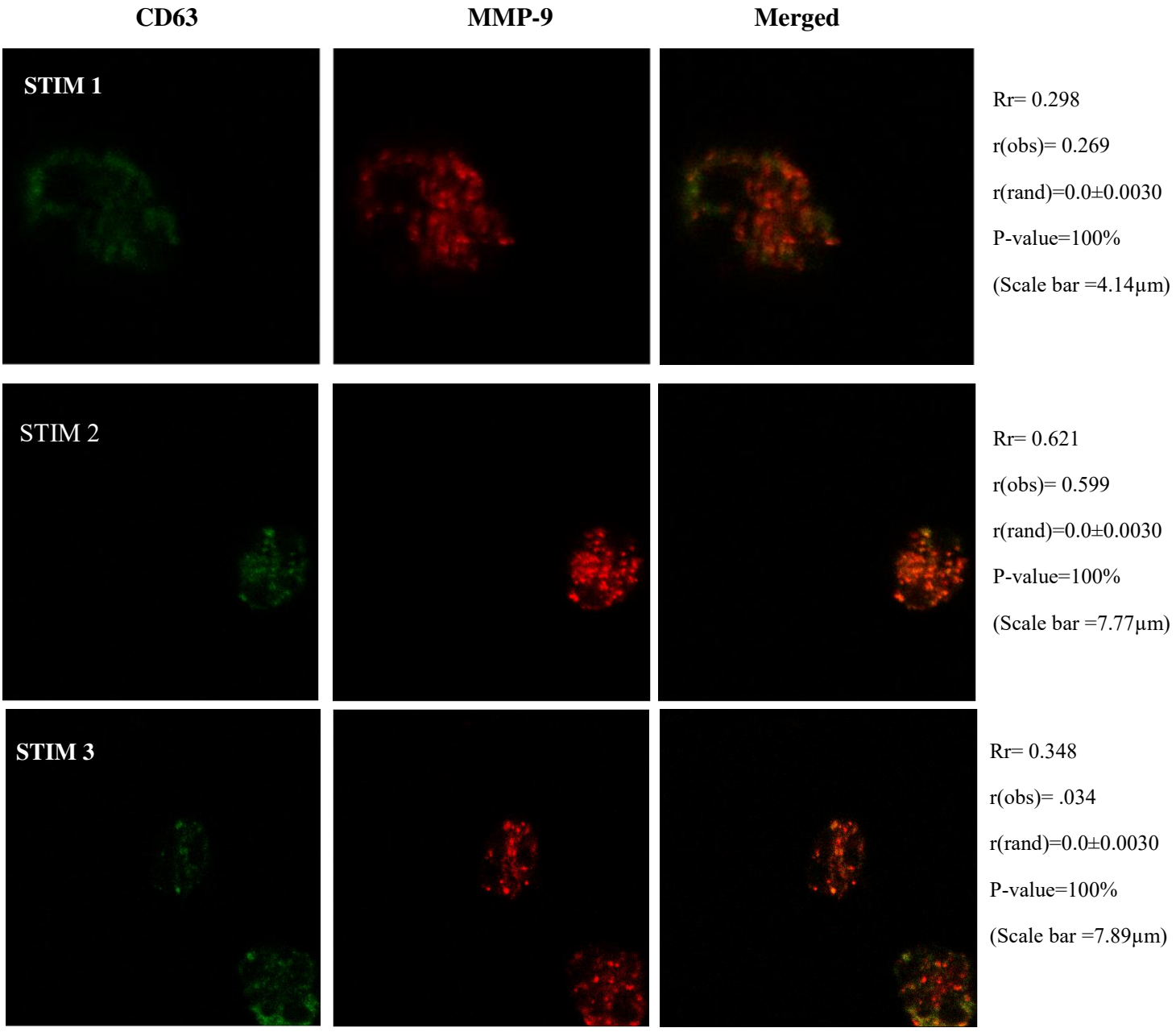


Figure 7.3 Confocal images to detect co-localisation of CD63 and MMP-9 in unstimulated (A; UN) and stimulated (B; STIM) neutrophils. MMP-9 was labelled using indirect immunofluorescence with CY3 conjugated antibody and CD63 was detected using FITC-conjugated monoclonal antibody. Rr or r = Pearson coefficient of correlation.

7.3.4 No co-localisation of CD177 with CD63

To evaluate the co-localization between CD177 and CD63, neutrophils were stained with unconjugated mouse anti-human CD177 and detected by CY3-conjugated donkey anti-mouse antibody and CD63 was detected by FITC-conjugated monoclonal CD63. The results showed that in unstimulated cells there was minimal co-localization between the two proteins (r(obs)=0.5). In response to stimulation, co-localization was reduced further, with the r(obs) decreasing to 0.129.

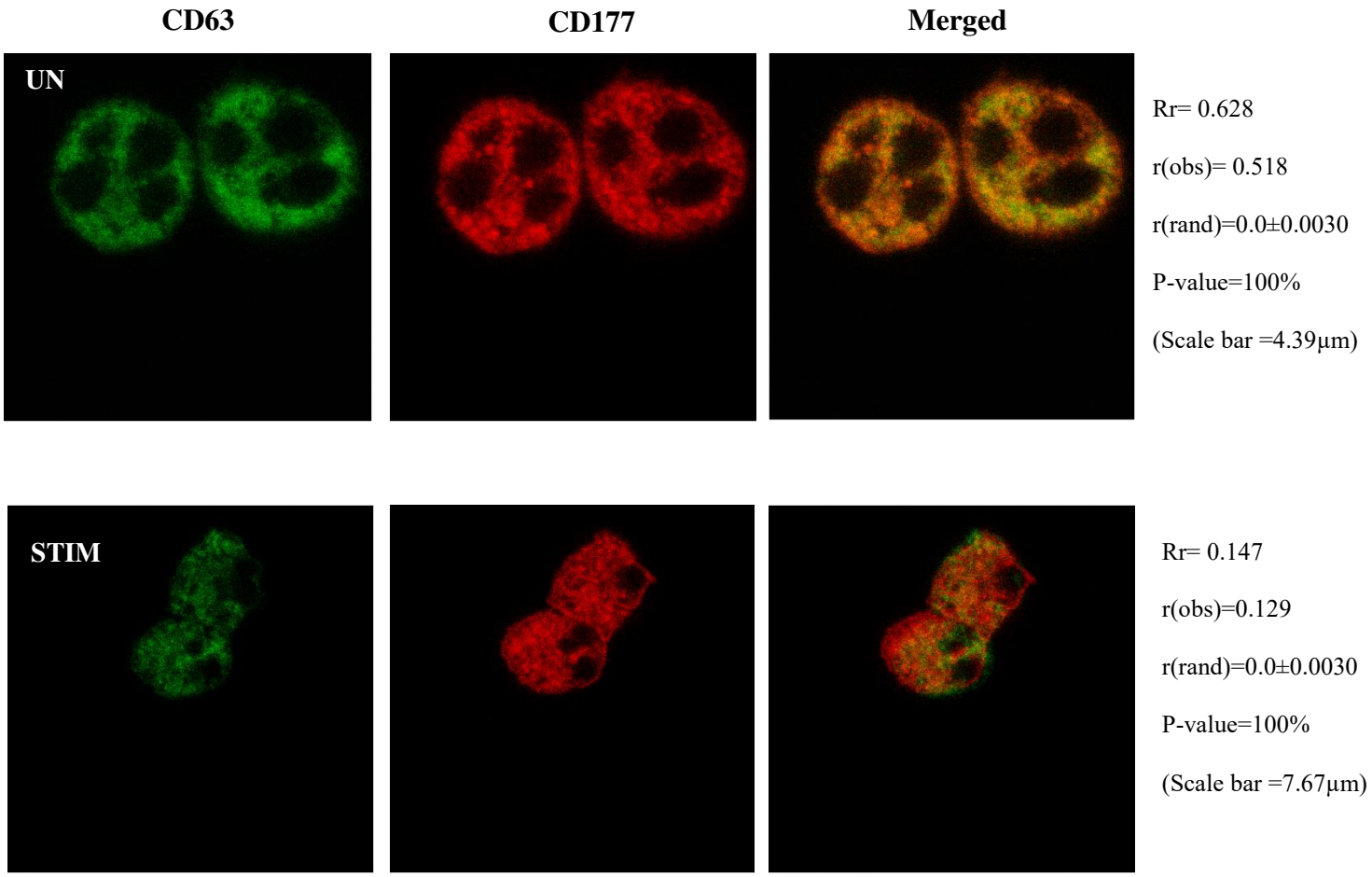


Figure 7.4 Co-localisation of CD63 and CD177 in unstimulated (UN) and stimulated (STIM) neutrophils. Human neutrophils were isolated and left without stimulation or stimulated with cytochalasin B (cytoB) followed by addition of fMLP for 15 min, after which they were fixed, permeabilized, and stained for CD177 and CD63.

7.3.5 Co-localisation of CD177 with CD66b

CD177 is a glycosyl-phosphatidylinositol (GPI) anchored glycoprotein (a high affinity Pr3 receptor) that is expressed on neutrophil plasma membranes and secondary granules. In the absence of stimulation, CD177 and CD66b were co-localised in neutrophil (fig 7.5). However, the results showed that after stimulation, this co-localisation was lost. The co-localisation parameter falling from 0.856 in resting cells to 0.5-0.6 after stimulation (fig 7.5).

7.3.6 Co-localisation of Pr3 with CD66b

CD66b is reported to be localized within the membrane of specific granules (Jost *et al.,* 1991). However study by Mollinedo *et al.,* demonstrated that CD66b is present in the membranes of both specific and tertiary granules. Pr3 is thought to be localized in the specific granules, but not in tertiary granules (Witko-Sarsat *et al.,* 1999). However, confocal microscopy images show minimal co-localisation between Pr3 and CD66b in the absence of neutrophil stimulation (r=0.634 and 0.577). In particular, there were often prominent granules staining with Pr3 that were not identified by CD66b (see open arrows in fig 7.6) and the occasional large CD66b staining granule with little Pr3 positivity (closed arrow fig 7.6). The co-localisation between the two proteins in response to maximum stimulation was similar, being slightly reduced compared to unstimulated cells (fig 7.6).

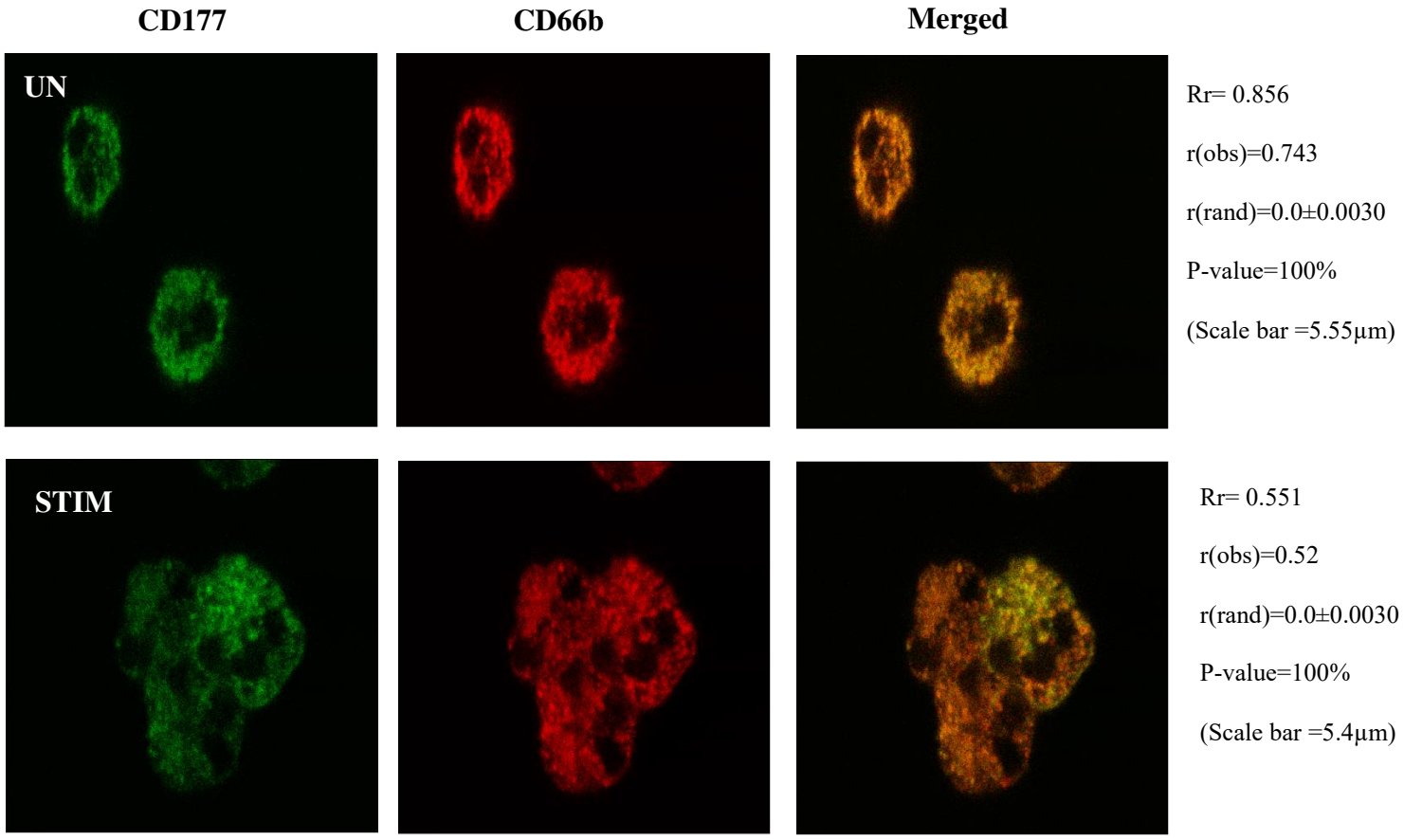


Figure 7.5 Co-localisation of CD66b and CD177 in unstimulated (UN) and stimulated (STIM) neutrophils. CD66b was labelled using indirect immunofluorescence with CY3 conjugated antibody and CD177 was detected using FITC-conjugated monoclonal antibody. Rr or r = Pearson coefficient of correlation.

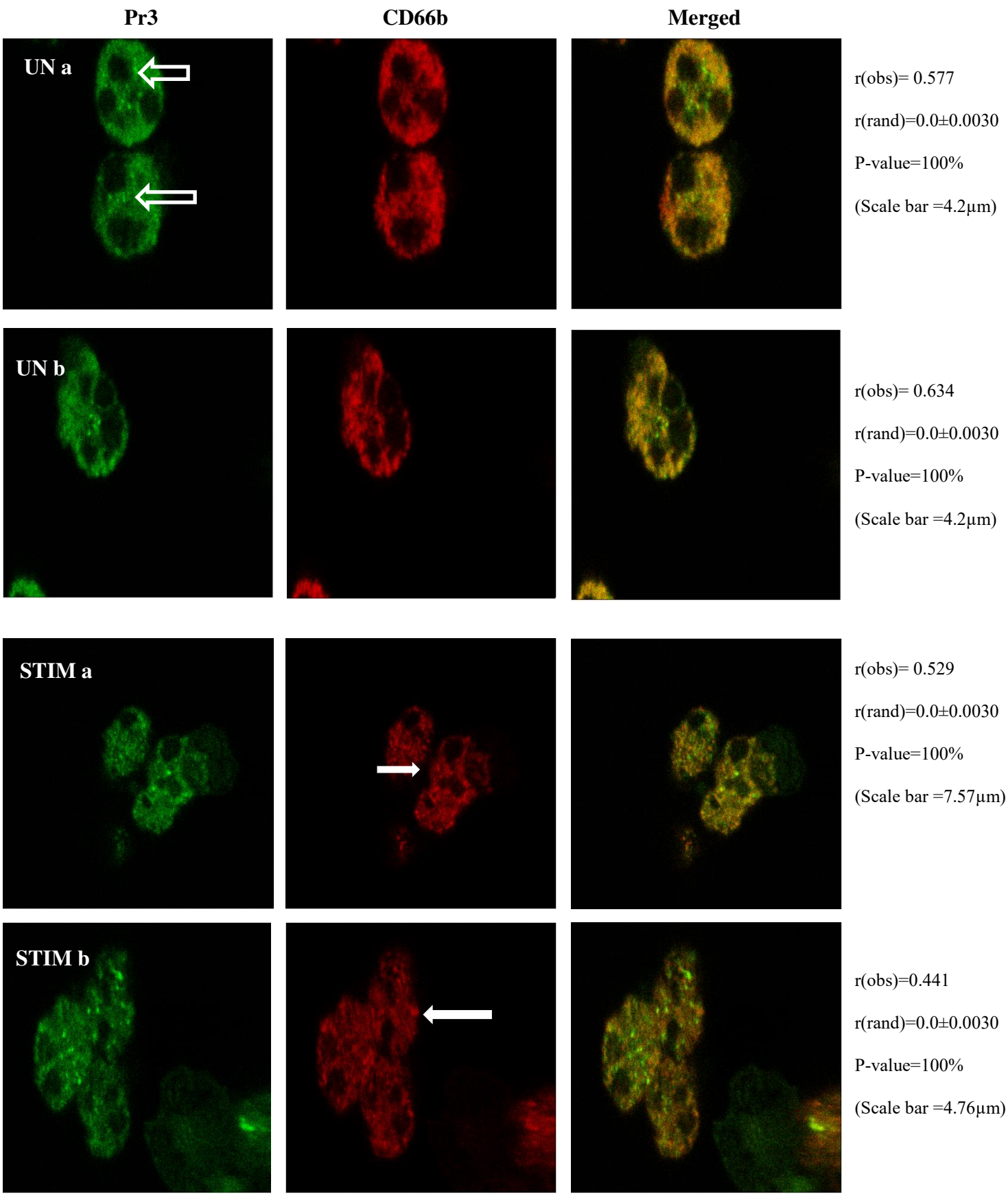


Figure 7.6 Confocal microscopy analysis of colocalization between Pr3 (green) and CD66b (red) immunofluorescence in human neutrophils. Neutrophils were left without stimulation (UN) or stimulated (STIM) with cytochalasin B (cytoB) followed by addition of fMLP for 15 min, after which they were fixed, permeabilized, and stained for CD166b and Pr3.

7.3.7 No co-localisation between Pr3 and MMP-8

There was no co-expression of Pr3 with MMP-8 either before or after stimulation, the Pearson coefficient being less than 0.5 in either case (fig 7.5). This result is consistent with the established location of MMP-8 in secondary granules within neutrophils from where it is secreted as an inactive enzyme, and activated by autolytic cleavage.

7.3.8 Co-localisation between Pr3 and MMP-9

In the absence of stimulation, there is co-expression between Pr3 and MMP-9, the Pearson coefficient being about 0.7. This was surprising as the majority of MMP-9 is thought to be in tertiary granules formed in the later stages of neutrophil maturation; whereas there is no evidence that Pr3 is located in the tertiary granules. However, in response to stimulation, the co-localisation was lost, the Pearson coefficient decreasing to between 0.5 and 0.4. This is consistent with release from separate granule pools. However, there were examples of granules near the cell edge, which may have undergone exocytosis in which there appeared to preferential loss of Pr3 and retention of MMP-9 (see for example, box in image 7.8B).

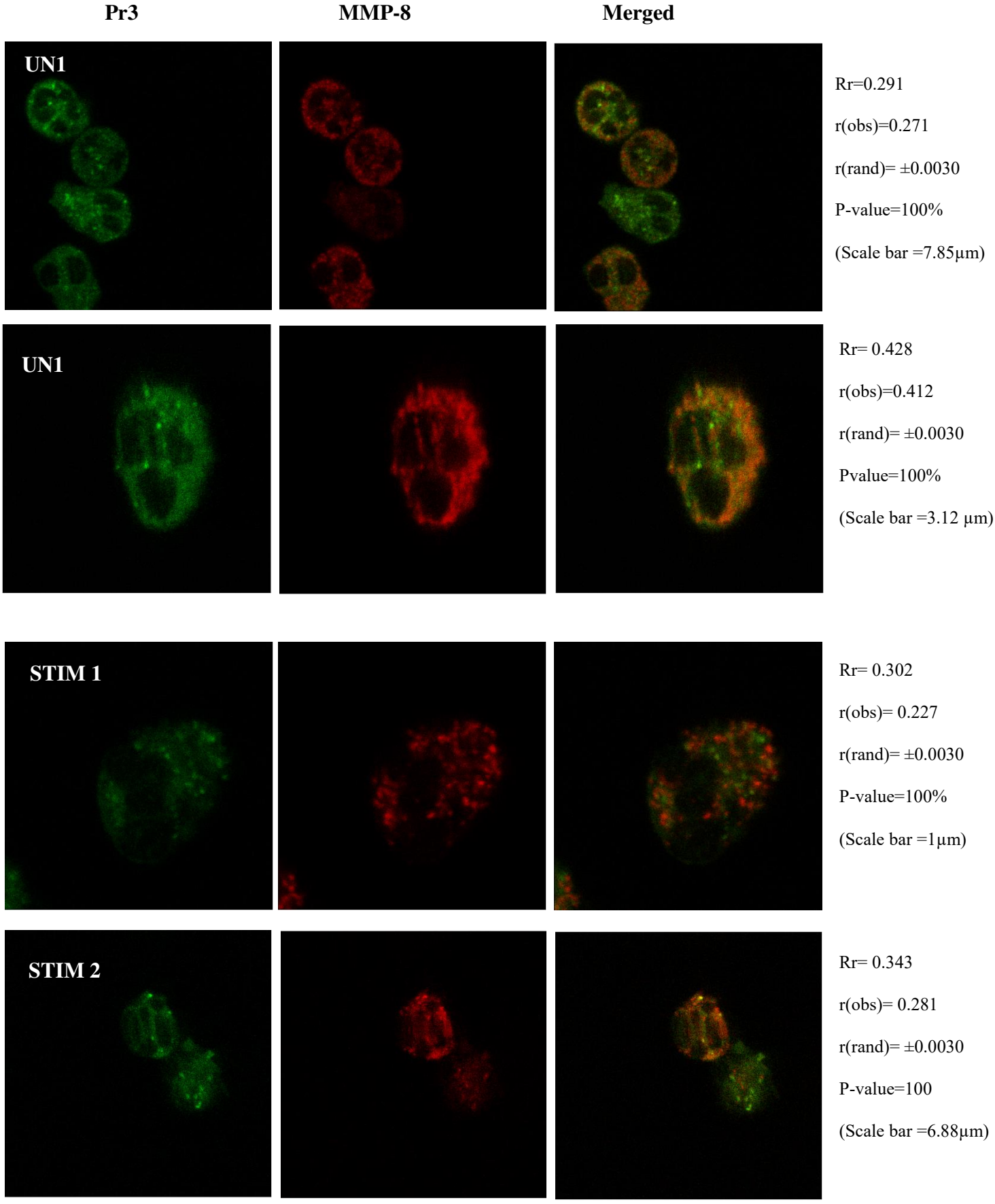
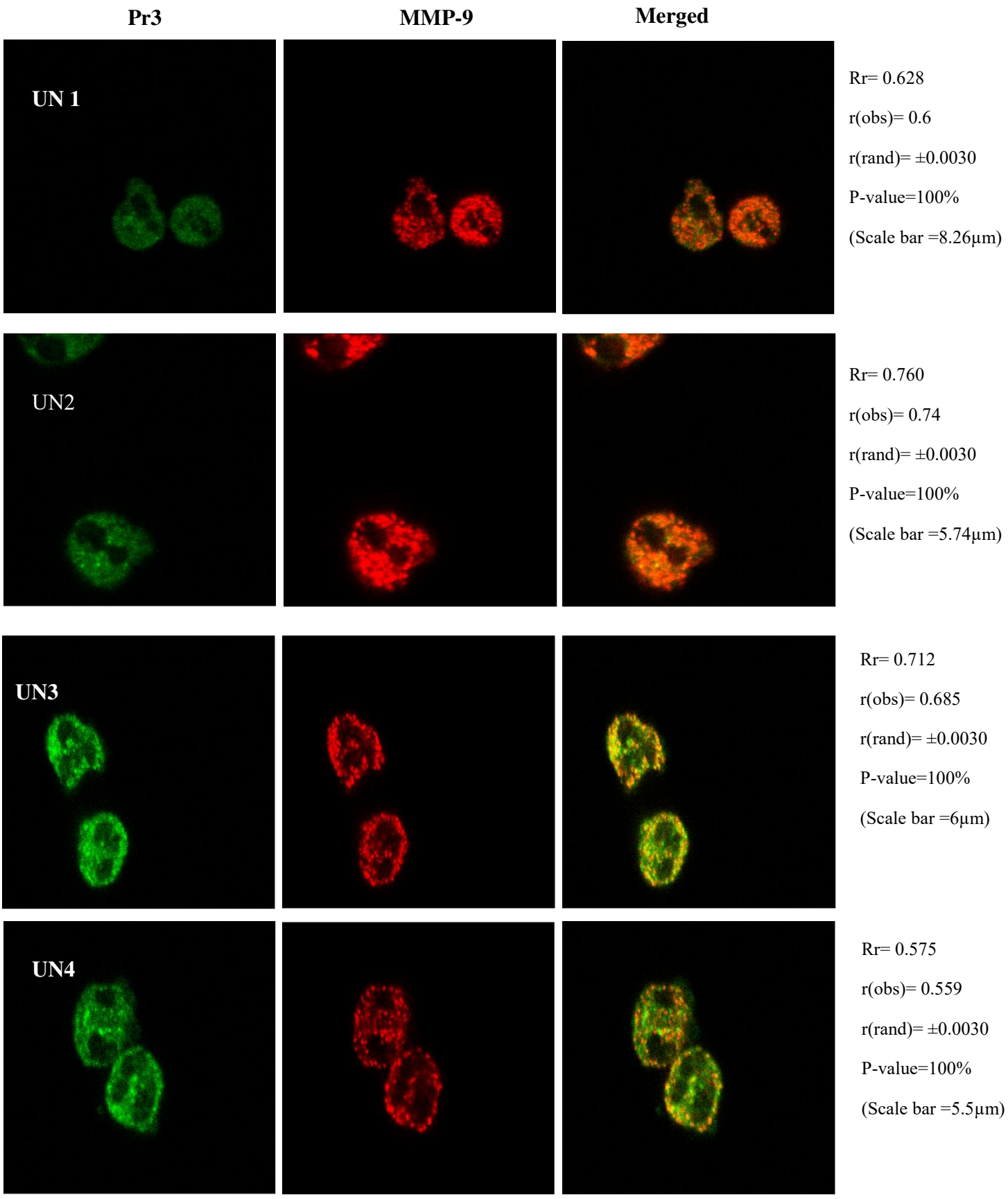


Figure 7.7 Co-localisation between Pr3 and MMP-8 in unstimulated (UN1 &UN2) or stimulated (STIM1 & STIM2) neutrophils. The cells were imaged using confocal laser scanning microscopy. The merged images show no colocalisation between Pr3 and MMP-8, determined using ImageJ software.

(A)


Pr3
MMP-9
Merged
UN 1
Rr= 0.628
r(obs)= 0.6
r(rand)= ±0.0030
P-value=100%
(Scale bar =8.26µm)
UN2
Rr= 0.760
r(obs)= 0.74
r(rand)= ±0.0030
P-value=100%
(Scale bar =5.74µm)
UN3
Rr= 0.712
r(obs)= 0.685
r(rand)= ±0.0030
P-value=100%
(Scale bar =6µm)
UN4
Rr= 0.575
r(obs)= 0.559
r(rand)= ±0.0030
P-value=100%
(Scale bar =5.5µm)

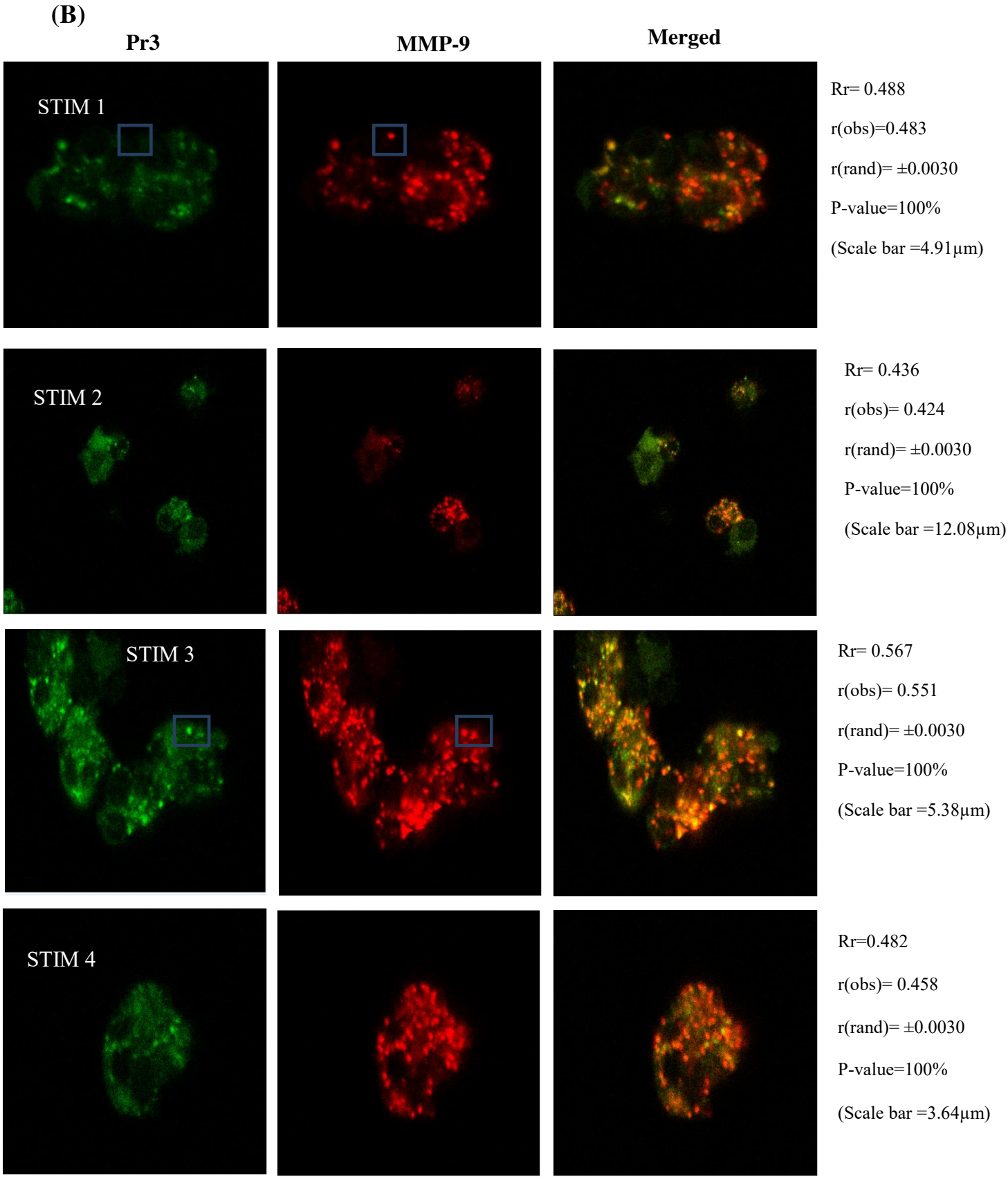
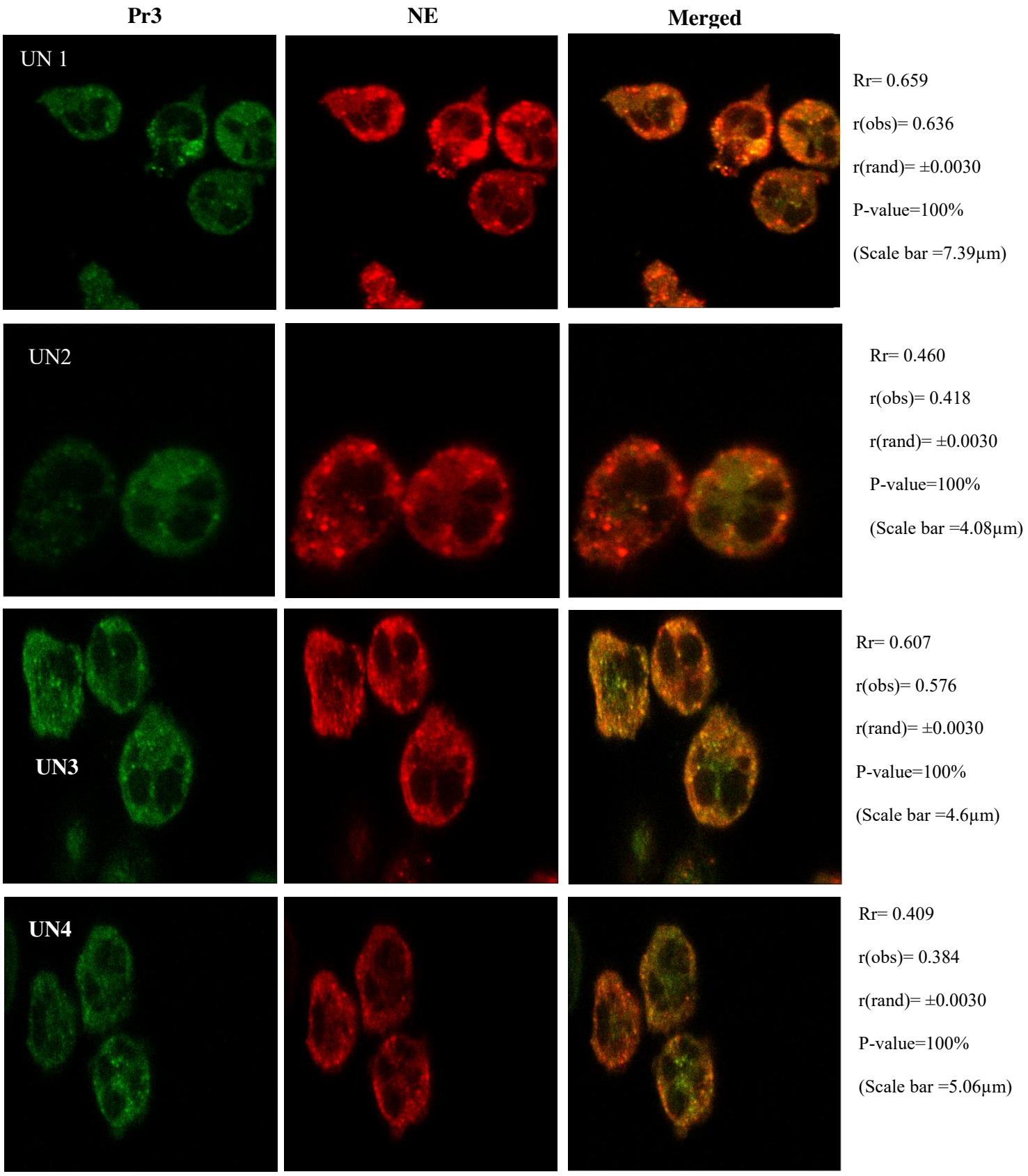


Figure 7.8 Co-localisation of Pr3 with MMP-9 in neutrophils. Cells were left unstimulated (A) or incubated with cytoB followed by addition of fMLP for 15 min (B) after which they were fixed, permeabilized, and stained for MMP-9 and Pr3. The merged images for the two proteins are shown on the right, with yellow indicating co-localisation between them determined using ImageJ software.

7.3.9 Co-localisation between Pr3 and NE

There was minimal co-localisation between Pr3 and NE in absence of stimulation (figure 7.9 A). However, stimulation of neutrophils caused an increase in the association between the two proteins, with the Pearson coefficient increasing from 0.513 to 0.791. These data are consistent with previous reports that while Pr3 and NE are stored in primary granules, Pr3 has also been reported to be present in neutrophil secondary granules, secretory vesicles and on the plasma membrane. This would explain the lack of correlation in unstimulated cells and the increase in correlation after stimulation if preferential release from secondary and secretory vesicles occurred.

(A)


Pr3
NE
Merged
UN 1
Rr= 0.659
r(obs)= 0.636
r(rand)= ±0.0030
P-value=100%
(Scale bar =7.39µm)
UN2
Rr= 0.460
r(obs)= 0.418
r(rand)= ±0.0030
P-value=100%
(Scale bar =4.08µm)
UN3
Rr= 0.607
r(obs)= 0.576
r(rand)= ±0.0030
P-value=100%
(Scale bar =4.6µm)
UN4
Rr= 0.409
r(obs)= 0.384
r(rand)= ±0.0030
P-value=100%
(Scale bar =5.06µm)

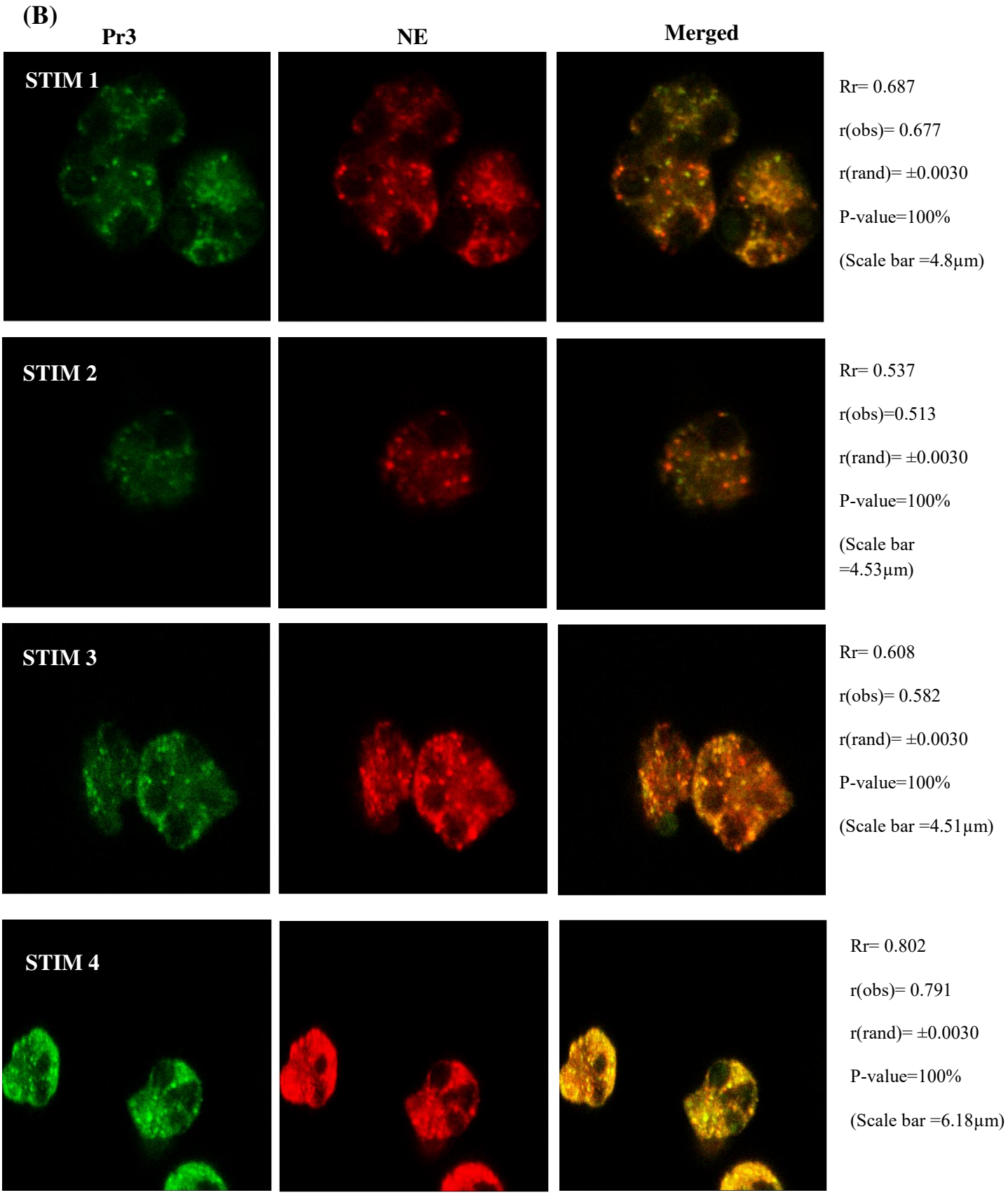


Figure 7.9 Co-localization analysis of Pr3 and NE by confocal microscopy. Unstimulated (A) or stimulated (STIM; B) neutrophils were incubated with the primary antibody (mouse anti-NE) followed by secondary antibody (CY3-conjugated donkey anti-mouse antibody) and finally incubated anti-Pr3 (FITC-conjugated monoclonal antibody). Shown are representative images of two experiments performed with cells from different donors (n=2).

7.3.10 No colocalisation between selected proteinases and granules markers in salivary neutrophils

Oral neutrophils were also investigated as they represent a human neutrophil post-transendothelial phenotype. These cells were isolated from the saliva of healthy individuals and stained for CD177, NE and MMP-9 with granule markers CD63 or CD66b with Pr3 or Pr3 with NE. There was no co-localization between the majority of these molecules. The azurophilic granule marker, CD63 was not co-localised with CD177 (r=0.218) or NE (r=0.184), although there was minimal co-localization with MMP-9 (fig 7.10). Similarly, the secondary granule marker CD66b was not co-localised with Pr3 (r=0.22), but Pr3 and NE showed minimal co-localization (r=0.547) in salivary neutrophils.

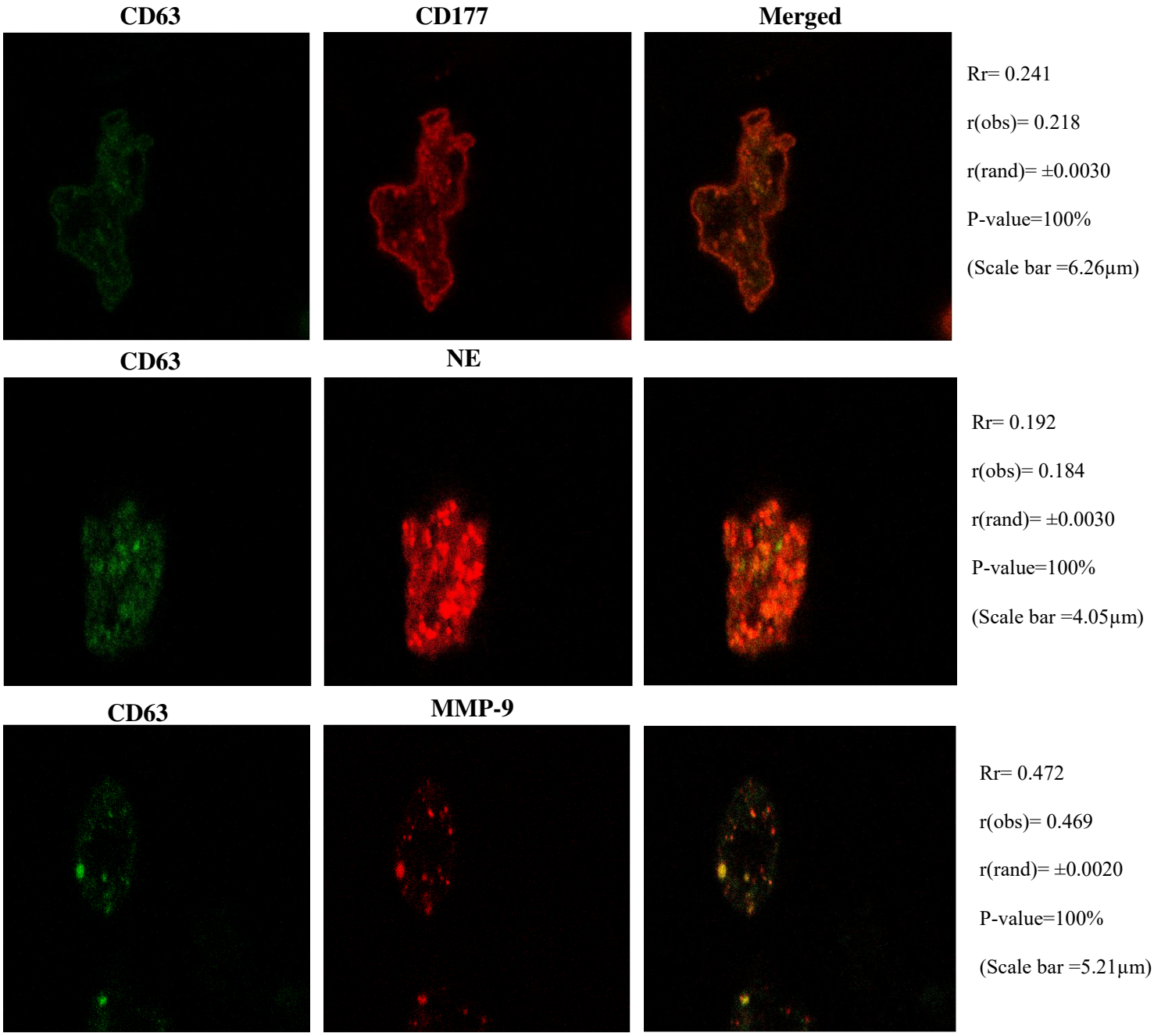


Figure 7.10 Confocal microscopy analysis for co-localisation of CD177, NE and MMP-9 with granule marker CD63 in salivary neutrophils. Human salivary neutrophils were isolated then fixed, permeabilized, and stained for CD177, NE and MMP-9 individually with granule marker CD63 (n=2).

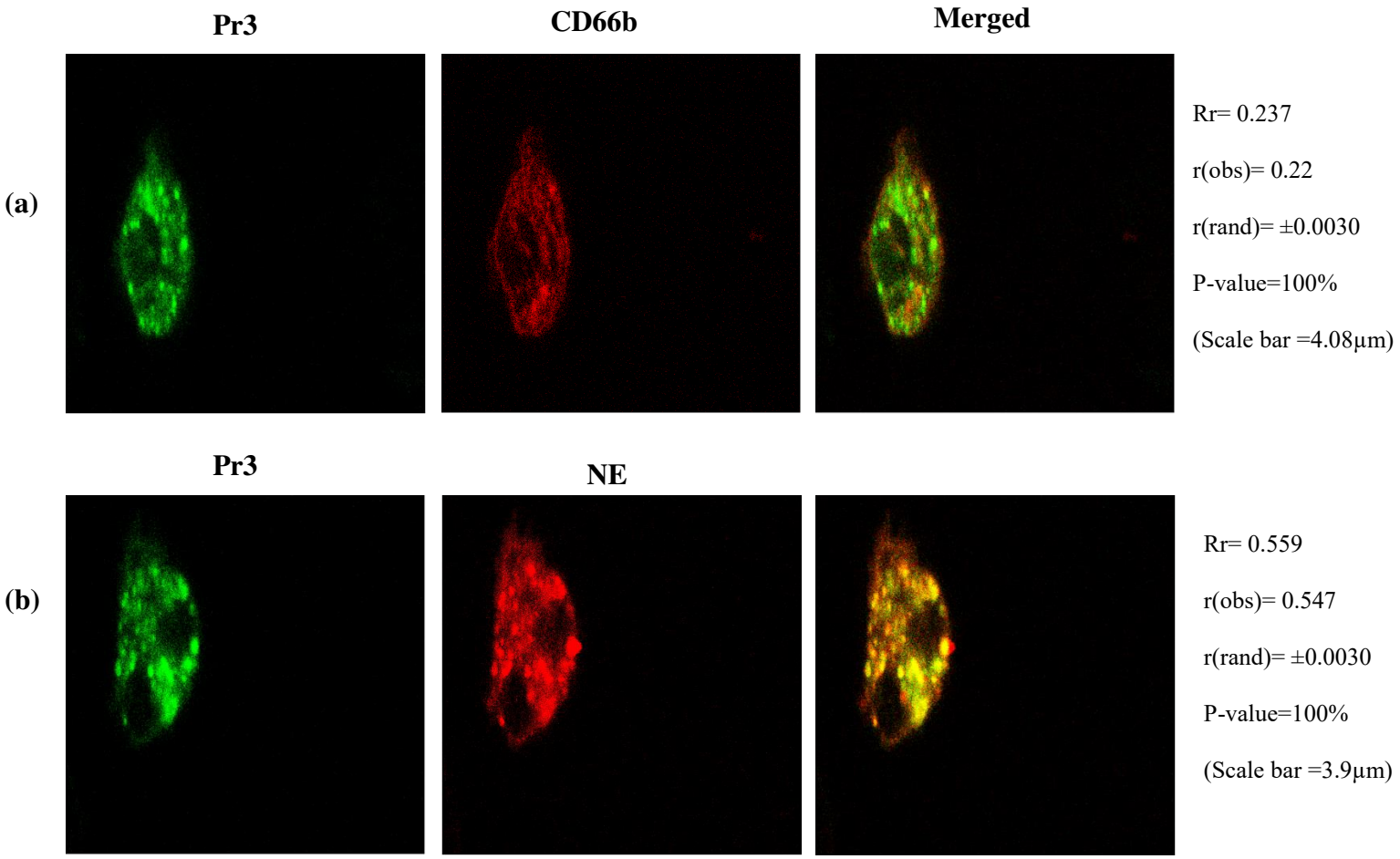


Figure 7.11 Confocal microscopy analysis for co-localisation of CD66b and Pr3 (a) and Pr3 and NE (b) in salivary neutrophils. Salivary neutrophils stained with primary antibody (mouse anti-NE) or primary antibody (mouse anti-CD66b) followed by secondary antibody (CY3-conjugated donkey anti-mouse antibody) and finally incubated with anti-Pr3 (FITC-conjugated monoclonal antibody).

7.4 Discussion

In this study, I analysed the co-expression of granule markers with selected neutrophil proteinases and also the co-expression of some of these proteinases with each other. The data presented here identified the co-localization using neutrophils that were purified using Dextran sedimentation only instead of separating the neutrophils by density gradient, prior to analysis, as the latter may activate the neutrophils and change the composition of each granule subset.

Circulating neutrophils are capable of undergoing a sequence of phenotypic changes that result in their alteration from dormant and poorly responsive cells to effector cells following exposure to pro-inflammatory stimuli. These phenotypic changes involve the relocation of receptors, proteins, adhesion molecules, and signalling molecules from the intracellular storage granules into the plasma membrane or phagosomal membranes and the release of proteases and other host- defence proteins into phagosomes or the surrounding tissue. Therefore these phenotypic changes may affect the co-localisation of these proteins or molecules inside the cells or their expression on the plasma membrane.

Neutrophil granule subsets are different in their content and capacity for mobilization in response to stimulation. These granule subsets undergo hierarchical stimulated exocytosis (Sengelov *et al.,* 1993; Jog *et al.,* 2007). Exocytosis, (also known as degranulation in neutrophils), is the release of granule-derived mediators from granules. Neutrophils granule contents are extremely cytotoxic, thus their release is highly regulated by specific signals to minimize irregular degranulation.

Primary granules are the main storage site of the most potent toxic mediators, including NSP, myeloperoxidase and defensins. Their membranes express CD63 (or lamp-3), which is a useful marker for immunofluorescence and flow cytometry. The secondary granules contain

lactoferrin and matrix metalloprotease 8, while tertiary granules contain the majority of metalloproteinase 9, among other substances. The secretory vesicles in human neutrophils contain human serum albumin. The secondary and tertiary granules have some overlapping components but can be distinguished by their intrinsic buoyant densities when centrifuged on Percoll density gradient (Kjeldsen *et al.,* 1994). The secondary granules possess a lipid-anchored protein, CD66b in their membranes but tertiary granules lack CD66b. CD66b is not a useful as CD63 as a granule marker as it is also found on the surface of unstimulated neutrophils, while CD63 is only found on the surface following mobilisation of the primary granules. The function of the specific granule types is different, as are the signal transduction pathways leading to their release.

When neutrophils are stimulated *in vitro* by fMLP, the result is an exocytosis of greater than 60% of secretory vesicles, 30% of gelatinase (tertiary) granules, 15% of specific (secondary) granules, and less than 5% of azurophilic (primary) granules. Exudative neutrophils analysed in skin windows in human volunteers demonstrated 100% release of secretory vesicles, 38% of gelatinase granules, 21% of specific granules, and 7% of azurophilic granules (Sengeløv *et al.,* 1993; Sengeløv *et al.,* 1995; Luerman *et al.,* 2010).

CD63, (a member of the tetraspanin superfamily) is an activation marker in neutrophils and one of the membrane proteins of azurophilic granules, which has been shown *in vitro* to be expressed on the cell surface following neutrophil activation in the presence of fMLP following cytochalasin B priming. Phorbol esters (like PMA) and fMLP alone are known to prompt minimal translocation of CD63 (Cham *et al.,* 1994). A previous study indicated that CD63 and NE are located in a complex, highly indicative of a role for CD63 in the targeting of NE to primary granules (Kallquist *et al.,* 2008).

NE is stored in the azurophilic granules, and also reported to be located in a nuclear envelope as shown by immunostaining and electron microscopy (Clark *et al.,* 1980; Benson *et al.,* 2003). NE is secreted by neutrophils during overwhelming inflammatory stimulation which destroys virulence factors and kills bacteria (Lehrer and Ganz, 1990; Belaaouaj *et al.,* 2000; Weinrauch *et al.,* 2002). Neutrophils were found to be a principal but not the only, source of MMP-8 and MMP-9. MMP-8 is stored in specific granules and secreted as inactive pro-proteins that are activated by autolytic cleavage. The function of MMP-8 is to degrade type I, II and III collagens. MMP-8 is released by PMN as well as by, monocytes, macrophages, and fibroblasts.

My results showed that there was no colocalization between CD63 and MMP-8 in the presence or absence of stimulation. Nevertheless, the result showed colocalization between CD63 and MMP-9 in neutrophils in the absence of stimulation. Following stimulation the colocalization between CD63 and MMP-9 was reduced and the Pearson coefficient ranged between 0.269 and 0.599. The data suggested that MMP-9 is localised in the same compartment with CD63 in resting neutrophil.

MMP-9 is rapidly released following stimulation (by different mediators, such as fMLP, TNF-α, and IL-8), and has been used as a marker for tertiary granule release (Chakrabarti *et al.,* 2005). It is probably that release of MMP-9 from tertiary granules that is crucial for facilitating migration of neutrophils across basement membranes. A report by Lominadze et al., utilising proteomic analysis of separated neutrophil granules reported that 5 % of the total cellular MMP-9 was located in the CD63-positive primary granules (Lominadze *et al.,* 2005). However, I would have expected the correlation to increase following stimulation as the tertiary granules release their MMP-9 following stimulation and the remaining cellular MMP-9 would be concentrated in the primary granules.

CD177 is a neutrophil surface receptor that belongs to the family of Ly-6 GPI-linked molecules and has been identified as a potential partner of Pr3. CD177 is not present in primary granules, but is found is on neutrophil plasma membranes and in secondary granules (Stroncek *et al.,* 1990; Goldschmeding *et al.,* 1992). Confocal microscopy images showed that CD177 and CD63 had a small amount of co-localisation in the absence of neutrophil stimulation but no co-localisation was observed after stimulation. However CD177 and CD66b were co-localised to the same compartment both in the absence of or following stimulation.

CD66b is a well-known as marker for neutrophil activation and exocytosis of specific granules. Under normal conditions, the majority of CD66b is located in the secondary granules, with lower amounts on the plasma membranes (Ducker and Skubitz, 1992 and Zhao *et al.,* 2004). Mollinedo *et al.,* showed that CD66b is also present in the membranes of tertiary granules (Molinedo *et al.,* 2003).

Following activation, CD66b expression on the plasma membranes is quickly up regulated by mobilization from intracellular pools (Kuroki *et al.,* 1992 and Zhao et al., 2004). Pr3, a serine proteinase is stored within primary, and specific granules as well as secretory vesicles. Herein, I evaluated the co-expression between Pr3 and CD66b in resting and stimulated neutrophils. Although Pr3 and CD66b are both reported to be located in specific granules, I found a minimal colocalization in resting neutrophils and neutrophils that had been stimulated with cytochalasin B and fMLP showed even lower colocalization levels.

As mentioned previously MMP-8 is also present in the specific granules, but my results showed no colocalization between Pr3 and MMP-8 in absence or presence of stimulation. However, I did find a colocalization between Pr3 and MMP-9 in unstimulated neutrophils, despite the lack of evidence in literature about the presence of Pr3 in the tertiary granules.

The Pearson coefficient averaged about 0.7 in the absence of stimulation and after stimulation it ranged from 0.5-0.4. These results go against dogma or granule contents and require examination by an alternate methodology.

Pr3 and NE are two abundant NSP involved in antimicrobial protection which are considered to have both similar localization and specificity. Both are closely related enzymes, with overlapping and potentially redundant substrate specificities (Kessenbrock *et al.,* 2008). They share 55% amino acid homology and many structural and functional characteristics (Wiesner *et al.,* 2005; Rao *et al.,* 1991; Jenne DE 1994). However, Pr3 has different properties from NE; Pr3 is already present at the plasma membrane in the absence of stimulation and it is also the main target for the autoantibodies referred to as ANCA. The mechanisms responsible for this specific autoimmunization against human Pr3, and not against its homologs human NE, remain unclear. However, a minority of ANCA are directed against another azurophilic granule protein, myeloperoxidase (Kallenberg *et al.,* 1994; Jennette and Falk 1997). Interestingly, my results showed minimal or no (r (obs)= 0.6-0.4) subcellular co-localization between Pr3 and NE in absence of stimulation, but after stimulation the co-localization between the proteins was increased and Pearson coefficient ranged from 0.8-0.5.

Modification of neutrophil phenotype and function is associated with their migration from blood into tissue. Thus, I investigated co-expression of some proteinases with granule markers in salivary neutrophils to compare to my results for blood neutrophils. Several remarkable functional changes are supposed to occur in neutrophils when they migrate from blood into various tissues, as part of their host defence response, where they may become more reactive to a number of stimuli (Kanamori *et al.,* 1997).

In this study, it was found that CD63 and CD177 were up regulated on the surface of the salivary neutrophils (as seen in figure 7.9) but there was no co-localization between them. In

addition, no colocalization was observed between CD63 and NE, whereas CD63 and MMP-9 show very low colocalization. Pr3 and CD66b also showed no colocaization. Pr3 and NE in salivary neutrophils showed minimal colocalization. This result suggested migration of neutrophils to oral cavity was accompanied by overt degranulation. Table 7.1 illustrates a summary of co-localisation analysis of the selected molecules.

My findings raise a number of questions. Neutrophils are easily activated during isolation, this raises the question of whether this affects the composition of neutrophil granules or not? The slight activation of neutrophil may cause the mobilization or fusion of secretory vesicles into the plasma membrane, thus this may change the composition of these granules and the expression of some molecules on the surface of neutrophils. The fixation and permeabilization of neutrophils may affect composition of each granule. The permeabilization of neutrophils allows the antibodies to bind their targets inside the cells but it may in itself cause artefacts affecting the final result of co-localization analysis. The relocation of neutrophil granules *in vivo* may be also different than those observed *in vitro*. During *in vivo* stimulation, the neutrophils communicate with other types of blood and immune cells which may not mediate effects during *in vitro*.

In this study I found that there was difference in the level of co-localization between cells under unstimulated and stimulated conditions which may due to the difference in size and density of each granule.

The changes in co-localisation after stimulation probably reflect the location of proteases in granules which are differentially secreted during stimulation. If two proteases reside within the same granule, then the co-localisation index before and after stimulation would remain unchanged (See figure 12.A). Similarly, if the proteases reside in separate granules, secretion would not change the co-localisation (see figure 7.12B). However, if the segregation of

proteases is not total, with some segregation and some overlap of proteases, there are two possible outcomes. (I) If the granules containing only one of the two proteases are the more likely to be secreted, then co-localisation is increased during secretion. (II) On the other hand, if the granules containing both proteases are more likely to be secreted, co-localisation is reduced after stimulation (See fig 7.12C and D).

Table 7.1 Conclusion of co-localisation analysis

Protein of interest (blood PMNs)	**CD63**	**CD66b**	**Pr3**
Unstim NE	Yes	-	*
Stim	Yes	-	Yes
Unstim MMP-8	No	-	No
Stim	No	-	No
Unstim MMP-9	Yes	-	Yes
Stim	*	-	*
Unstim CD177	Yes	Yes	-
Stim	No	Yes	-
Unstim Pr3	-	Yes	-
Stim	-	*	-
Protein of interest (salivary PMNs)	**CD63**	**CD66b**	**Pr3**
CD177	No	-	-
NE	No	-	Yes
MMP-9	No	-	-
Pr3	-	No	

(*) Variable result ranged from minimal to no co-localisation, (-) not tested.

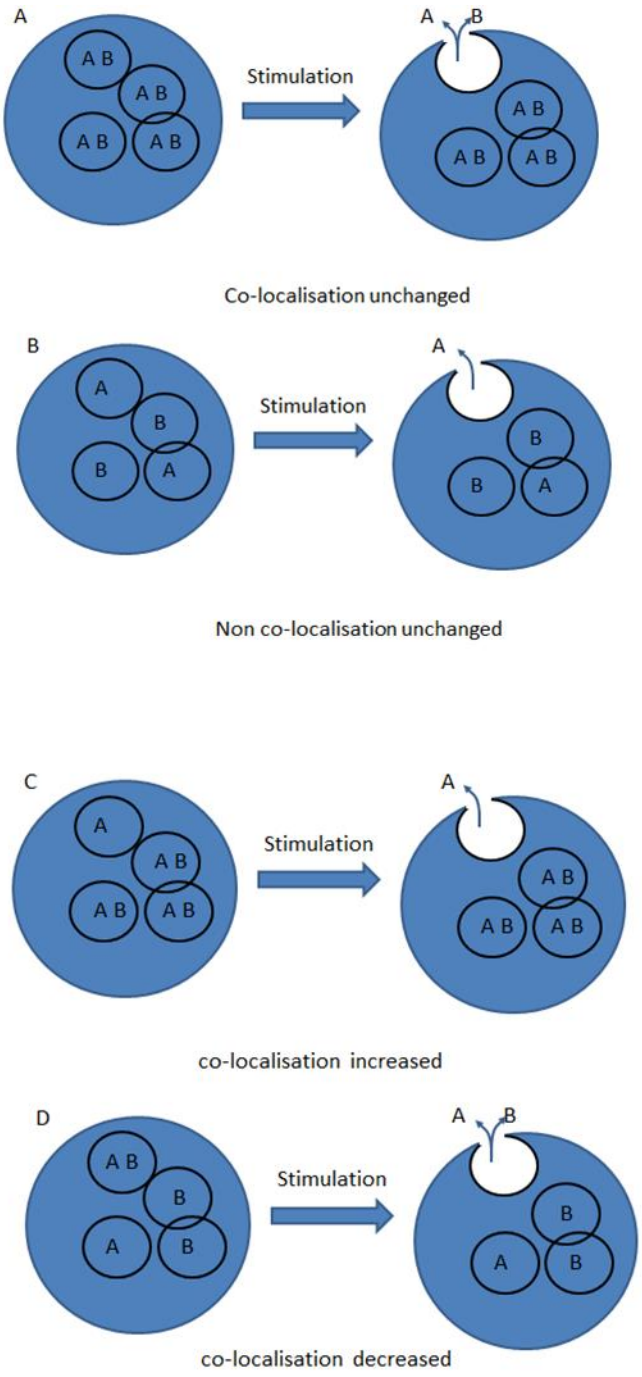


Figure 7.12 The possible mechanisms of co-localisation and non co-localisation of proteinases in granules in absence and presence of stimulation.

7.5 Summary

In this chapter I investigated the intracellular distribution of neutrophil granules markers and neutrophil proteinases. The investigation revealed some discrepancies with previous reports. Some neutrophil proteins were co-localized after neutrophil stimulation which suggested that after degranulation they were present in the same compartment (possibly the phagolysosome) or they may share the same transportation pathway during degranulation.

CHAPTER 8

GENERAL DISCUSSION

8.1 Overview

Neutrophil proteinases are important molecules of immune and inflammatory responses. In this thesis I have sought to investigate the change in expression of selected neutrophil proteinases following *in vitro* neutrophil transmigration and stimulation with microbial components (cytochalasin B, fMLP). I have sought to examine the differences in neutrophil surface and intracellular expression of Pr3, CD177 (a surface receptor of Pr3), MMP-8 and MMP-9. In addition I have examined the expression of these neutrophil proteinases and surface neutrophil markers (CD16 and CD63) in sPMNs (*in vivo* low inflammation transmigration model) and compare it to that in bPMNs. In this thesis, I have paid particular attention to the effect of serum serine-proteinase inhibitors (alpha-1-antitrypsin) on removing Pr3 from the high affinity Pr3 surface receptor (CD177) using isolated neutrophils *ex vivo* stimulation models and *in vitro* Pr3 capture assays using recombinant soluble CD177 and monoclonal antibodies. Finally, I have assayed the distribution of neutrophil proteinases between granules using confocal microscopy. I have focused on co-localization of selected neutrophil proteinases with markers of primary granules (CD63), and secondary granules (CD66b) and the co-localization of enzymes known to primarily reside in the tertiary granules (MMP-9) and primary granules (elastase) with Pr3.

8.2 Neutrophil transmigration and proteinases

Many previous studies have focused on the role of neutrophil proteases in neutrophil transmigration but this still remains a contentious issue (Young *et al.,* 2007; Yadav *et al.,* 2003; Shapiro 2002). Therefore in this thesis I studied the change in surface expression and intracellular levels of Pr3, MMP-8 and MMP-9 prior to and following neutrophil stimulation or transmigration. Moreover, I studied the expression of CD177 which has been identified as an important molecule in neutrophil transmigration through the interaction with PECAM-1 and Pr3 (Sachs *et al.,* 2007; Kuckleburg *et al.,* 2012). CD177 is a specific neutrophil antigen,

up regulated during bacterial infection, sepsis and during pregnancy as well as in newborns. CD177 also plays a role in different diseases such as immune mediated neutropenia, polycythemia vera and Wegener's disease (Göhring *et al.,* 2004; Wolff *et al.,* 2006; Caruccio *et al.,* 2003). The expression of CD177 varies between individuals where CD177-negative subpopulations range from 0-100%. Pr3 is the main auto-antigen in anti-neutrophil cytoplasmic antibody-associated vasculitis (Wegener's disease) and its surface expression on neutrophils has pathogenic importance. I have demonstrated that neutrophils expressed both low and high (or negative and positive) sub-populations of mPr3 and CD177 after *in vitro* transmigration, but these findings are in contrast with the study by Sachs *et al.,* 2007 in which they showed that CD177-positive neutrophils tended to transmigrate more readily than CD177-negative neutrophils.

Herein, I detected that the expression of CD177 was significantly increased after *in vitro* transmigration whereas Pr3 showed no significant change, but in some individuals (n=3) the migrated neutrophils lacked the mPr3 negative subpopulation. However, the increased expression of CD177 on CD177-positive cells (as the ratio of CD177 doesn't change), could be explained by the effects of the fMLP chemoattractant effects in the absence of migration. In contrast, a study by Kuckleburg *et al,* (2012), reported that Pr3 expression and activity were significantly increased on CD177-positive neutrophils following transmigration, while neutrophils lacking CD177 demonstrated no increase in Pr3, thus they suggested that both molecules are required for neutrophil migration. However, they also reported that CD177-deficient individuals have no impaired transmigration compared to normal individuals. In addition, Sachs *et al.,* did not mention that CD177-negative neutrophils were not able to migrate. These conflicting results may be attributable to the endothelial cell line used or the culture conditions (such as coating of inserts).

In this study I confirmed the previous observation that blocking antibodies against CD177 decreased neutrophil transmigration across endothelial monolayers (Sachs et al., 2007; Kuckleburg *et al.,* 2012), but I also demonstrated that CD177-positive neutrophils were able to migrate across the HUVEC cell layer despite being coated with MEM166 antibody and that no significant loss of MEM166 occurred during the transmigration.

In contrast, by examining the characteristics of salivary neutrophils, I have demonstrated that these cells expressed only the positive subsets of Pr3 and CD177 and the expression levels on salivary neutrophils were significantly higher than on blood neutrophils. The expression of these molecules on salivary neutrophils has not been published before. Neutrophils pass from the connective tissue to the gingival sulcus via the intercellular spaces of the junctional epithelium, thus the results suggest that *in vivo* Pr3 and CD177 are required for efficient extravasation. Moreover, the oral environment may preferentially require the presence of surface Pr3 and CD177 expression to interact with the endogenous microbes. While I was unable to observe decreased intracellular Pr3 levels for in vitro transmigration, for the in vivo equivalent I found sPMN were lower in intracellular Pr3 content compared to bPMNs. Furthermore unlike *in vitro* conditions incubation of migrated neutrophils, further upregulation of Pr3 on the surface of sPMN by cytochalasin B and fMLP was not possible. These results imply that sPMN represent a condition following greater and/or more prolonged stimulation, but that only a defined proportion of the intracellular Pr3 can be mobilised outside of the cell. However, more Pr3 may be released when neutrophils enter into the phase of NET formation.

Due to a time limitation (the application for ethics exceeded my study time) I could not extend these investigations of sPMNs to other inflammatory conditions such as idiopathic pulmonary fibrosis which may represent higher inflammatory conditions for comparison.

Further study is necessary to determine if there is a site-specific requirement for mPr3 expression for all types of inflammation *in vivo*.

In the present work, I demonstrated that the high number of the neutrophils in saliva are apoptotic or necrotic, which may account for their failure to release additional intracellular Pr3 or further increase their surface expression of CD177 or Pr3 with additional stimulation. Nonetheless, I note that these cells still illustrate the capacity to actively shed CD16 and to externalise a small additional amount of CD63, which suggested that these cells are still capable of eliciting further degranulation of primary granules and releasing the enzymes responsible for shedding CD16. There was also some variation in the levels of apoptosis/necrosis from volunteer to volunteer which may reflect the nature of inflammation (i.e. gingivitis) in their oral cavity.

I have demonstrated that salivary neutrophils have released almost all of their MMP-8 and -9. In contrast, *in vitro* transmigration studies showed that blood neutrophils were more prone to release a proportion of their MMP-9 stores, (but less than salivary neutrophils) and none of their MMP-8 stores, during migration through endothelial cells. These differences in results between *in vitro* and *in vivo* models are likely to reflect differences in the composition of chemoattractants and stimulants as well as the non-sterile environment in the oral cavity. However, the oral cavity is a unique environment and the heterogeneity of the oral cavity and related microenvironments might influence the release and the expression of these proteins by salivary neutrophils.

8.3 Subcellular localization and distribution of neutrophil proteinases

Since the majority of the neutrophil functions are dependent on the mobilization and release of their granules and their content (such as proteinases), assessment and determination of location, distribution and co-localisation of neutrophil proteinases are of major interest for

understanding their role during transmigration or inflammation. In this thesis I identified the co-localisation of selected neutrophil proteinases with granule markers or between different proteases before and after maximum stimulation to detect the effect of degranulation on the distribution and co-localisation of these proteins. In previous reports, the localization of some of these proteins in neutrophils have been determined by electron microscopy, some by subcellular fractionation and some by mobilization, assuming that proteins that are mobilized together also localize together (Borregaard *et al.,* 1997). Interestingly our investigation revealed some discrepancies with previous reports. I have demonstrated that in the absence of stimulation, MMP-9 and CD63 were co-localised in neutrophils and after stimulation, the co-localization between the two proteins was lost or significantly reduced. This finding was surprising, as it is well established that the majority of neutrophil MMP-9 is stored in tertiary granules, but CD63 is a membrane marker of azurophilic granules. In addition, there was co-expression between Pr3 and MMP-9 in resting neutrophils but, in response to stimulation, the co-localisation was lost. It is well known that subcellular fractionation of resting neutrophils shows that the major intracellular store of Pr3 (like CD63) is the azurophilic granules. Lesser amount of Pr3 has been reported in the secretory vesicles and in specific granules (Witko-Sarsat *et al.,* 1999), but no evidence has been reported for Pr3 to be located in tertiary granules. Pr3 is the most abundant of the neutrophil proteases with each adult neutrophil estimated to store 3 pg of Pr3 compared to 1.1pg of NE and 0.85pg of CatG (Campbell *et al.,* 2000). Thus, this may reflect the importance of this enzyme as an essential molecule in neutrophil function and in the inflammatory process.

NE and Pr3 are homologous proteases that belong to the chymotrypsin superfamily of serine proteases. NE and Pr3 are located in azurophilic granules and NE is also localized in the nuclear envelope, as revealed by immunostaining and electron microscopy (Clark *et al.,* 1980; Benson *et al.,* 2003). In resting neutrophils, I detected NE co-localised with CD63 and

showed a variable level of co-localisation with Pr3. In response of stimulation, a similar level of co-localisation between NE and CD63 was observed and the level of co-localisation between Pr3 and NE was increased. This implies that degranulation caused mobilisation of Pr3 and NE to a common intracellular compartment along with CD63. Alternatively these results may show that the non-CD63 associated Pr3 is released from the cell and only the CD63-associated Pr3 remains (with coincident increase in co-localisation with NE). In salivary neutrophils I have demonstrated that the co-localisation between Pr3 and NE remained high, whereas the co-localisation of other examined proteins was lost, which is in agreement with the hypothesis of increased co-localisation through degranulation of these cells.

I have demonstrated that there was minimal co-localisation between CD177 and CD63 in absence of stimulation, which was completely lost following stimulation. In contrast, CD177 was co-localised with CD66b both in the presence and absence of stimulation. This suggests that the majority of CD177 is located in the secondary granules.

In this work I have identified that MMP-8 was not located with CD63 which is consistent with previous reports that MMP-8 is not located in azurophil granules and located in specific granules. However, I found in both conditions for blood neutrophils that there was no association between MMP-8 and Pr3, both of which have been reported in specific granules in a previous electron microscopy study (Witko-Sarsat *et al.,* 1999). In contrast, the confocal images showed poor co-localisation between Pr3 and CD66b in the absence of stimulation, and after stimulation there was no co-localization.

8.4 The surface expression of CD177 and mPr3 and proteinase inhibitors (AAT)

Initially, I have confirmed the finding of previous studies that unstimulated neutrophils, isolated from healthy individuals, expressed varying levels of Pr3 and CD177 on their cell surface. I have also demonstrated that the expression of mPr3 on resting cells in some individuals was low and monomodal. However the same individuals showed bimodal expression of CD177. Previous studies have suggested that CD177 acts as a receptor of mPr3, by viewing that mPr3 expression is highly correlated with CD177 expression and mPr3 co-localises with CD177 on the neutrophil membrane (Bauer *et al.,* 2007; von Vietinghoff *et al.,* 2007). I have found that the pattern of mPr3 expression changed to bimodal in response to maximum stimulation (cytoB followed by addition of fMLP), in agreement with previous study by Hu *et al.,* 2009, although they used TNF-α in their stimulation methods. The mechanism leading to expression of low level mPr3 on the surface of CD177-negative neutrophils is not fully understood yet. These results point out that CD177 may not be a sole binding partner of mPr3, and other binding site(s) may exist as well on neutrophils and facilitate a low amount of mPr3 expression. It has been reported that several other membrane partners of Pr3 such as CD16/FcγRIIIb or the adhesion molecule CD11b/CD18 (β2 integrin), may play role in mPr3 expression on CD177-negative cells. Membrane-bound Pr3 is sensitive to inhibition by physiologic inhibitors including AAT. Pr3 deleterious action is theoretically prevented by AAT. In the present work I used autologous serum as a physiological source of AAT to investigate its effect on Pr3 and CD177 expression on unstimulated and stimulated neutrophils. Assessment of surface expression of mPr3, CD177 and CD16 was performed using flow cytometery. The percentage of Pr3 and CD177 expression was still detectable in the presence of serum in resting and stimulated neutrophils. The levels of surface CD177 showed no change in expression in the presence of serum, compared with absence of serum, on unstimulated or stimulated neutrophils. I have

demonstrated that mPr3 is still detectable on the surface of neutrophils in the presence of serum, but it showed a marked decrease relative to levels bound in the absence of serum. Levels of mPr3 on unstimulated neutrophils in the absence and presence of serum were similar (no significant difference), although a significant reduction in mPr3 is noted upon stimulation in serum when compared to cells stimulated in the absence of serum. However, I demonstrated that stimulation of neutrophils in the presence of serum showed a significant increase in the $mPr3^{high}$ population, which suggested that regardless of the presence of physiological inhibitors, increased surface Pr3 would still occur with stimulation and AAT may be is not able to inhibit or remove all inducible mPr3.

In this thesis inhibition of mPr3 by AAT was also analysed using cells (CHO) that stably expressed the CD177 receptor produced by recombinant technology. My data enhance the earlier finding that addition of purified AAT to Pr3-bound CD177 transfected CHO cells removed Pr3 from the surface of CD177 receptor-expressing CHO cells (Korkmaz *et al.,* 2008). In this study I used Pr3 (stimulated neutrophil supernatant and commercially purified) and AAT (commercial purified from serum and recombinant as well as autologous serum) from different sources. I have demonstrated that there was no difference in the ability of both types of Pr3 to bind CD177 on the surface of CHO cells. Moreover both types of AAT were equally capable of removing Pr3 that bound to CD177 expressed on transfected CHO cells.

8.5 Neutrophil isolation methods and their surface marker expression

In this thesis I compared two methods of neutrophil isolation from peripheral blood with respect to mPr3 expression and the expression of CD16. I have demonstrated that Pr3 was detected on unstimulated neutrophils from both techniques but the level on unstimulated Percoll-separated cells appeared higher (no significant difference) than on dextran-separated cells. Neutrophils isolated from both methods showed a significant increase in mPr3

expression following stimulation compared to unstimulated cells with higher expression on dextran-separated cells compared to Percoll-separated cells. Nevertheless, neither of these differences achieved statistical significance it was only when the ratio of MCF mPr3 between stimulated neutrophils was compared between these two methods that this obvious difference was confirmed by statistical analysis. The data suggested that Percoll-gradients increased the baseline levels of mPr3 on the surface of unstimulated neutrophils, but that the levels of mPr3 achieved much greater levels if the cells were purified by dextran sedimentation.

I observed that the level of CD16 on unstimulated dextran-sedimented cells was higher than that on unstimulated Percoll-separated cells, but the levels of CD16 after maximal stimulation-induced shedding results in similar levels on both methods. These results revealed that Percoll may cause low activation of neutrophils in absence of stimulation and Percoll-separated cells did not respond to stimulation to the same extent as dextran-derived cells. It is important to note that the dextran-sedimentation doesn't remove monocytes or lymphocytes and the effects of these cells on neutrophil responses, cannot be ruled out. Further study is necessary to find the most appropriate isolation method to study neutrophils.

8.6 Future research

From this thesis a number of questions have been raised and suggest some interesting areas for future research.

In this work I have shown that the expression of CD177 and Pr3 was higher on salivary neutrophils than that in blood neutrophils and these cells were all CD177-positive. However, *in vitro* study showed both positive and negative sub populations of CD177 and Pr3 could migrate. In addition *in vitro* transmigration showed that neutrophils are more prone to release a proportion, but not all, of their MMP-9 stores, and none of their MMP-8 stores, during migration through endothelial cells. By comparison, migrated salivary neutrophils had

released almost all of their MMP-8 and MMP-9 prior to collection and measurement. Therefore it would be interesting if further investigation including extending the findings for salivary neutrophil degranulation to other inflammatory conditions and to gingival neutrophils (the other type of neutrophil in oral cavity). Moreover, it would be useful to study the expression of Pr3 with other serine proteinases (NE and Cat G) on salivary neutrophils and on *in vitro* transmigrated neutrophils using transwell assay with endothelial and epithelial cells. It would be interesting to study the expression of other Pr3 membrane partners on neutrophils (bPMNs and sPMNs) from normal and null-CD177 individuals during transmigration and microbial infection to investigate other mechanisms leading to the expression of Pr3.

I have noted that salivary neutrophils are not able to release Pr3 after maximum stimulation despite there being detectable levels of intracellular Pr3 still present, thus it would be interesting to study the limitation of Pr3 release from blood neutrophils using different stimulant and microbial components.

Inflammatory diseases mediate destruction of the tooth supporting tissues (periodontium) which occur as a result of collateral damage caused by the enzymes released by neutrophils as they attempt to contain the bacterial infection. Further research to study the expression of neutrophil proteases on salivary neutrophils isolated from normal individuals compared to individuals with defined periodontal diseases would also be interesting.

The results from this thesis and other studies showed that CD177 is not a sole binding partner of mPr3. Further in depth study is needed to evaluate the effect of proteinase inhibitors (including AAT and other serine protease inhibitors) on other categories of membrane partners of Pr3 (such as CD16/FcγRIIIb and CD11b/CD18) by creating cell lines expressing these receptors, as well as expressing recombinant Pr3 on CHO cells alone.

The data presented in this thesis demonstrated that the co-localisation of some proteinases with each other or with granule markers revealed some discrepancies with previous reports. Therefore, further studies using alternative methods [such as Fluorescence resonance energy transfer (FRET)] of determining co-localisation would be required to confirm the validity of these results. Further investigations are also needed to include the co-localisation of neutrophil proteases with all granule markers (including secretory vesicles markers). It would be interesting to study the co-localisation of these molecules in neutrophils purified by different methods to determine if this influences the findings.

8.7 Final summary

Neutrophils proteases are complex and have a very significant effect on the inflammatory reactions. In this thesis I sought to investigate the effect of protease inhibitors on the expression of mPr3 and CD177. I have shown that despite the ability of purified AAT to inhibit Pr3 binding to CD177, significant surface Pr3 was still found on the surface of CD177-positive neutrophils when stimulated in the presence of 100% autologous serum, but only following maximal stimulation of neutrophils.

In this thesis I sought to investigate the role of neutrophil proteinases on neutrophil transmigration. I have demonstrated that transmigration (*in vitro* transmigration) alone does not result in a significant increase in mPr3 expression. Moreover, all CD177-positive and negative neutrophils were able to migrate through the endothelial cells. Neutrophils were more prone to release MMP-9, but not MMP-8, during migration through endothelial cells.

I have sought to compare intracellular and surface proteinase levels between bPMNs and sPMNs. Migrated salivary neutrophils have released almost all of their MMP-8 and MMP-9 prior to collection and measurement. Only CD177-positive PMNs were found in the saliva,

despite being bimodal in the periphery, and CD177 bound significant levels of Pr3 on the surface of these cells.

In this thesis I also sought to study the co-localization and distribution of some neutrophil proteases before and after stimulation using confocal microscopy. I have shown that CD63 was co-localized with some MMP-9 and Pr3 was co-localized with MMP-9 in absence of stimulation. These results are in conflict with accepted dogma of granule contents and further investigations are needed.

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APPENDIX

ABSTRACTS AND PUBLICATIONS FROM THIS WORK

Poster- I3 IRG Annual Meeting, Thornhill Cardiff, Wales (September 2011)

26th Annual Postgraduate Research Day (November 2011).

The changes of extracellular levels of CD177 and Pr3 and intracellular levels of MMP-8 and MMP-9 after neutrophils transmigration.

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Background: Neutrophil transmigration is a crucial event in host defense and inflammation. Neutrophils contain several different proteases which are thought to play a role in aiding in transendothelial cell migration. It is unknown if transmigration of neutrophils results in significant release of these proteinases, significant intracellular re-organisation of the proteinases or induces surface expression of Pr3/CD177 (NB1) complex. Pr3 and NB1 show a bimodal expression pattern with expression on 0-100% of neutrophils in the population.

Methods: Human umbilical vein endothelial cells (HVEC line) were cultured on transwell membranes coated with matrigel® and either not treated or treated with TNF-α. Freshly isolated neutrophils were applied to the upper chamber. fMLP was used as stimulant for control cells and as chemoattractant by adding into the lower chamber. The expression of Pr3, CD177, MMP-8, and MMP-9 were analysed by flow cytometery.

Results: Neutrophil transmigration through the HVEC monolayer was significantly increased when the endothelial cells were pre-treated with TNF-α, showing that the mechanism of transmigration mimicked the physiological process. Although Pr3 was up-regulated to the cell membrane (mPr3) following stimulation with fMLP before transmigration (control cells) this was only slightly increased ($p>0.05$) by transmigration. The percentage of CD177 (and Pr3) expression neutrophils was same for both pre- and post- migration. The level of MMP-9 decreased to about 75% of the unstimulated level after fMLP stimulation. Transmigration decreased this significantly further to about 40%. In contrast, pre and post transmigration MMP-8 levels showed that no significant difference.

Conclusion: The findings indicate that transmigration alone does not result in a significant increase in PR3 expression. Moreover, all CD177- negative neutrophils were able to migrate through the endothelial cells. Neutrophils with surface Pr3-bound to CD177 do not have an advantage for transmigration, as no enrichment for CD177 cells is seen post-migration for either un-stimulated and TNF-α treated endothelia. However, neutrophils are more prone to release MMP-9, but not MMP-8, during migration through endothelial cells.

Poster- British Society for Immunology Congress, Liverpool (BSI; December 2013)

28th Annual Life Sciences Postgraduate Research Day (November 2013)

The comparison of surface and intracellular proteinase between bPMNs and sPMNs.

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Background: Peripheral blood neutrophils (bPMNs) and salivary neutrophils (sPMNs) are important cells that play an essential role in immunity and inflammation. In the oral cavity sPMNs have a major role against invading oral microorganisms with high numbers of sPMNs constantly migrate from the blood stream through the gingival crevice into the oral cavity. Their main function is to protect the oral environment from pathogens and prevent infection. Neutrophils contain several different proteases which are thought to play a role in aiding in transendothelial cell migration and are implicated in antimicrobial defence. The aim of this study was to compare intracellular and surface proteinase levels between bPMNs and sPMNs.

Methods: bPMNs and sPMNs were collected from healthy donors. The cells counted and stimulated with cytochalasin B followed by addition of fMLP, and compared to unstimulated controls. The expression of proteinase 3 (Pr3), CD177, metalloproteinase-8 (MMP-8), and MMP-9 were analysed by flow cytometery.

Results: The results showed that the percentages of surface Pr3 and CD177 expression on unstimulated bPMNs ranged from 0 to 100% in a given individual, but unstimulated sPMNs expressed only positive populations of CD177 and Pr3. The surface Pr3 expression on sPMNs was significantly higher than that on bPMN ($P=0.001$). Moreover, Stimulation of bPMN and sPMNs from matched donors showed an 11-fold increase in surface Pr3 expression on bPMN, but no significant change in expression on sPMNs compared to unstimulated cells. The levels of intracellular Pr3 in sPMNs were detectable however, they were significantly lower ($P=0.0002$) than levels in bPMNs. The levels of MMP-8 and MMP-9 in sPMNs were almost negligible, indicating that most of these proteinases are released either through transmigration or due to contact with oral microbes. The intracellular level of Pr3 was significantly higher than the levels of MMP-8 and MMP-9 in sPMNs ($P< 0.0001$).

Conclusion: Migrated salivary neutrophils have released almost all of their MMP-8 and MMP-9 prior to collection and measurement. Only CD177-positive PMNs were found in the saliva, despite being bimodal in the periphery, and CD177 bound Pr3 on the surface of these. While intracellular levels of MMP-8 and MMP-9 were completely depleted in sPMN, detectable levels of intracellular Pr3 were still present, although they couldn't be mobilised by further cell stimulation.

Poster- British Society for Immunology Congress, Liverpool (BSI; December 2013)

The effect of serum on mPr3 and CD177 expression on neutrophils

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Background: Proteinase 3 (Pr3) is a serine protease that is stored primarily in azurophilic granules and secretory vesicles in neutrophils. The high affinity Pr3 receptor, CD177, is expressed on a subset of neutrophils (ranging from 0-100%), but usually only half of the circulating neutrophils express CD177 in most normal individuals. As a serine proteinase, Pr3 is controlled by a variety of inhibitors, including alpha-1-antitripsin (AAT), which is present in serum at 1.5-3.5 g/L. We investigated if the surface expression of Pr3 and CD177 was affected by stimulation of neutrophils in the presence or absence of serum.

Methods: Neutrophil cells were isolated from healthy donors. The cells counted and stimulated with fMLP only or cytochalasin B followed by addition of fMLP, and compared to unstimulated controls, in the presence or absence of 100 % autologous serum. The expression of Pr3 and CD177 was analysed by flow cytometery.

Results: The expression of membrane bound Pr3 (mPr3) by neutrophils was still detectable in the presence of serum however, the expression was reduced by 6-fold on both mPr3low and mPr3high cells in comparison to cells incubated in the absence of serum. No increase was observed in mPr3 expression following stimulation with fMLP in either the presence or absence of serum. In comparison, stimulation with cytochalasin B combined with fMLP resulted in a 9-fold increase ($P<0.0001$) in the Pr3high cells compared with unstimulated cells in the absence of serum. This increase was only 3-fold ($P< 0.05$) in the presence of serum.

Conclusion: The expression of membrane bound Pr3 (mPr3) by neutrophils was still detectable in the presence of serum however, the expression was reduced by 6-fold on both mPr3low and mPr3high cells in comparison to cells incubated in the absence of serum. No increase was observed in mPr3 expression following stimulation with fMLP in either the presence or absence of serum. In comparison, stimulation with cytochalasin B combined with fMLP resulted in a 9-fold increase ($P<0.0001$) in the Pr3high cells compared with unstimulated cells in the absence of serum. This increase was only 3-fold ($P< 0.05$) in the presence of serum.